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MANGANESE SULFATE

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Major and trace element content of cattle hair as an indicator of calcium, magnesium, phosphorus, potassium, sodium, iron, zinc, manganese, copper, molybdenum, and cobalt supply.

Communication No. 6

Incorporation and elimination of orally administered ^{52}Mn in the hair, blood, and milk of cattle.

1. Introduction and Objective

In a series of studies [20, 4, 5, 6, 19, 18, 15, 16], the Mn content of cattle hair was related to the Mn supply of these animals. Although it could be demonstrated that (1) additional administrations of Mn, also in the form of salts, elevate the amount of Mn contained in the pigmented body hair significantly [4], (2) there are elements acting synergistically

and antagonistically [4,11], and (3) the pigmented body hair of cattle reflects the supply of Mn better than the crown hair, it still remained unknown in what period of time and to what extent the Mn passed into the hair. It appeared equally useful to compare the capability of the hair to reflect the supply of Mn with that of the blood and of the milk. In tests with calves, the Mn content of the blood bore no relationship to the Mn supply [5].

The present experiments were conducted, just as with the laying hen [7], using $^{52}MnSO_4$, $^{52}MnCl_2$, and $^{52}MnO_{1.12}$ (reduced MnO_2).

2. Material and Method

In the experiment, there were 15 Holstein cows composed of five animals each in three even groups with respect to age and milk production. Advance feeding of the cows with $MnSO_4$, $MnCl_2$, and $MnO_{1.12}$ covered a period of 11 days, using a winter ration consisting of alfalfa hay, beets*, feed rye silage, and concentrated feed.

At the beginning of the experiment, five cows each, at 15 minute intervals, were administered 150 mg ^{52}Mn in the form of $^{52}MnSO_4$, $^{52}MnCl_2$, and $^{52}MnO_{1.12}$, using a probang**. The ^{52}Mn was conveyed quantitatively with water into the rumen. The activity of the solution administered per cow amounted to 4.5 m Ci.

Samplings of black body hair, blood from the jugular vein, and milk were taken every 6, 12, 18, 24, 48, and 96 hours after administration of the ^{52}Mn preparation. Excrement and urine were removed immediately after excretion, in order to avoid contamination of the hide and udder. The

* Also "beetroot, turnips".
** Esophageal tube.

excrement remained active* from the 16th to the 240th hour after administration of the ^{52}Mn. No detectable activity** could be found in the urine by the Geiger counter.

The black body hair was cut using a machine clipper set at 1/10 mm. Cleaning of the hair was done with ether and water [2]. Activity in the ash was determined following dry incineration at 550°C. Milk and blood were analyzed similarly after drying at 105°C. Activity measurement was conducted using the same apparatus and the same methods as described in the experiments with laying hens [7].

3. Results of the investigations

3.1. Blood

The Mn content of the cattle blood is extremely low and varies between 4.6 and 8 μg per 100 ml total blood; this corresponds to an Mn concentration of less than 0.6 ppm dry substance [13, 10, 9]. On the basis of these low values and the experience gained from the laying hen tests [7], a high ^{52}Mn activity in the blood of the milchcows was not anticipated. Only by using very large samples (50 ml blood) was it at all possible to to obtain reasonably certain measurement results. In the case of the first sampling 6 hours following administration, it was nonetheless impossible to obtain satisfactory reproducible values.

As far as the ^{52}Mn activity of the blood is concerned, no significant differences ($P>0.05$) existed between the groups ($^{52}MnSO_4$, $^{52}MnCl_2$, and $^{52}MnO_{1.12}$). The values were combined for this reason (Table 1).

* Radioactive?
** Radioactivity?

Table 1

The ^{52}Mn activity of the blood in counts/min per gram dry substance.

Hours	12	18	24	48	96
$\bar{x}$	3.2	4.0	5.0	8.5	10.8
s	2.8	2.4	3.0	7.0	9.5

$\bar{x}$ = mean value
s = standard deviation

During the period from the 12th to the 96th hour after administration of the ^{52}Mn, the ^{52}Mn activity rose continuously. The maximum ^{52}Mn concentration was found 48 and 96 hours after administration ($P<0.05$, 0.01). Admittedly, individual variations are very large, as evidenced also in the high standard deviation.

3.2 Milk

The Mn content of the cattle milk exhibits about the same order of magnitude as in the blood; it varies between 20 and 130 ug per liter [8, 1, 14]. In spite of drying and incineration of large quantities of milk, it was not possible to demonstrate ^{52}Mn with certainty in any sampling, neither in the whole milk nor in the skim milk, or the cream.

3.3 Black body hair

The Mn content of cattle hair, which is dependent upon the Mn supply, kind of hair, age of hair, age of the animal, and the Zn and P supply, is subject to large variations. In more than 600 samples of the black body hair, in the mean of the individual sites, these variations lay between 2.9 and 26.3 ppm [6].

As early as six hours aften the ^{52}Mn dosages, ^{52}Mn could be demonstrated in all hair samples (Table 2).

Table 2

The ^{52}Mn activity of the black body hair in counts/min per gram dry substance.

Hours		6	12	18	24	48	96
$MnSO_4$	$\bar{x}$	9	15	17	56	33	42
	s	4	3	19	60	23	33
$MnCl_2$	$\bar{x}$	16	23	35	53	139	33
	s	9	17	14	31	134	26
$MnO_{1.12}$	$\bar{x}$	18	60	92	86	151	44
	s	9	64	41	16	78	23

$\bar{x}$ = mean value
s = standard deviation

Activity rose up to 24 hours after administration and at this time attained its maximum value in the cows of the $MnSO_4$ group, whereas this value was reached in animals of the $MnCl_2$ group and the $MnO_{1.12}$ group only after 48 hours.

There were no significant differences between the $MnSO_4$ group and the $MnCl_2$ group, whereas the difference between the $MnSO_4$ and $MnO_{1.12}$ group was significant ($P<0.001$, 0.05).

In the cows of the $MnCl_2$ group and the $MnO_{1.12}$ group, the ^{52}Mn activity of the black body hair rose continuously up to 18 hours following the ^{52}Mn dosage. After 96 hours, the ^{52}Mn activity of the hair of all groups had dropped to 33-44 counts/min per gram dry substance.

Thus, it can be demonstrated that the Mn of the $MnO_{1.12}$ and $MnCl_2$ is stored in the cutaneous appendages of the milchcow, like the broiler [12] and the laying hen [7], to at least the same extent as the Mn of the $MnSO_4$, which in these tests actually exhibited the lowest concentration in the hair 48 hours after dosage.

4. Discussion of the results

The results obtained demonstrate that the absorbed Mn is concentrated much more heavily in the hair than in the blood and the milk. The Mn content of the body fluids is in no way suited for testing for the level of supply with this element, a fact which, in the case of blood, was also discovered in experiments with stable Mn [5, 9, 10]. Only SAWHNEY and KEHAR [17] found differing Mn amounts in the blood corresponding to differing supplies of Mn. Their values, however, are double those of the literature, so that it is possible that in these findings problems of methodology could have exerted an in fluence. What has been said of the blood holds true to the same extent for the milk.

Contrastingly, in the mean of all samples up to 48 hours (or even longer), the ^{52}Mn activity of the hair rose almost continuously following ^{52}Mn dosaging; it then began to fall again gradually (Fig 1).

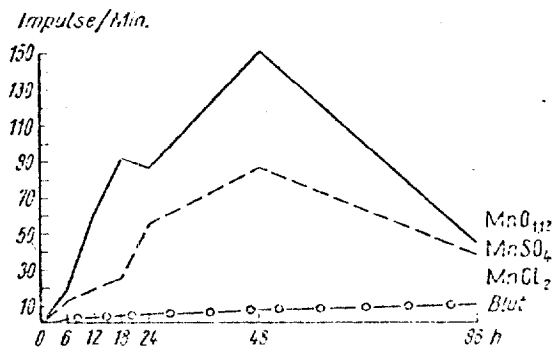

Fig. 1 - The ^{52}Mn activity of the body hair as a function of time.

Thus, in principle, the same picture emerges as was found in the case

of the plumage of the laying hen [7]. To be sure, in the case of cattle the rise in ^{52}Mn activity is less rapid. This could be connected with the conversions of the rumen contents by the rumen flora and fauna, which contribute to a slower absorption of the ^{52}Mn.

In any event, hair and plumage play an active role in the Mn metabolism of the body, and, as was demonstrated for plumage, serve as short-term Mn storage sites that later release Mn to the skeleton and ovary.

The Mn content of the cattle hair is in close relationship to the Mn supply via the fodder.

Summary

The present study has been conducted to investigate the incorporation and elimination of Mn in the hair, blood and milk of cows after the oral administration of $Mn^{52}SO_4$, $Mn^{52}Cl_2$ and $Mn^{52}O_{1,12}$.

1. No Mn^{52} was found in the milk; in blood the Mn^{52} values were found to increase continously ($P < 0.01$) until the end of the experiment i. e. 96 hour after the administration of Mn^{52}. No significant differences were observe between the different groups receiving $Mn^{52}SO_4$, $Mn^{52}Cl_2$ and $Mn^{52}O_{1,12}$. The Mn content of the milk and blood cannot be used as an indicator of the level of Mn supply.
2. Maximum values for the Mn^{52} activity in black body hair were 10–18 times higher than those in blood. For short periods Mn is stored in the hair. Hence the Mn content of hair may be used to serve as an indicator of the Mn supply of the animal.
3. No difference in the Mn^{52} content of black body hair was noted between the two groups receiving $MnSO_4$ or $MnCl_2$. The hair of cows receiving $MnSO_4$ or $MnCl_2$ was found to contain significantly less Mn^{52} than that of cows fed $MnO_{1,12}$($P < 0.011, 0.05$).
4. Both $MnSO_4$ and $MnCl_2$ or $MnO_{1,12}$ may be utilized as Mn source for animals.

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Der Mengen- und Spurenelementgehalt des Rinderhaares als Indikator der Calcium-, Magnesium-, Phosphor-, Kalium-, Natrium-, Eisen-, Zink-, Mangan-, Kupfer-, Molybdän- und Kobaltversorgung

6. Mitteilung

Der Ein- und Ausbau von oral verabreichtem Mangan-52 in das Haar, das Blut und die Milch des Rindes

1. Einleitung und Aufgabenstellung

In einer Reihe von Arbeiten [20, 4, 5, 6, 19, 18, 15, 16] wurde der Mn-Gehalt des Rinderhaares in Beziehung zur Mn-Versorgung dieser Tiere gebracht. Obwohl bewiesen werden konnte, daß zusätzliche Mn-Gaben, auch in Form von Salzen, den Mn-Anteil des pigmentierten Deckhaares signifikant erhöhen [4], daß es synergistisch und antagonistisch wirkende Elemente gibt [4, 11], und daß das pigmentierte Deckhaar des Rindes die Versorgung mit Mn besser widerspiegelt als das Schopfhaar [4], blieb unbekannt, in welcher Zeit und in welchem Umfang das Mn in das Haar einwandert. Im gleichen Maße erschien es nützlich, das Widerspiegelungsvermögen der Mn-Versorgung durch das Haar mit dem des Blutes und der Milch zu vergleichen. Bei Versuchen mit Kälbern stand der Mn-Gehalt des Blutes in keiner Beziehung zur Mn-Versorgung [5].

Die vorliegenden Versuche wurden ebenso wie beim Legehuhn [7] mit Mn-52-SO_4, Mn-52-Cl_2 und Mn-52-$O_{1,12}$ (reduziertes MnO_2) durchgeführt.

2. Material und Methode

Im Versuch standen 15 Kühe des Schwarzbunten Rindes in drei hinsichtlich Alter und Milchleistung ausgeglichenen Gruppen zu je fünf Tieren. Die Vorfütterung der Kühe mit $MnSO_4$, $MnCl_2$ und $MnO_{1,12}$ erstreckte sich bei einer Konzentration aus Luzernehen, Rüben, Futterroggensilage und Kraftfutter über den Zeitraum von 14 Tagen.

Zu Versuchsbeginn erhielten je fünf Kühe in Abständen von 15 Minuten 150 mg Mn-52 in Form von Mn-52-SO_4, Mn-52-Cl_2 und Mn-52-$O_{1,12}$ mit der Schlundsonde verabreicht. Das Mn-52 wurde mit Wasser quantitativ in den Pansen überführt. Die Aktivität der je Kuh eingegebenen Lösung betrug 4,5 mCi.

Die Probenahme bei schwarzem Deckhaar, Blut aus der Halsvene und Milch erfolgte jeweils 6, 12, 18, 24, 48 und 96 Stunden nach der Verabreichung des Mn-52-Präparates. Kot und Harn wurden sofort nach der Exkretion entfernt, um eine Verschmutzung des Felles und Euters zu vermeiden. Der Kot war von der 16. bis 240. Stunde nach der Mn-52-Gabe aktiv. Im Harn konnte keine mit dem Geiger-Müller-Zähler nachweisbare Aktivität gefunden werden.

Das schwarze Deckhaar wurde mit einer $^1/_{10}$ mm tief schneidenden Haarschneidemaschine abgeschnitten. Die Reinigung des Haares erfolgte mit Äther und Wasser [2]. Die Aktivität wurde nach trockener Veraschung bei 550 °C in der Asche ermittelt. Milch und Blut kamen nach der Trocknung bei 105 °C auf dem gleichen Wege zur Analyse. Die Aktivitätsmessung geschah mit denselben Geräten und nach den gleichen Methoden, wie sie bei den Versuchen mit Legehennen [7] beschrieben wurden.

3. Ergebnisse der Untersuchungen

3.1. Blut

Der Mn-Gehalt des Rinderblutes ist außerordentlich niedrig und schwankt zwischen 4,6 und 8 µg je 100 ml Gesamtblut, das entspricht einer Mn-Konzentration von weniger als 0,6 ppm in der Trockensubstanz [13, 10, 9]. Auf Grund dieser niedrigen Werte und der bei Legehennen gesammelten Erfahrungen [7] wurde keine hohe Mn-52-Aktivität im Blut der Milchkühe erwartet. Nur durch große Einwaagen (50 ml Blut) war es überhaupt möglich, einigermaßen sichere Meßergebnisse zu bekommen. Bei der ersten Probenahme sechs Stunden nach der Applikation konnten trotzdem keine hinreichend reproduzierbaren Werte erhalten werden.

In der Mn-52-Aktivität des Blutes bestanden zwischen den Gruppen (Mn-52-SO_4, Mn-52-Cl_2, Mn-52-$O_{1,12}$) keine signifikanten Unterschiede ($P > 0,05$). Die Werte wurden deshalb vereinigt (Tabelle 1).

Tabelle 1

Die Mn-52-Aktivität des Blutes in Impulsen/Minute je Gramm Trockensubstanz

Stunden	12	18	24	48	96
$\bar{x}$	3,2	4,0	5,0	8,5	10,8
s	2,8	2,4	3,0	7,0	9,5

$\bar{x}$ = Mittelwert s = Standardabweichung

In der Zeit von der 12. bis 96. Stunde nach der Verabreichung des Mn-52 stieg die Mn-52-Aktivität kontinuierlich an. Die höchste Mn-52-Konzentration wurde 48 und 96 Stunden nach der Verabreichung gefunden ($P < 0,05$, 0,01). Die individuellen Schwankungen sind allerdings sehr groß. Das drückt sich auch in der hohen Standardabweichung aus.

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3.2. Milch

Der Mn-Gehalt der Rindermilch liegt etwa in der gleichen Größenordnung wie beim Blut. Er schwankt zwischen 20 und 130 μg je Liter [8, 1, 14]. Trotz der Trocknung und Veraschung großer Milchmengen konnte bei keiner Probenahme, weder in der Vollmilch noch in der Magermilch oder Sahne Mn-52 sicher nachgewiesen werden.

3.3. Schwarzes Deckhaar

Der Mn-Gehalt des Rinderhaares ist in Abhängigkeit vom Mn-Angebot, der Haarart, dem Haaralter, dem Tieralter und der Zn- und P-Versorgung starken Schwankungen unterworfen [3], die bei mehr als 600 Proben des schwarzen Deckhaares im Mittel der einzelnen Standorte zwischen 2,9 und 26,3 ppm lagen [6].

Bereits sechs Stunden nach den Mn-52-Gaben konnte in allen Haarproben Mn-52 nachgewiesen werden (Tabelle 2).

Tabelle 2

Die Mn-52-Aktivität des schwarzen Deckhaares in Impulsen/Minute je Gramm Trockensubstanz

	Stunden	6	12	18	24	48	96
$MnSO_4$	$\bar{x}$	9	15	17	56	33	42
	s	4	3	19	60	23	33
$MnCl_2$	$\bar{x}$	16	23	35	53	139	33
	s	9	17	14	31	134	26
$MnO_{1,12}$	$\bar{x}$	18	60	92	86	151	44
	s	9	64	41	16	78	23

$\bar{x}$ Mittelwert, s Standardabweichung

Die Aktivität stieg bis 24 Stunden nach der Verabreichung an und erreichte zu dieser Zeit bei den Kühen der $MnSO_4$-Gruppe ihren Höchstwert, während dieser bei den Tieren der $MnCl_2$- und $MnO_{1,12}$-Gruppe erst nach 48 Stunden erzielt worden war.

Zwischen der $MnSO_4$- und $MnCl_2$-Gruppe bestanden keine signifikanten Unterschiede, während die Differenz zwischen der $MnSO_4$- und $MnO_{1,12}$-Gruppe und der $MnCl_2$- und $MnO_{1,12}$-Gruppe signifikant war ($P = 0{,}001 - 0{,}05$).

Die Mn-52-Aktivität des schwarzen Deckhaares erhöhte sich bei den Kühen der $MnCl_2$- und $MnO_{1,12}$-Gruppe bis 48 Stunden nach der Mn-52-Gabe kontinuierlich. Nach 96 Stunden hatte sich die Mn-52-Aktivität des Haares in allen Gruppen auf 33–44 Impulse/Minute und Gramm Trockenmasse verringert.

Es kann somit festgestellt werden, daß von der Milchkuh ähnlich wie vom Broiler [12] und der Legehenne [7], das Mn des $MnO_{1,12}$ und $MnCl_2$ zumindest im

gleichen Umfang in den Hautanhängen eingelagert wird, das des $MnSO_4$, welches in diesen Versuchen 48 Stunden nach der Gabe die geringste Konzentration im Haar aufwies.

4. Diskussion der Ergebnisse

Die erhaltenen Ergebnisse demonstrieren, daß sich das absorbierte Mn viel stärker im Haar als im Blut und in der Milch anreichert. Der Mn-Gehalt der Körperflüssigkeiten eignet sich keinesfalls zur Testung des Versorgungsgrades mit diesem Element, was beim Blut auch in Versuchen mit stabilem Mn festgestellt wurde [5, 9, 10]. Lediglich SAWHNEY und KEHAR [17] fanden bei unterschiedlicher Mn-Versorgung auch verschiedene Mn-Mengen im Blut. Ihre Werte liegen jedoch doppelt so hoch wie in der Literatur angegeben, so daß methodische Probleme bei diesen Erhebungen Einfluß genommen haben können. Für die Milch gilt das beim Blut Gesagte im gleichen Umfang.

Demgegenüber stieg die Mn-52-Aktivität des Haares im Mittel aller Proben bis 48 Stunden (oder auch noch länger) nach der Mn-52-Gabe nahezu kontinuierlich an, um dann wieder allmählich abzusinken (Abb. 1).

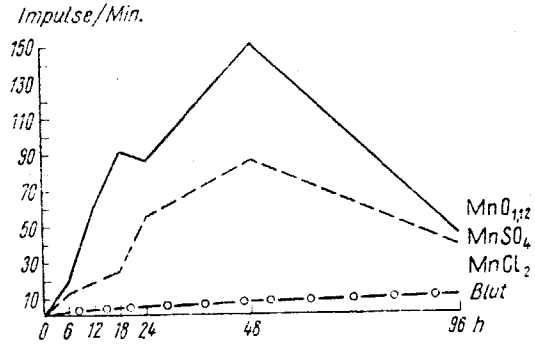

Abb. 1. Die Mn-52-Aktivität des Deckhaares in Abhängigkeit von der Zeit

Im Prinzip ergibt sich damit das gleiche Bild, wie es beim Gefieder der Legehennen gefunden wurde [7]. Allerdings ist beim Rind der Anstieg der Mn-52-Aktivität weniger rasch. Das dürfte mit den Umsetzungen des Pansen-Inhaltes durch die Pansenflora und -fauna in Verbindung stehen, die zu einer langsameren Absorption des Mn-52 beitragen.

Auf jeden Fall nehmen Haar und Gefieder aktiv am Mn-Stoffwechsel des Körpers teil und dienen, das wurde beim Gefieder bewiesen [7], kurzfristig als Mn-Speicher, die das Mn später an das Skelett und Ovar abgeben.

Der Mn-Anteil des Rinderhaares steht in enger Beziehung zur Mn-Versorgung über das Futter.

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Zusammenfassung

In der vorliegenden Arbeit wurde der Ein- und Ausbau des Mn in Haar, Blut und Milch nach oralen Gaben von Mn-52-SO_4, Mn-52-Cl_2 und Mn-52-$O_{1,12}$ bei der Kuh untersucht.

1. In der Milch konnte kein Mn-52 nachgewiesen werden, im Blut stiegen die Mn-52-Werte bis zum Versuchsende, 96 Stunden nach der Gabe, kontinuierlich an ($P < 0{,}01$). Es bestanden keine signifikanten Unterschiede zwischen den Gruppen, die Mn-52-SO_4, Mn-52-Cl_2 und $MnO_{1,12}$ erhielten.
   Der Mn-Gehalt von Milch und Blut ist als Indikator der Mn-Versorgung ungeeignet.
2. Die Mn-52-Aktivität des schwarzen Deckhaares war bei den Höchstwerten 10–18mal stärker als im Blut. Das Haar dient kurzfristig als Mn-Speicher. Der Mn-Gehalt des Haares eignet sich als Indikator der Mn-Versorgung.
3. Im Mn-52-Gehalt des schwarzen Deckhaares bestand zwischen der $MnSO_4$- und $MnCl_2$-Gruppe kein Unterschied. Die $MnSO_4$- und $MnCl_2$-Kühe besaßen signifikant weniger Mn-52 im Haar als die mit $MnO_{1,12}$ gefütterten ($P < 0{,}001$, $0{,}05$).
4. Sowohl $MnSO_4$ als auch $MnCl_2$ und $MnO_{1,12}$ eignen sich als Mn-Quelle für Nutztiere.

Резюме

В работе исследовался приток и отход Mn в волосах, крови и молоке коров после дачи им в рот Mn-52-SO_4, Mn-52-Cl_2 и Mn-52-$O_{1,12}$.

1. В молоке не было найдено наличия Mn-52, в крови же количество Mn-52 непрерывно повышалось до конца опыта, т. е. 96 часов после дачи ($P < 0{,}01$). Между группами, получавшими Mn-52-SO_4, Mn-52-Cl_2 и $MnO_{1,12}$, значительной разницы обнаружено не было.
   Содержание Mn в молоке и крови животных не может служить в качестве индикатора по обеспечению Mn.
2. Активность Mn-52 черных покровных волос была при максимальных величинах в 10–18 раз выше, нежели в крови. Таким образом, волосы на короткое время аккумулируют Mn. Содержание Mn в волосах является пригодным в качестве индикатора по обеспечению Mn.
3. В отношении содержания Mn-52 в черных покровных волосах по группам $MnSO_4$ и $MnCl_2$ не было обнаружено никакой разницы. У коров группы $MnSO_4$ и $MnCl_2$ содержание Mn-52 в волосах было значительно ниже, чем у группы, получавшей $MnO_{1,12}$ ($P < 0{,}001$, $0{,}05$).
4. Для составления смесей минеральных веществ являются пригодными в качестве источников Mn как $MnSO_4$, так и $MnCl_2$ и $MnO_{1,12}$.

Summary

The present study has been conducted to investigate the incorporation and elimination of Mn in the hair, blood and milk of cows after the oral administration of $Mn^{52}SO_4$, $Mn^{52}Cl_2$ and $Mn^{52}O_{1,12}$.

1. No Mn^{52} was found in the milk; in blood the Mn^{52} values were found to increase continously ($P < 0.01$) until the end of the experiment i. e. 96 hour after the administration of Mn^{52}. No significant differences were observed between the different groups receiving $Mn^{52}SO_4$, $Mn^{52}Cl_2$ and $Mn^{52}O_{1,12}$.
   The Mn content of the milk and blood cannot be used as an indicator of the level of Mn supply.
2. Maximum values for the Mn^{52} activity in black body hair were 10–18 times higher than those in blood. For short periods Mn is stored in the hair. Hence the Mn content of hair may be used to serve as an indicator of the Mn supply of the animal.
3. No difference in the Mn^{52} content of black body hair was noted between the two groups receiving $MnSO_4$ or $MnCl_2$. The hair of cows receiving $MnSO_4$ or $MnCl_2$ was found to contain significantly less Mn^{52} than that of cows fed $MnO_{1,12}$ ($P < 0.011$, 0.05).
4. Both $MnSO_4$ and $MnCl_2$ or $MnO_{1,12}$ may be utilized as Mn source for animals.

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stand 12 hours. Filter, and wash with hot water until free from chlorides. Break the point of the filter with a stirring rod and wash the precipitate into the beaker with hot water. Add 10 cc. of dilute sulfuric acid (1 + 1), heat nearly to boiling, and titrate with 0.1 *N* potassium permanganate solution. Finally add the filter to the solution and complete the titration. One cc. of 0.1 *N* potassium permanganate is equivalent to 0.0028 gram of calcium oxide (CaO). Report as percentage of calcium oxide (CaO).

7 MAGNESIUM[2].—TENTATIVE.

To the combined filtrate and washings from the calcium determination, **6**, add 25 cc. of strong nitric acid and evaporate to dryness. Heat on a hot plate or sand bath until the ammonium salts are decomposed. Take up with dilute hydrochloric acid (1 + 4) and make to a volume of about 100 cc. with water. Add 5 cc. of a 10 per cent sodium citrate solution and 10 cc. of a 10 per cent sodium hydrogen phosphate solution or enough to precipitate all the magnesium. Add dilute ammonium hydroxide (1 + 1), with constant stirring, until the solution is faintly alkaline; then add about 25 cc. of strong ammonium hydroxide and allow to stand in a cool place overnight. Filter and wash with dilute ammonium hydroxide solution (1 + 1). Dissolve the precipitate in dilute hydrochloric acid (1 + 4) and reprecipitate as before. Allow to stand several hours, filter, wash free of chlorides with the dilute ammonium hydroxide solution (1 + 9), ignite, and weigh as magnesium pyrophosphate ($Mg_2P_2O_7$). Calculate and report the result as percentage of magnesium oxide (MgO).

8 MANGANESE[4].—OFFICIAL.

To an aliquot of solution *A*, under **2**, representing about 0.5 gram of ash, add 15 cc. of concentrated sulfuric acid and evaporate to about 50 cc. Add 5-10 cc. of strong nitric acid and continue the evaporation. It is neither necessary nor advisable to evaporate until dense fumes appear, since the ferric sulfate then dissolves with difficulty. Nitric acid may be present, but not hydrochloric. Add water, a little at a time; heat until the iron salts have dissolved; and dilute to about 150 cc. Add 0.3 gram of potassium periodate, boil for a few minutes, and allow to cool.

The standard is prepared in the following manner: To a volume of water equal to the sample, add 15 cc. of concentrated sulfuric acid and sufficient pure ferric nitrate, free from manganese, to equal approximately the quantity of iron in the sample. Add a measured quantity of 0.1 *N* potassium permanganate solution, until the color is slightly darker than the sample, and then add 0.3 gram of potassium periodate and boil for a few minutes. When cool, transfer the sample and standard to 250 cc. flasks and dilute to the mark with water. If the color is weak it may be necessary to dilute to smaller volumes. Compare the colors in any standard colorimeter. Calculate and report the results as percentage of manganomanganic oxide (Mn_3O_4).

SODIUM AND POTASSIUM.

9 *Method I.—Official.*

Saturate 10 grams of the sample with concentrated sulfuric acid and ignite in a muffle at a low red heat to destroy organic matter. Heat the residue on a steam or water bath with 5-10 cc. of strong hydrochloric acid and about 50 cc. of water. Transfer to a beaker and add strong ammonium hydroxide, drop by drop, until the precipitate formed requires several seconds to dissolve, thus leaving the solution but faintly acid. Heat nearly to boiling, and add strong ammonium hydroxide to precipitate all the iron, aluminum, etc. Boil in a covered beaker for about 1 minute; remove; and if no

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faint pink (pH 5.0), and let stand at least 4 hr. Filter, and wash with H_2O at room temp. until filtrate is oxalate-free. Break point of filter with Pt wire, and wash ppt into beaker in which the Ca was pptd, using stream of hot H_2O. Add ca 10 ml H_2SO_4 (1+4), heat to ca 90°, add ca 50 ml hot H_2O, and titr. with 0.05*N* $KMnO_4$. Finally add filter paper to soln and complete titrn.

6.012 *Micro Method (6)*

Weigh 2 g sample into small crucible and ignite in muffle at 500–550°. Dissolve ash in HCl (1+4) and transfer to 100 ml beaker. Add 5 ml HCl and evap. to dryness on steam bath to dehydrate SiO_2. Moisten residue with 5 ml HCl, add ca 50 ml H_2O, heat few min. on steam bath, transfer to 100 ml vol. flask, cool quickly to room temp., dil. to vol., shake, and filter, discarding first portion of filtrate.

Pipet 15 ml aliquot into conical-tip centrifuge tube contg 2 ml satd $(NH_4)_2C_2O_4$ soln and 2 drops Me red, 2.042(i). Add 2 ml HOAc (1+4), rotating tube to mix contents thoroly. Add NH_4OH (1+4), while intermittently rotating tube, until soln is faintly alk.; then add few drops of the HOAc from dropper until color is faint pink (pH 5.0). (It is important at this point to rotate tube so that last bit of liquid in conical tip has required color.) Let stand at least 4 hr; then centrifuge 15 min. (Ppt should be in firm lump in tip of tube.) Remove supernatant, using suction device, Fig. 6.1, taking care not to disturb ppt. Wash ppt by adding 2 ml NH_4OH (1+49), rotating tube to break up ppt. (It may be necessary to jar tube sharply.) Centrifuge 10 min., again remove supernatant, and wash with reagent as before. Repeat this operation of washing ppt 3 times.

After last supernatant has been removed, add 2 ml H_2SO_4 (1+4) to tube, break up ppt as before, heat on steam bath to 80–90°, and titr. in tube with 0.02*N* $KMnO_4$, rotating liquid during titrn to attain proper end point. If tube cools to <60° during titrn, as indicated by slow reduction of $KMnO_4$, reheat in steam bath few min. and complete titrn. Perform blank on identical quantity H_2SO_4 in similar tube heated to same temp. to det. quantity of the $KMnO_4$ soln necessary to give color of end point. Subtract this value from buret reading. 1 ml 0.02*N* $KMnO_4$ = 0.000400 g Ca. Report as % Ca.

6.013 Magnesium (7)—Official, Final Action

To combined filtrate and washings from Ca detn, **6.011**, add 50 ml HNO_3 and evap. to dryness to decompose NH_4 salts. Take up with 5 ml HCl and dil. to ca 100 ml with H_2O. Add 5 ml *10% Na citrate soln* and 10 ml *10% $(NH_4)_2HPO_4$ soln*, or enough to ppt all the Mg. Add NH_4OH (1+4)

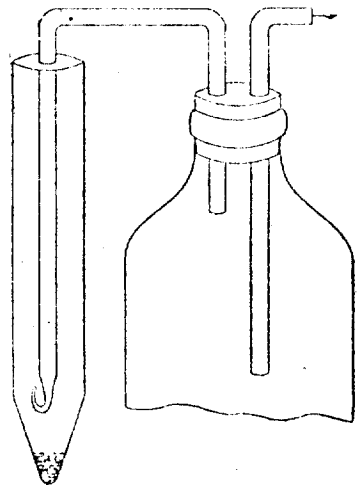

FIG. 6.1.—SUCTION DEVICE USED IN MICRO METHOD FOR DETERMINATION OF CALCIUM

with constant stirring (using policeman) until soln is faintly alk. and ppt forms; then add 25 ml NH_4OH, stir vigorously until ppt is granular, and keep in cool place overnight. Filter, and wash free from Cl with cold NH_4OH (1+10). Ignite in muffle at 500–550° until all C is oxidized, then at 900–950° ca 4 hr to form $Mg_2P_2O_7$. Cool, and weigh as $Mg_2P_2O_7$. (If sample is excessively high in Mn, dissolve ignited ppt in HNO_3, det. Mn as in **6.014**, and correct $Mg_2P_2O_7$ for $Mn_2P_2O_7$.) Report as % Mg.

6.014 Manganese (8)—Official, Final Action

To aliquot of Soln A, **6.005**, equiv. to 0.2–0.5 g ash, add 15 ml H_2SO_4 and evap. to ca 30 ml. Add 5–10 ml HNO_3 and continue evapn. (Do not evap. until dense fumes appear, because $Fe_2(SO_4)_3$ then dissolves with difficulty. HNO_3 may be present, but not HCl.) Add H_2O, little at time, heat until Fe salts dissolve, and dil. to ca 150 ml. Add 0.3 g *KIO_4*, or its equiv. in HIO_4, in small portions, boil few min. or until color of $KMnO_4$ shows no further increase in intensity, and let cool.

Prep. std as follows: To vol. H_2O equal to sample add 15 ml H_2SO_4 and enough pure $Fe(NO_3)_3$, free from Mn, to equal ca quantity of Fe in sample. Add measured quantity of 0.1*N* $KMnO_4$ until color is slightly darker than sample, then add 0.3 g KIO_4, and boil few min. When cool, transfer sample and std to 250 ml flasks and dil. to mark with H_2O. (If color is weak, it may be necessary to dil. to <250 ml.) Compare colors in colorimeter. (Photoelec. colorimeter with 525–550 mμ light filter may be used.) Report results as % Mn.

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Some Effects of Acute Manganese Excess in Rats.* (30351)

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The chronic effects of a high dietary manganese intake have been studied in a number of animals(1-14) and chronic manganese intoxication has been described in man(15-20). However, the effects of an acute manganese load on the organism have not been extensively investigated. To our knowledge only Paterni(21) has reported on this problem.

In the present study rats have been injected subcutaneously with various dose levels of manganese and observations of the effects on a number of physiological parameters made at various time periods.

Materials and methods. Albino Holtzman rats weighing 100 to 550 g were used throughout the study. Observations reported for any specific time period and or dose levels are compared to controls of equal weight, age and sex. Manganous chloride was diluted in normal saline to give dose levels ranging from 5 to 150 mg of manganese. Controls received an equal volume of normal saline only. At various time periods following subcutaneous administration of manganese, animals were anesthetized with ether and 4 ml blood samples taken from the inferior vena cava. All

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TABLE I. Alterations in Blood Hemogram and Chemistry Values 20 Hours Following Acute Manganese Administration (15 mg/100 g Body Weight).

	Controls	Mn injected	
Hgb	13.7 ± .9 g %	16.3 ± 1.1	p <.001
Hct	42.7 ± 2.8 %	53.9 ± 2.9	p <.001
RBC	6.97 ± 1.90 (×10⁶) cells/mm³	6.66 ± 1.99 (×10⁶)	
M.C.V.	65.0 ± 2.5 μ³	72.8 ± 3.2	p <.001
M.C.H.	20.5 μμg	22.6	
M.C.H.C.	32.0 g %	31.0	
Serum protein	5.65 g %	5.77	
Blood volume	22.0 ml	23.0	
Plasma volume	11.8 ml	11.5	
Serum Mg	2.36 ± .39 mg %	3.29 ± .60	p <.01
" Cl	104 ± 4 mEq/l	123 ± 6	p <.001
" P	2.64 ± .16 mg %	3.26 ± .24	p <.01
" Ca	4.53 ± .60 mEq/l	3.14 ± .38	p <.001
" Fe	3.62 ± 1.02 μg/ml	.81 ± .20	p <.001

samples were heparinized with the exception of those samples used for serum electrophoresis. All measurements were made using standard techniques except where otherwise noted. Blood and plasma volumes were determined using iodine[131] albumin dilution methods. Serum magnesium values were determined using the molybdivanadate method of Simonsen, Westover and Wertman(22). All animals were maintained on identical diets but were not pair fed.

Results. As shown in Table I, hemoglobin, hematocrit and mean corpuscular volume were significantly increased in animals receiving 15 mg/100 g body weight of manganese, as were serum chloride, phosphorus and magnesium. Serum calcium and iron were markedly depressed.

The peak increase in hematocrit and hemoglobin occurred between 12 and 18 hours (Fig. 1), and was maintained for several hours. Maximum response was induced by a dosage of 17 to 30 mg of manganese per 100 g of body weight and a measurable response was observed at levels of 5 mg per 100 g of body weight.

Examination of peripheral blood smears, following administration of 30 mg manganese per 100 g of body weight, revealed anisocytosis, basophilic stippling and hypochromia of red blood cells. Alterations in leucocytes were not observed. Necrotic changes were noted in hepatic tissue 18 hours following the injection of a single manganese dose of 17 mg per 100 g of body weight or more. As seen in Fig. 2, these changes are most pronounced in the periphery of the hepatic lobule. Microscopic examination revealed an apparent increase in liver and spleen iron content in several rats 48 hours following injection of a single dose of 30 mg manganese per 100 g of body weight. However, quantitative observations of iron content will be necessary to confirm this finding.

Rats weighing 140 ± 10 g maintained on an elevated dietary manganese intake (1% of manganese) failed to demonstrate an elevation of hematocrit or hemoglobin when compared to pair fed controls.

Discussion. The possibility of an hematinic action of manganese has been considered by several previous workers. Indeed, elevated hemoglobin values have been reported in

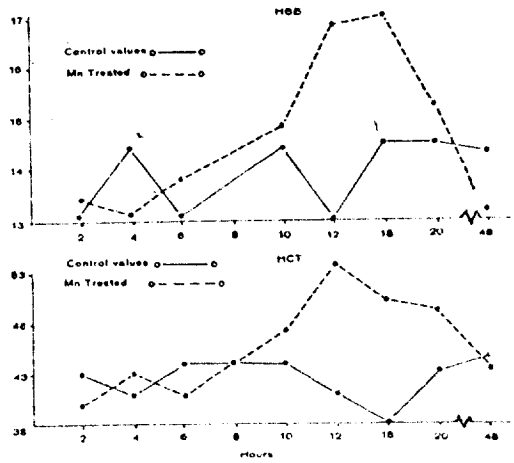

FIG. 1. Effect of acute administration of 50 mg of manganese on blood hemoglobin and hematocrit in 160 g rats. Each value represents the mean of at least 5 rats. These values are not included in Table I because of the difference in dosage of manganese.

workers exposed to fumes containing a high level of manganese(20). However, the elevated hemoglobin and hematocrit values found in the present study cannot be considered as evidence supporting the role of manganese in hematopoiesis. It is difficult, indeed, to consider that elevated manganese levels are capable of stimulating activity of bone mar-

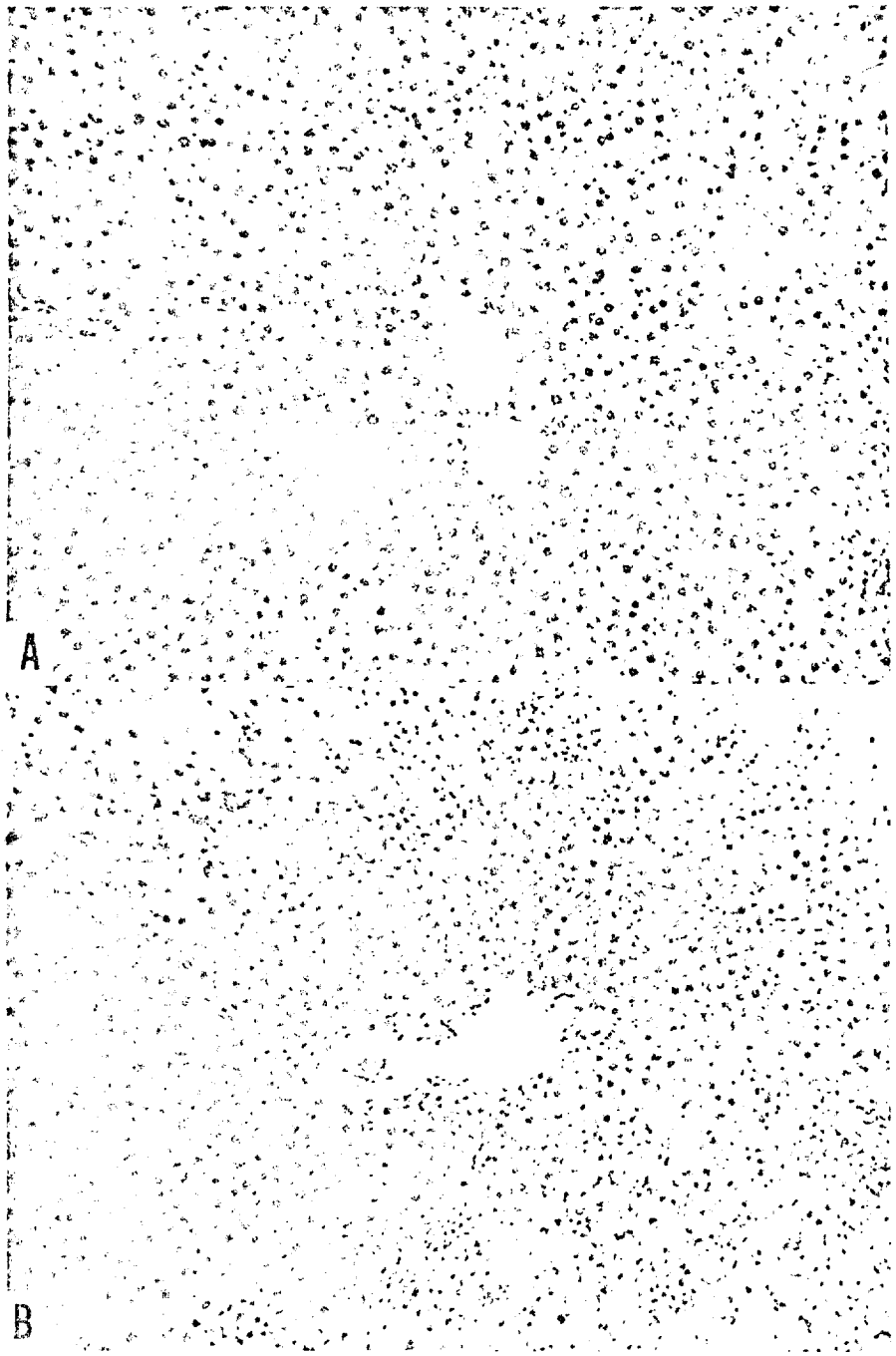

FIG. 2. Necrotic lesions in the liver of a manganese treated rat. A. Control. B. MN Treated.

row within the time period involved in our study. Lack of alteration in blood or plasma volume in manganese-treated animals, as well as the relatively unchanged serum protein values, virtually eliminates the possibility of fluid loss from the vascular space as being the primary cause of the elevation of hematocrit and hemoglobin. The possibility remains that a sudden release of erythrocytes from an extramedullary site, such as the spleen, is responsible for the erythrocytosis. In fact, preliminary studies have revealed an approximate 30% decrease in splenic weights in the manganese-treated group(23). Whether this finding is due to a primary effect of manganese or to a manganese-induced release of epinephrine is being studied.

In contrast to the elevated serum magnesium levels observed in our acute study, decreased serum magnesium values have been reported in sheep(7) and cows(4,8,9) chronically maintained on elevated dietary manganese intake. Both elevated and depressed magnesium levels are reported for rabbits maintained on high dietary manganese(6). It would then appear that the influence of manganese excess upon serum magnesium levels varies with the duration of effect, mode of manganese administration, or both. The elevated magnesium levels reported for the rabbit were observed at early stages in the course of intoxication. The relationship of manganese to magnesium in various *in vitro* and *in vivo* systems has been studied(24) but further studies will be necessary to determine the exact relationship of elevated manganese levels to magnesium metabolism.

The elevated serum phosphorus and depressed serum calcium observed may well be related to the same mechanism producing the hypermagnesemia. The interrelationship of these 3 elements and their role in animal metabolism has been well documented. Chornock (3) has shown that an excess oral intake of manganese resulted in increased fecal losses of calcium. However, it seems unlikely that subcutaneous manganese administration would have a similar effect. Conrad and Baxter have recently demonstrated a doubling of intracellular uptake of calcium^{45} in myocardium following subcutaneous administration of manganese(25). The increased calcium was found in the microsomal fraction of the myocardial cells. The rapid appearance of hypermagnesemia and hyperphosphatemia suggests that there is an outflux of these cations from soft tissue cells, bone or both. Whether this is a general catabolic effect of manganese excess cannot be determined from the present study.

Available knowledge of iron metabolism and its relationship to other metals of biological significance offers little basis for speculation concerning the mechanism responsible for the depressed values observed in this study. Somers and Shive(26) suggest that an excess of manganese may induce signs of iron deficiency in plants by oxidizing the available iron into the physiologically inactive ferric form.

Hartman and co-workers(7) found that high dietary intake of manganese in lambs interfered with iron absorption. Again, the rapidity of the effect in the present study using injected manganese makes an explanation based on a gastrointestinal absorption defect untenable. The demonstrable increase in hepatic and splenic iron content concurrent with the fall in serum values suggests the possibility of an intracellular shift of the metal. Further studies will be necessary to clarify the actual mechanisms involved.

Summary. Rats were injected subcutaneously with 5 to 120 mg of manganese as the chloride and various parameters were studied at periods from 1 to 72 hours. Blood hemoglobin, hematocrit and erythrocyte mean corpuscular volume increased within 10 hours after the injection, with a peak effect between 12 and 18 hours. Serum magnesium, chloride and phosphorus also increased but serum calcium and iron decreased. Blood volume was unchanged. Peripheral blood revealed anisocytosis, basophilic stippling and hypochromia of erythrocytes. Necrotic areas in the liver were observed within 18 hours and an apparent increase in iron content of liver and spleen was noted.

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CHANGES IN ELECTRICAL ACTIVITY AND REFLEX EXCITABILITY OF THE SPINAL CORD UNDER THE INFLUENCE OF MANGANESE

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UDC 612.831.014.423.4+612.833].014.46:546.711

Injection of manganese chloride (0.01-1 mg/kg) into the general circulation of anesthetized cats causes changes in the electrical activity of the spinal cord recorded from the center for the flexor muscles.

* * *

Significant morphological and functional changes arising in the central nervous system in manganese poisoning have been described in the literature [4, 5, 6]. Changes in excitability of peripheral and central nervous structures have been reported following administration of manganese compounds to animals in "biological concentrations" [7, 8, 11], and a definite correlation has been established between the course of the fundamental nervous processes and the variations in the content of manganese and other trace elements in the central nervous system [1, 2, 9, 10].

The object of this investigation was to study the effect of manganese on the electrical activity and reflex excitability of the spinal cord.

EXPERIMENTAL METHOD

Experiments were conducted on 52 cats anesthetized with chloralose (40 mg/kg) and Nembutal (15 mg/kg) injected intraperitoneally. To study the electrical activity of the spinal cord potentials were picked up from its dorsal surface by means of platinum needle electrodes and from deep inside the cord (center of the flexor muscles) by means of a focal microelectrode (Nichrome wire, 30 μ in diameter, with glass insulation). Mono- and polysynaptic responses were recorded from the central part of the divided deep peroneal nerve. Test stimulation, in the form of single square pulses of current, was applied to the central end of the divided dorsal root of L_6 from an electronic stimulator with radiofrequency output. An ac amplifier and CRO were used for recording.

Solutions of manganese chloride (0.0001-0.01 M) were injected into the femoral vein of the cats in doses of 0.01-1 mg of the pure metal per kg body weight [3], and were also applied locally to the place where the potentials were detected.

EXPERIMENTAL RESULTS

After intravenous injection of manganese chloride in a dose of 0.1-1 mg/kg, no visible changes in amplitude or frequency of the potentials recorded from the dorsal surface of the spinal cord were observed (Fig. 1).

When the microelectrode was buried into the gray matter of the ventral horn of the spinal cord to a depth of 2.0-2.8 mm, in the first 2-3 min after injection of manganese (0.1 mg/kg) the amplitude of the spikes increased appreciably from their background level (Fig. 1, A, B). By the 5th minute the initial amplitude of the potentials was restored (Fig. 1, C); starting with the 15th-20th minute the amplitude of the potentials again increased and remained high for 20-30 min (Fig. 1, D, E). By the 60th-90th minute the electrical activity had gradually returned to its initial background level (Fig. 1, F). The amplitude of the potentials recorded from the center for the flexor muscles was increased after administration of manganese in a dose of 1 mg/kg for the first 1-3 min, but later (after the 5th minute) it fell below the background level. By the 20th-30th minute the electrical activity had returned to its initial level.

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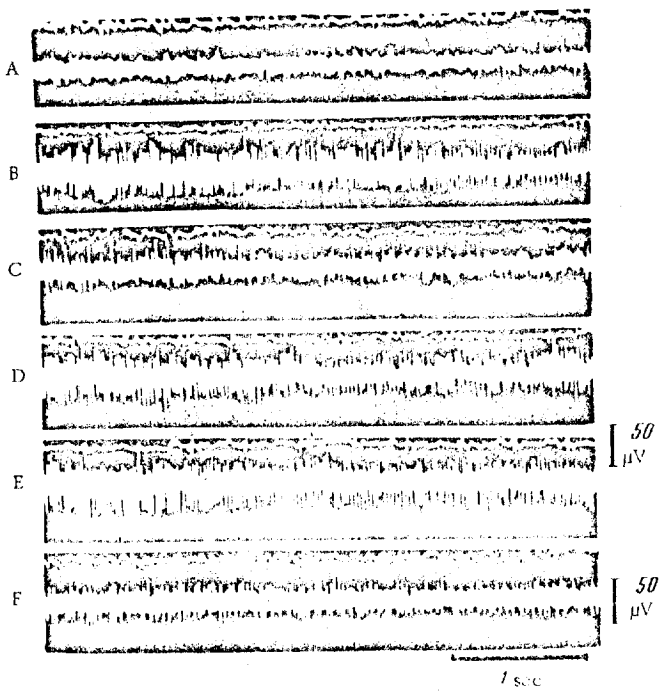

Fig. 1. Effect of manganese (0.1 mg/kg) on spontaneous electrical activity of the cat's spinal cord. A) Initial background; B-F) 30 sec and 5, 15, 30, and 60 min respectively after intravenous injection of manganese. Significance of curves (from top to bottom): potentials recorded from dorsal surface of spinal cord; potentials recorded from microelectrode buried in region of motor nuclei of tibialis muscle.

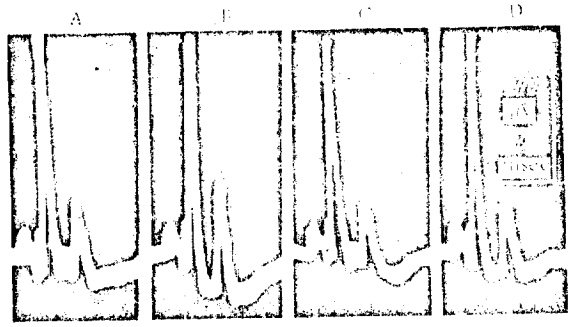

Fig. 3. Changes in mono- and polysynaptic reflex discharges after application of manganese chloride solution to dorsal surface of a cat's spinal cord. A) Initial background; B, C) 1 and 20 min respectively after application of manganese solution; D) 10 min after rinsing surface of spinal cord with physiological saline.

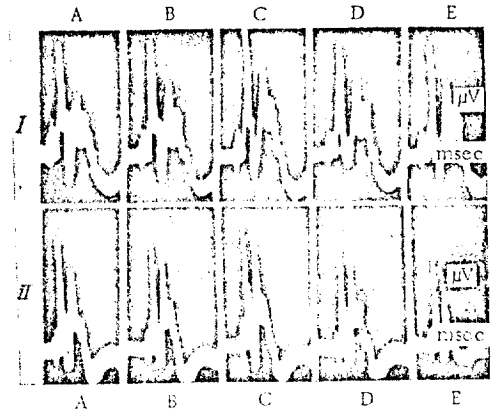

Fig. 2. Changes in mono- and polysynaptic discharges of the cat's spinal cord after injection of manganese (0.1-1 mg/kg). I) A: initial background B-E: 30 sec and 10, 30, and 60 min respectively after intravenous injection of manganese in a dose of 0.1 mg/kg. II) A: initial background; B-E: 30 sec and 10, 30, and 60 min respectively after intravenous injection of manganese in a dose of 1 mg/kg.

Experiments on spinal animals, in which the cord was preliminarily divided under deep ether anesthesia at the level T_1-T_2, showed that intravenous injection of manganese caused the same changes in electrical activity of the spinal cord as in animals with an intact nervous system.

After application of manganese chloride solution to the dorsal surface of the spinal cord (for a period of 30-45 min), in the first 2-3 min the amplitude of the potentials recorded from the flexor center increased by 30%. By the 5th minute the amplitude of the potentials had returned to its initial level, but after the 6th minute it increased again and remained high for 20-25 min. After the surface of the spinal cord had been washed with physiological saline, no appreciable changes in electrical activity were observed.

The state of the spinal centers after injection of magnesium chloride into the general circulation was judged from recordings of mono- and polysynaptic spinal cord reflexes.

Intravenous injection of manganese chloride in a dose of 0.01-0.1 mg/kg caused an initial increase in the amplitude of the monosynaptic reflex discharge (MRD) (Fig. 2, I, A, B, C). Later, starting with the 10th-20th minute, the amplitude of the MRD fell slightly and remained low until the end of the experiment (Fig. 2, I, D, E). The polysynaptic reflex discharges (PRD) increased after the 30th second (Fig. 2, I, A, B, C, D), but from the 40th minute until the end of the experiment they differed only slightly from the initial indices, or were a little higher (Fig. 2, I, E). After injection of manganese in a

dose of 1 mg/kg the amplitude of the MRD at first fell slightly (Fig. 2, II, A, B), but by the 10th minute they were higher than initially (Fig. 2, II, C). After the 30th-40th minute the amplitude of the MRD again fell, and by the end of the experimental it was 60-70% of its initial value (Fig. 2, II, D, E). Under these conditions the polysynaptic responses were usually increased after the 30th second (Fig. 2, II, A-C) but by the end of the experiment the amplitude of the PRD was usually lower than initially (Fig. 2, II, D, E).

Experiments on spinal animals showed that the MRD increased in amplitude in the first 30 min after injection of manganese, but after the 30th-45th minute they decreased slightly. The amplitude of the PRD also increased after the 30th second to reach a maximum by the 20th-30th minute. After the 30th-40th min the increase in PRD was replaced by a decrease in their amplitude (by 30-40%).

The study of the local action of magnesium chloride solutions on the dorsal surface of the spinal cord showed that the MRD underwent no appreciable changes throughout the period of action of manganese (30-45 min) or they were very slightly depressed (Fig. 3, A, B). After application of manganese solution the PRD were increased after the 1st minute (Fig. 3, B), returning to their initial level only by the 20th minute (Fig. 3, C). After the surface of the cord had been washed with physiological saline no visible change took place in the amplitude of the MRD and PRD (Fig. 3, D).

In a series of control experiments (intravenous injection of 0.9% sodium chloride solution) no appreciable changes were observed in the background electrical activity or in the mono- and polysynaptic reflex responses of the spinal cord.

The results obtained demonstrate that injection of manganese chloride into the general circulation of an animal in a dose of 0.01-0.1 mg/kg evokes an initial increase in the background electrical activity of the spinal cord, recorded from the center for the flexor muscles, and an increase in the amplitude of the mono- and polysynaptic reflex responses, followed by a return to their initial level. It might be considered that these changes are due to the facilitatory influence of suprasegmental portions of the central nervous system on the spinal cord. However, the results of experiments on spinal animals showed that injection of manganese under such conditions caused changes in the electrical responses identical with those obtained in animals with an intact nervous system. In addition, no significant differences were found in the direction of the changes in the electrical responses when manganese solution was applied to the dorsal surface of the spinal cord. The changes in the electrical responses of the spinal cord are evidently dependent principally on the direct influence of manganese salts on its functional state, and they are presumably associated with stimulation of oxido-reductive processes taking place after injection of manganese [12].

At the same time it is worth noting that the polysynaptic reflexes were modified (increased in amplitude) to a more marked degree than monosynaptic. This indicates that manganese ions have a greater influence on the interneurons than on the motoneurons of the spinal cord. Injection of large doses of manganese (1 mg/kg) causes, after the initial stimulation of electrical activity and reflex responses of the spinal cord, a fairly prolonged depression of these functions. In this case manganese may evidently give rise to functional changes in spinal cord activity of a parabiotic character.

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THE DETERMINATION OF MANGANESE IN BIOLOGICAL MATERIAL BY ACTIVATION ANALYSIS, WITH A NOTE ON THE GAMMA SPECTRUM OF BLOOD

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Abstract—A method is described for the determination of manganese in biological material using the technique of activation analysis. The limit of detection of the method is 0·1 mμg. Human blood contains about 2·4 μg of manganese per 100 ml, and the element is about equally distributed between cells and serum. Part of the γ spectrum of neutron-activated blood is described, and it is suggested that γ spectroscopy could be used for routine measurements of sodium and chlorine in blood.

INTRODUCTION

MANGANESE is an essential element in living matter, where it is probably an activator for several enzyme systems. Unfortunately, it is generally present in extremely small amounts, so that special techniques have to be used to determine it. Among these special techniques the more sensitive are modifications of the catalytic method of SZEBELLEDY and BARTFAI (1936) (KUN, 1947; FORE and MORTON, 1952a), which can detect 0·4–5 mμg, and the microbiological assay described by NICHOLAS (1952), which can be made sensitive to 0·2 mμg of the element. The full sensitivity of these methods can only be obtained by skilled workers, and they are unlikely to be improved greatly by future research.

Natural manganese consists wholly of Mn^{55}, which has a large cross-section for activation by thermal neutrons to 2·6-hour Mn^{56}. Activation analysis using this reaction has been described by CLARK and OVERMAN (1947) to determine microgram amounts of manganese in aluminium. The activation method described below can be used in a routine fashion to determine millimicrogram amounts of manganese, and with the higher neutron fluxes soon to be available at Harwell it will be possible to determine as little as 1 $\mu\mu$g of the element.

EXPERIMENTAL

As in all experimental work involving submicrogram quantities, most of the problems arise from contamination of the sample. The activation method is unique in that contamination, prior to activation only, is important, as subsequent operations will not introduce radioactive manganese. It was found that cellophane and polystyrene sheet on which blood samples might be collected contain 0·3 and 0·2 p.p.m. of manganese respectively, and fresh polythene tubes were found to transfer 2–3 mμg of manganese to their contents. Stainless-steel instruments, which may contain up to 2% of manganese, were never allowed to touch samples, nor were glassware and ordinary chemical reagents, though redistilled water does not produce serious contamination. Samples were generally collected in small weighed polythene tubes which had been thoroughly washed with nitric and hydrochloric acids, rinsed with redistilled water, and dried in a dust-free atmosphere. Even so, the tubes gave rise to a "blank" correction of 0·3 mμg of manganese per sample.

Blood samples of approximately 0·1 ml were obtained by pricking subjects with a sharp fragment of silica, after the skin near the proposed puncture had been thoroughly washed and dried. The

blood was allowed to drop into clean, weighed polythene tubes. If serum was required, the blood was allowed to clot and the tubes were centrifuged at high speed. The serum was withdrawn from the cells with a clean polythene pasteur pipette and sealed in a fresh tube. The tubes were reweighed, sealed, and packed, together with a standard of 2–10 μg spec. pure manganese in solution, in a polythene bag inside an aluminium can. The can was then irradiated for 2·5 hours in the Harwell pile BEPO in a flux of about 10^{12} thermal neutrons/cm^2/sec. After irradiation, the tubes were unpacked

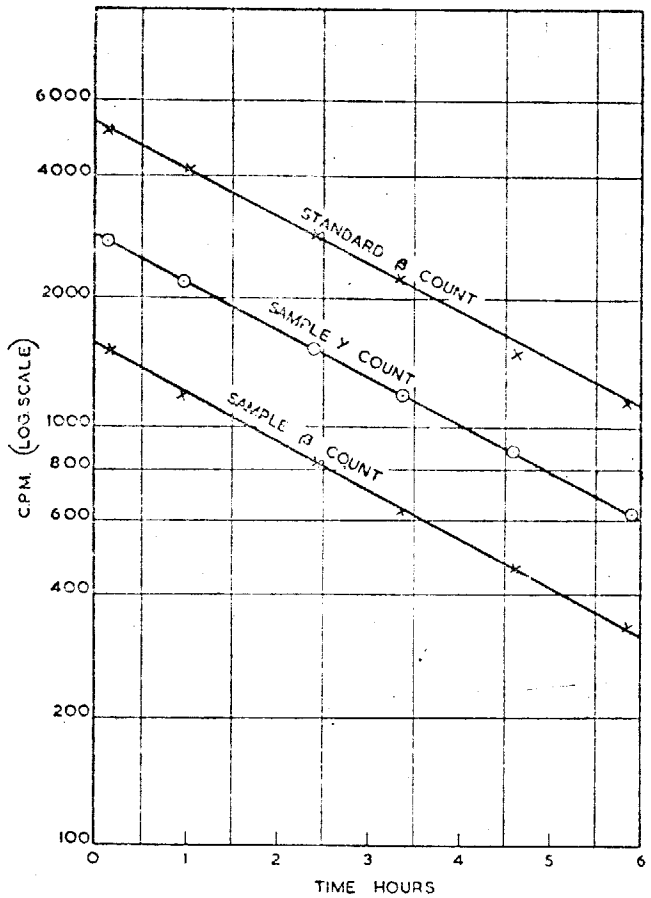

FIG. 1.—Decay curves for Mn^{56} from blood sample and spec. pure manganese nitrate standard.

and their outsides thoroughly washed with concentrated hydrochloric acid and very dilute manganese nitrate solution. They were then cut open and their contents dissolved in hot fuming nitric acid containing 50 mg manganese carrier and "hold back" carriers of bromide, chloride, cobalt, chromium, copper, iron, nickel, sodium, potassium, yttrium and zinc. This and all subsequent operations were carried out in a fume cupboard to avoid the hazard of breathing radioactive HCl and HBr evolved at any stage, since all precipitations were done in a boiling-water bath. Manganese was precipitated as the dioxide by adding concentrated sodium chlorate, taking care not to let the nitric acid concentration fall below 50%, and the precipitate was spun down, washed twice with water, and dissolved in acidified hydrogen peroxide. Excess peroxide was boiled out with the additions of a little ferric chloride, and the manganese precipitated with sodium carbonate solution. The precipitate was washed with water and dissolved in dilute hydrochloric acid; ferric chloride, ammonium phosphate and ammonium acetate were added, and ferric hydroxide was precipitated through the solution at

pH 7 with dilute ammonia. This ferric hydroxide scrub was repeated with the supernatant and washings from this stage, to remove all traces of phosphate, and the manganese was then precipitated with sodium carbonate to reduce the volume. The manganese carbonate was dissolved in 2 N nitric acid and manganese dioxide precipitated by adding saturated sodium bromate solution.

The dioxide was washed three times with water and once with acetone, and then slurried onto a weighed counting tray with acetone, dried under an infra red lamp and weighed to determine the chemical yield. The tray was then covered with scotch tape and counted with an E.H.M.2 end-window Geiger counter. The whole procedure took about one half-life (2·5 hours) for eight samples.

Losses of manganese and decontamination from sodium and phosphorus were tested, using Mn^{54}, Na^{24}, and P^{32}. The purity of the separated Mn^{56} was tested by plotting a decay curve (see Fig. 1) and by counting the sample after 24 hours to ensure that no long-lived activity was present.

During the course of this work it was found that Mn^{56} could be counted by Geiger or scintillation methods with about equal efficiency. Hence the γ spectrum of irradiated blood was examined in an attempt to find an Mn^{56} peak and so determine the element without chemical separation.

RESULTS

The elementary composition of blood is fairly well known, and it is a simple matter to calculate the activity to be expected from any individual element activated under known conditions. In Table 1 are listed the theoretical activities from 0·1 ml of whole blood after activation for 2 hours in a flux of 10^{12} neutrons/cm²/sec, assuming zero decay-time and 10% counting efficiency. Isotopes with half-lives less than 20 min are omitted, so that elements like magnesium and aluminium do not appear in the table.

It is quite clear from this table that the major interfering activities in blood come from sodium, potassium, chlorine, bromine, iodine, and phosphorus, and fortunately all these elements can be separated from manganese by simple chemical methods. The measured losses of manganese and decontamination from sodium and phosphorus are shown in Table 2.

Chemical yields were generally in the range 60–90%, and the recovery of added manganese was satisfactory, considering the levels used.

While the samples used here were not ashed, it was established by tracer techniques that no loss of manganese takes place on ashing biological material at 500°C. 98% of the manganese in a plant sample can be recovered by three extractions of the ash with 6 N nitric acid.

The following sources of error may be pointed out:

(1) The uncertainty in the blank correction for polythene tubes was $\pm 0{\cdot}1$ mμg.
(2) The accuracy of pipetting the standard was $< \pm 1\%$.
(3) The neutron flux was homogeneous to within $\pm 2\%$.
(4) The uncertainty in the chemical composition of the final manganese dioxide precipitate was estimated as $\pm 10\%$.
(5) It was established that under the conditions used, the count rate was independent of the weight of the final precipitate: statistical counting errors were less than $\pm 2\%$.
(6) Long-lived impurities in the separated Mn^{56} accounted for $<0{\cdot}3\%$ of the count.
(7) Production of Mn^{56} by reactions other than $Mn^{55}(n,\gamma)Mn^{56}$ may occasionally be important. The only reactions described in the literature are the fast-neutron reactions, $Fe^{56}(n,p)Mn^{56}$ and $Co^{59}(n,\alpha)Mn^{56}$. The cross-sections

for these reactions have not been accurately measured because of interfering manganese impurities in the iron and cobalt used, but they are unlikely to exceed 0·005 barns and are probably about 0·001 barns. The cross-section of the $Mn^{55}(n,\gamma)$ reaction is 12·6 barns. Hence these reactions will only interfere if iron or cobalt are present in large excess. In most biological materials iron, cobalt and manganese are microconstituents at about the same concentration. However, in blood and red blood cells, iron is an important constituent, and it is estimated that about 20% of the Mn^{56} actually measured was produced from this iron. This estimate can only be made roughly, because the fast-neutron flux in BEPO is not accurately known.

TABLE 1.—THE CALCULATED MAJOR ACTIVITIES FROM 0·1 ML OF WHOLE BLOOD IRRADIATED FOR 2 HOURS WITH 10^{12} NEUTRONS /CM²/ SEC

Element	Isotope	$T^{1/2}$	μg/ml	Authority	calc. c.p.m.
Na	24	15 h	1600	HAWK, OSER, and SUMMERSON (1947)	1,000,000
P	32	14 d	400	HAWK, OSER, and SUMMERSON (1947)	3,200
S	35	87 d	4000	HAWK, OSER, and SUMMERSON (1947)	400
Cl	38	37 m	3000	HAWK, OSER, and SUMMERSON (1947)	4,000,000
K	42	12 h	2000	HAWK, OSER, and SUMMERSON (1947)	160,000
Ca	45	150 d	50	HAWK, OSER, and SUMMERSON (1947)	2
Cr	53	2 h	0·001	URONE and ANDERS (1950)	2
Mn	56	2·6 h	0.024	BOWEN (this work)	800
Fe	59	45 d	500	HAWK, OSER, and SUMMERSON (1947)	100
Co	60	5·2y	0·0002	THERS, WILLIAMS, and YOE (1955)	0·002
Ni	65	2·6 h	<0·8	SEITCH (pers. comm.)	<50
Cu	64	13 h	1·1	KEFOE, CHOLAK, and STORY (1940)	200
Zn	69	14 h	7	HOCH and VALLEE (1949)	60
As	76	1·1 d	0·05	KESOSLEY and SCHAEFERF (1951)	50
Br	80	4·6 h	13	WINSLOW and SMITH (1937)	20,000
Rb	86	19 d	~24	estimated	~200
Sr	87	2·8 h	0·03	HARRISON, RAYMOND, and TRETHEWAY (1955)	6
Mo	93	6·7 h	1·0	DICK and BINGLEY (1951)	2
Sn	121	1·1 d	0·12	KEFOE, CHOLAK, and STORY (1940)	0·2
I	128	25 m	0·15	HAWK, OSER, and SUMMERSON (1947)	2400
Cs	134	3·1 h	~0·5	estimated	~1300
Pb	209	3·3 h	0·4	CHOLAK and STORY (1938)	0·05
U	239	24 m	0·015	NEUMAN (1949)	60

TABLE 2.—LOSSES IN THE CHEMICAL STEPS

Chemical step	% Mn lost	% Na left	% P left
MnO_2 pptation with $NaClO_3$	4·7	0·1	76
$MnCO_3$ pptation	0·1	0·7	40
Two $Fe(OH)_3$ Scrubs	10·0	100	0·025
$MnCO_3$ pptation	0·1	0·7	40
MnO_2 pptation with $NaBrO_3$	2·8	0·1	7·4
Total	17·7	5×10^{-9}	0·0022

The main limitation in accuracy is the error in the final weighing of manganese as the dioxide, which is known to be a nonstoichiometric substance with a large adsorbent capacity. The last traces of sodium bromate could never be washed out from the dioxide, for example. However, a simple stoichiometric derivative of manganese,

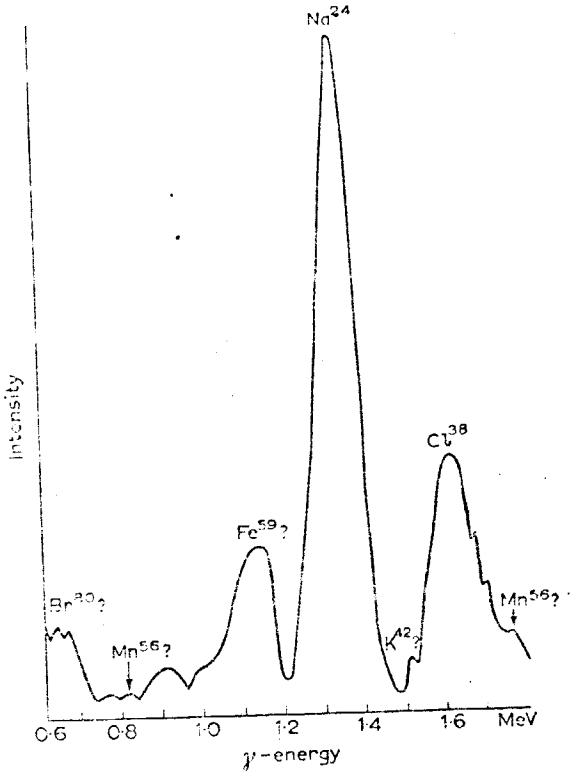

FIG. 2.—Part of the γ spectrum of blood irradiated with pile neutrons. The large peaks caused by Na^{24} and Cl^{38} have roughly the calculated intensity, but other features are more difficult to assign to any particular isotope, though tentative assignments are given. The third largest peak is too large to be caused entirely by Fe^{59}, and may be a secondary peak from a more energetic Na^{24} gamma.

which can be precipitated and dried rapidly (without ignition in a muffle furnace), does not seem to be known. In view of this limitation, it is considered that the overall accuracy of the method is no better than ±10%.

TABLE 3.—RECOVERY OF MANGANESE ADDED TO 0·1 ML OF BLOOD

μg Mn added	μg Mn found	% recovery
0·02	0·020	100
0·05	0·056	112
0·2	0·211	106
0·5	0·535	107
1·0	[illegible]	94

The main analytical results obtained by the activation method are summarized in Table 4. In this table the whole-blood and blood-cell results have not been corrected for the $Fe^{59}(n,p)$ reaction described above.

TABLE 4.—MANGANESE CONTENT OF BLOOD AND HAIR

Material	Obtained from	Mean Mn content
Whole blood	3 rabbits	3·90 ± 0·80 μg/100 ml
Whole blood	2 guinea pigs	6·50 ± 2·50 μg/100 ml
Whole blood	2 rats	2·50 ± 0·80 μg/100 ml
Whole blood	7 men	2·45 ± 0·91 μg/100 ml
Whole blood	11 women	2·40 ± 0·78 μg/100 ml
Blood cells	2 humans	1·06 ± 0·40 μg/100 ml whole blood
Blood serum	2 humans	0·97 ± 0·40 μg/100 ml whole blood
Hair	3 humans	1·56 ± 0.10 μg/g

Part of the γ spectrum of activated whole blood is shown in Fig. 2. The source was scanned with the double-crystal scintillation spectrometer described by PEIRSON (1955), and the range of energies investigated was from 0·6 to 1·9 MeV. Only the Na^{24} and Cl^{38} peaks at 1·38 and 1·60 MeV respectively show up clearly, but small bumps at about 1·81 and 0·82 MeV might have been caused by gamma rays from Mn^{56} with energies of 1·77 and 0·82 MeV. It is clear that sodium and chlorine could be determined very easily in blood samples of 0·1 ml or less by this technique, but for other elements some kind of chemical separation is required.

DISCUSSION

The activation method here described could be applied with minor modifications to a very wide range of materials, though in many applications it might prove too sensitive. The full sensitivity of the method has not been approached, since it is largely determined by the neutron flux available. Thus, if a neutron flux of 10^{14} n/cm²/sec were used, the limit of detection for manganese would be about .1 μμg, or even less if low background-counting techniques were employed.

Fig. 2 shows that γ spectroscopy might well be used as a routine procedure to determine sodium and chlorine in 0·1-ml samples of irradiated whole blood. If larger amounts of blood were used, it is probable that these elements and potassium, bromine, iodine and manganese could also be determined simultaneously. The procedure is simple and could easily be learned by unskilled workers, and it is also relatively rapid. The main drawbacks to its use are the initial expense of the γ spectrometer and the necessary proximity to a nuclear reactor.

The results for the concentration of manganese in whole blood are somewhat lower than those reported by most of the earlier workers (BERTRAND and MEDIGRECIANU, 1912; REMAN and MINOT, 1920; KEHOE, CHOLAK, and STORY, 1940; BENTLEY, SNELL, and PHILLIPS, 1947), although they were obtained with much smaller samples which are more likely to be contaminated. The spectrometric results of WOLFF (1948) appear to be two orders of magnitude lower than any previously reported work, unless the units given are misrepresented.

Manganese is present in extremely minute traces in blood compared with its concentration in other organs. The fact that serum and cells contain roughly equal amounts suggests that it may be a naturally occurring impurity, though its concentration is remarkably similar in blood from different individuals. The element is known to be removed exceedingly rapidly from the bloodstream. Thus, HAMILTON, SCOTT, and CROWLEY (1950) have shown that less than 0·08% of a dose of labelled manganese was present in rat blood five hours after intravenous injection, and GREENBERG and CAMPBELL (1940) have given comparable figures for retention after oral administration. MAYNARD (1953) has found by tracer methods that manganese is chiefly associated with the mitochondrial fraction of liver cells, and since blood corpuscles contain very few mitochondria, one would not expect them to concentrate the element.

The values for manganese in human hair may be largely surface contamination, though they agree with the results of FORE and MORTON (1952b) for rabbit hair.

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An Improved Formaldoxime Method for the Determination of Manganese in Plant Material

Analyst 82: 254-59, 1957

By E. G. BRADFIELD

A method is described for the determination of manganese in plant material in which no preliminary separation of interfering elements is needed. Interference from iron and copper is eliminated by warming the solution, which results in decomposition of the formaldoxime complexes of these two metals, and precipitation of metal phosphates is prevented by using the sequestering agent N-hydroxyethylethylenediaminetriacetic acid. The method is as rapid as the normal periodate procedure and its sensitivity is about 5 times as great. The coefficient of variation is 1·2 per cent. for high concentrations of manganese and 2·3 per cent. for lower concentrations.

The manganese content of plant material is usually determined colorimetrically by oxidation with potassium periodate to form permanganic acid.[1] Compared with most colorimetric methods the sensitivity is poor and, when used for material of low manganese content, a sample weight of 5 to 10 g is required. This weight of sample may not be available if the amount of material is limited.

Formaldoxime, which forms an orange-red colour with manganese in alkaline solutions (Mellan[2]), has been used for the determination of manganese in plant material by Sideris[3,4] and in textiles by Hamlin.[5] Both authors note interference by phosphates, which are precipitated as alkaline-earth phosphates when the solution is made alkaline, and by iron, which forms an intense violet-red colour with formaldoxime. Sideris[4] recommends the precipitation of iron as ferric phosphate and phosphates as lead phosphate in acetic acid solution, while Hamlin[5] removes iron by extracting its cupferron complex with methylene dichloride and prevents precipitation of alkaline-earth phosphates by the addition of ammonium salts and sodium pyrophosphate. Both procedures involve prior separation of iron and this increases the time required for the determination.

Experimental

Elimination of interference by iron—

Sideris[3] has previously shown that interference by iron can be avoided by the formation of a complex with sodium cyanide. It is, however, necessary to compensate for the greenish-

yellow colour of the cyanide complex by adding iron to the standards in amounts roughly equivalent to the content of the test solutions, a difficult procedure when the iron content of the test materials is variable. A search was therefore made for alternative agents that would selectively complex iron and prevent its reaction with formaldoxime.

Five sequestering agents (*i*) ethylenediaminetetra-acetic acid (EDTA); (*ii*) N-hydroxyethylethylenediaminetriacetic acid (HEEDTA), (*iii*) NN'-dihydroxyethylethylenediaminediacetic acid (HEEDDA), (*iv*) diethylenetriaminepenta-acetic acid (DTPA) and (*v*) 1:2-diamino*cyclo*hexanetetra-acetic acid (CDTA) were tried. Of these, only CDTA prevented iron from forming a coloured complex with formaldoxime; unfortunately, it also prevented the formation of the manganese complex. During these experiments, it was noticed that the iron - formaldoxime complex decomposed on prolonged standing, ferric hydroxide being precipitated from solutions containing EDTA and DTPA, but not from solutions containing HEEDTA and HEEDDA. It was found that the rate of decomposition was increased by warming; the reaction was therefore investigated quantitatively.

Portions of solutions of manganese and iron containing 40 μg and 300 μg, respectively, were placed in six 50-ml calibrated flasks and diluted to about 30 ml with water. To each, 5 ml of a 10 per cent. w/v solution of HEEDTA, 1 ml of diluted formaldoxime reagent (prepared from hydroxylamine sulphate and paraformaldehyde) and 2 ml of a 10 per cent. w/v solution of sodium hydroxide were added. The flasks were placed in a water bath at 65° C for 0, 10, 20, 30, 60, 120 and 240 minutes and then removed and cooled; the contents of each were diluted to the mark and the absorptions of the solutions were measured. The experiment was repeated with, in one series, 40 μg of manganese and no iron; in another 300 μg of iron and no manganese; and in a third no manganese or iron. The results are

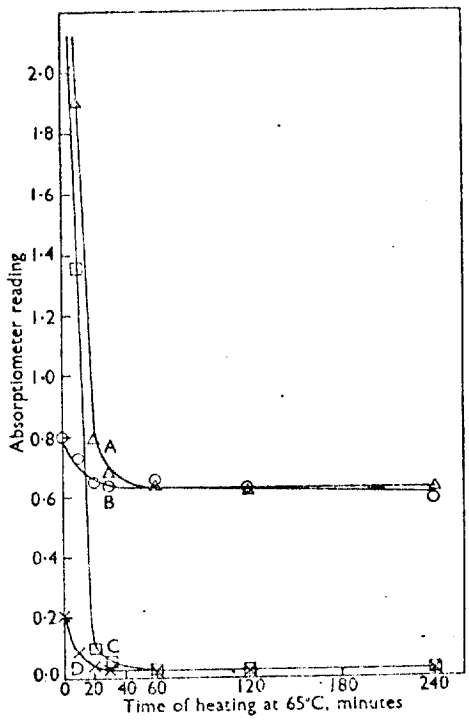

Fig. 1. Effect of heating iron and manganese with formaldoxime reaction mixture at 65° C: curve A, 40 μg of manganese + 300 μg of iron; curve B, 40 μg of manganese; curve C, 300 μg of iron; curve D, blank

shown in Fig. 1. It will be seen that, although the iron - formaldoxime complex is completely decomposed after 1 hour, the colour due to the manganese complex, after an initial

fall in intensity, is stable over the period of 1 to 3 hours. This initial fall is probably due to contamination of the reagents by iron, since the blank has a high initial absorption, which subsequently decreases on warming. The initial colour of the blank was pink, similar to that of the iron - formaldoxime complex. It is therefore possible to overcome interference by iron by warming the reaction mixture for 2 hours at 65° C before measuring the absorption.

EFFECT OF HEAT ON INTERFERING IONS—

In view of these results, the effect of warming the formaldoxime complexes of other interfering metal ions was investigated, the results being as follows—

Copper—Copper reacts with formaldoxime to give a violet coloured solution; interference can be overcome by addition of cyanide.[5] When a solution containing 100 μg of copper was warmed, the formaldoxime complex was rapidly decomposed and the solution became colourless.

Nickel—The golden brown solution produced by 100 μg of nickel with formaldoxime decomposed slowly on warming. After 2 hours at 65° C the absorption was equivalent to that of 2 μg of manganese.

Cobalt—The pale yellow-green colour of 100 μg of cobalt with formaldoxime slowly faded on warming: after 2 hours the absorption was equivalent to that of 2 μg of manganese.

Other elements—There was no colour with 100-μg amounts of zinc, cadmium, chromium, lead, molybdenum or titanium or 500 μg of aluminium, and consequently there was no interference in the determination of manganese.

EFFECT OF SEQUESTERING AGENTS ON THE PRECIPITATION OF ALKALINE-EARTH PHOSPHATES—

In plant material, calcium and magnesium are the predominant elements that form insoluble phosphates in alkaline solution and precipitation of these can be prevented by adding sufficient of a suitable chelating agent to sequester the metal ions. Experiments showed HEEDTA to be the most effective agent; 5 ml of a 10 per cent. w/v solution were adequate for the types of plant material analysed.

METHOD

REAGENTS—

HEEDTA solution—Dissolve 10 g of trisodium N-hydroxyethylethylenediaminetriacetate in water and dilute to 100 ml.

Formaldoxime reagent—Dissolve 20 g of paraformaldehyde and 55 g of hydroxylamine sulphate in boiling water and dilute to 100 ml. For use, dilute this solution 10 times.

Sodium hydroxide solution—Dissolve 10 g of analytical-reagent grade sodium hydroxide in water and dilute to 100 ml.

PROCEDURE—

Weigh 1 g of plant material, previously ground in a mortar and dried at 105° C, into a 250-ml tall Pyrex-glass beaker. Add 25 ml of concentrated nitric acid, cover with a watch-glass, and digest at low heat until no solid material remains. Then add 2·5 ml of 60 per cent. perchloric acid and continue digestion until fumes of perchloric acid are given off. If at this stage the digest is still coloured, add a further 5 ml of nitric acid and again heat until fumes of perchloric acid are given off. Uncover the beaker slightly and continue heating until nearly all the perchloric acid has been removed. Cool, add 25 ml of water, boil and filter the solution through a Whatman No. 540 filter-paper into a 100-ml Pyrex-glass calibrated flask. When cool, dilute to volume.

By pipette place an aliquot, containing 10 to 50 μg of manganese, in a 50-ml Pyrex-glass calibrated flask, dilute to about 30 ml and add 5 ml of HEEDTA solution. Add sodium hydroxide solution dropwise until any free perchloric acid is neutralised, using a wide-range pH paper as external indicator. Then add 1 ml of diluted formaldoxime reagent, followed immediately by 2 ml of sodium hydroxide solution. It is necessary to add the sodium hydroxide solution within 2 minutes of adding the reagent, since formaldoxime decomposes rapidly in dilute solutions. Remove the stopper from the flask and place it in a water bath at 65° C for 2 hours. At the end of this time remove the flask from the bath, cool the contents and dilute to 50 ml.

Measure the absorption of the solution at a wavelength of 450 mμ on a Unicam SP600 spectrophotometer, using 4-cm cells, and calculate the manganese content from a calibration graph, prepared by plotting absorption readings against the manganese contents of solutions containing 0 to 50 μg of manganese, added as a standard solution of manganese sulphate. Develop the colour of the standards in exactly the same manner as that of the samples.

Determine the blank by carrying out this procedure on the reagents only.

Results

The recovery of manganese was tested by the addition of 20 μg and 40 μg of manganese to 10-ml aliquots of a solution containing potassium, calcium, phosphate, magnesium, iron, copper, boron and zinc in such amounts that these aliquots were roughly equivalent in mineral content to an acid digest of 1·0 g of dried plant material. The results of triplicate determinations gave a mean recovery of 20·6 μg of manganese from a recovery range of 20·4 to 20·7 μg when 20 μg had been added to the aliquot. For the addition of 40 μg of manganese the mean recovery was 40·2 μg and the recovery range 39·8 to 40·6 μg.

The precision of the method was determined by carrying out a series of 10 determinations of manganese on a sample of apple leaves that had a low manganese content and a similar series on a sample of blackcurrant leaves that had a fairly high manganese content. For the apple leaves a mean content of 20·7 p.p.m. of manganese was found with a range of 20·2 to 21·6 p.p.m., the standard deviation being 0·5 and the coefficient of variation 2·3 per cent. For blackcurrant leaves the mean content was 132·1 p.p.m., with a range of 130·4 to 135·2 p.p.m., the standard deviation being 1·6 and the coefficient of variation 1·2 per cent. It will be seen that the coefficient of variation is low.

The manganese contents of a number of plant materials were then determined by the method described and compared with the values obtained by the periodate oxidation method given by Piper.[1] It will be seen from Table I, in which each result is the mean of three determinations, that there is good agreement between the two methods. For the determination of the small amounts of manganese present in apple and cauliflower leaves, only 1 g of plant material was required for the formaldoxime method whereas, for comparable accuracy, 5 g were required for the periodate method.

Table I

Manganese content of plant materials by formaldoxime and periodate methods

Material	Manganese found by formaldoxime method, p.p.m.	Manganese found by periodate method, p.p.m.
Blackcurrant leaves	129	130
Blackcurrant leaves	104	107
Apple leaves	21	20
Cauliflower leaves	19	18
Kale leaves	39	38
Wheat straw	49	51
Pasture herbage	106	109
Broccoli leaves	56	55
Strawberry leaves	325	318
Cacao leaves	564	582

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3. Sideris, C. P., *Ind. Eng. Chem., Anal. Ed.*, 1937, **9**, 445.
4. ----, *Ibid.*, 1940, **12**, 307.
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ch. Pathol. 10(6): 859-868, 1930

EXPERIMENTAL SUBACUTE AMYLOID NEPHROSIS IN RABBITS*

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In the course of studies on chronic metal poisoning, I noted a curious and interesting lesion in the kidneys of rabbits receiving manganese chloride subcutaneously. The lesion consisted of a marked subacute nephrosis involving the tubules and glomeruli, accompanied by the deposition of an amyloid-like substance in the glomerular tufts and about the tubular capillaries.

To my knowledge such an observation has not been recorded as a result of metal poisoning, and it was thought to be of sufficient interest to be made the subject of a report.

Manganese poisoning in man produces a peculiar combination of degenerative lesions in the basal ganglions of the brain and cirrhosis of the liver, a condition not unlike that of hepatolenticular degeneration as described by Wilson [1] and others. Mainly because of this fact, much experimental work has been done with manganese.

It is of interest to recall that Mella,[2] by injecting manganese into monkeys, reproduced the lesions described as characteristic of manganese poisoning in man. Later Findlay [3] reported cirrhotic changes in the livers of rabbits, guinea-pigs and rats poisoned with manganese.

Hurst and Hurst [4] summarized the literature and verified Findlay's observations, producing fibrous changes in the livers of guinea-pigs and rabbits with subcutaneous injections of manganese chloride. However, they were unable to demonstrate changes in the central nervous systems of their animals.

Primarily, my work was undertaken with the idea of disproving or substantiating the possibility that chronic manganese poisoning in rabbits gives rise to a similar deposition of pigment in the livers, as was found by Hall and myself [5] in our experiments confirmatory of Mallory's work with copper.

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* From the Department of Pathology, Stanford University Medical School.

1. Wilson: Brain **34**:290, 1911.
2. Mella: Experimental Production of Basal Ganglion Symptomatology in Macacus Rhesus, Arch. Neurol. & Psychiat. **11**:405, 1924.
3. Findlay: Brit. J. Exper. Path. **5**:92, 1924.
4. Hurst and Hurst: J. Path. & Bact. **31**:303, 1928.
5. Hall and Butt: Experimental Pigment Cirrhosis Due to Copper Poisoning: Its Relation to Hemochromatosis, Arch. Path. **6**:1, 1928.

It is perhaps not irrelevant to point out that this investigation acts as a control on our former work with copper. Furthermore, it must be stated that in our studies with both copper and manganese, carrots were purposely omitted from the diet.

Much work has been done on the production of amyloid disease. No attempt will be made to summarize the voluminous literature on the subject. In passing, mention will be made of the more recent work of Kuczynski,[6] further elaborated by Smetana,[7] in regard to the production of amyloid disease in mice by injections of nutrose. More recently Yokoi [8] reported that with intravenous injections of defibrinated blood, serum, or natrium silicate, amyloid disease may be produced in these animals. Letterer [9] has made a comprehensive study of the experimental development of amyloid in mice, producing the disease with injections of proteins, colloidal sulphur or colloidal selenium.

EXPERIMENTAL PROCEDURE

Young, healthy rabbits of about the same age, 6 months, were selected for the experiment. The animals were kept in separate cages and maintained on a diet consisting of barley, alfalfa and lettuce or cabbage leaves.

At irregular intervals, twenty-four hour samples of urine were collected, and quantitative tests for albumin and examinations of sediment were made. The method of Shevky and Stafford [10] was employed for the quantitative estimation of albumin.

Subcutaneous and intravenous injections were made, aseptic precautions and sterile solutions being used.

Autopsy was performed on the animals within twenty-four hours after death. Sections of the kidneys, liver, heart and adrenal glands were placed in Orth's solution and in 65 per cent alcohol. The tissues fixed in Orth's solution were examined for fatty changes. The alcohol-fixed tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin or van Gieson's stains. Methyl violet and iodine were used to identify the amyloid.

RABBITS RECEIVING SUBCUTANEOUS INJECTIONS OF MANGANESE CHLORIDE

Ten rabbits were given injections of manganese chloride two or three times a week. See table 1 for the details.

Rabbits 1, 2 and 3 were killed by an overdose of manganese after having received injections of smaller doses over a period of twelve weeks. Rabbit 4 had an injection of the same dose, but subsequently

6. Kuczynski: Virchows Arch. f. path. Anat. **239**:185, 1922.
7. Smetana: Bull. Johns Hopkins Hosp. **37**:408, 1925.
8. Yokoi: Tr. Jap. Path. Soc. **19**:343, 1929.
9. Letterer: Beitr. z. path. Anat. u. z. allg. Path. **75**:487, 1926.
10. Shevky and Stafford: Clinical Method for Estimation of Protein in Urine and Other Body Fluids, Arch. Int. Med. **32**:222, 1923.

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recovered. Gross examination of the organs revealed no abnormalities, except intense congestion of the lungs and mottling of the livers. Histologic examination of the tissues showed numerous small areas of necrosis in the livers, extensive hemorrhage and edema in the lungs

TABLE 1.—*Rabbits Receiving Subcutaneous Injections of Manganese Chloride*

Rabbit	Duration of Experiment, Wk.	Number of Injections	Total $MnCl_2$ Received, Gm.	Albumin in Urine, Mg. per 24 Hr. Specimen at Termination of Experiment	Lesions of Kidney	Amyloid in Kidney	Lesions of Other Organs	Amyloid in Other Organs
1	12	27	0.062	.....	Cloudy swelling, tubular necrosis	None	Liver: areas of necrosis, small amount of pigment	None
2	12	27	0.062	.....	Cloudy swelling, tubular necrosis	None	Liver: areas of necrosis, small amount of pigment	None
3	12	27	0.062	.....	Cloudy swelling, tubular necrosis	None	Liver: areas of necrosis, small amount of pigment	None
4	31	89	0.482	1010.0	Marked tubular and glomerular involvement, moderate interstitial scarring	+++	Liver: areas of necrosis, slight increase of periportal fibrous tissue, terminal pericarditis	None
5	39	77	0.500	1694.0	Marked tubular and glomerular involvement, moderate interstitial scarring	+++	Spleen: pigment Liver: small amount of pigment	None
6	48	107	0.876	86.0	Moderate tubular and glomerular involvement	++	Lung: bronchopneumonia Liver: moderate increase in periportal fibrous tissue Spleen: pigment	None
7	48	103	0.929	110.0	Moderate tubular and glomerular involvement	++	Lung: bronchopneumonia Spleen: pigment	None
8	17(F) 8(S)	(17) F 13 S	1.030 0.134	1090.0	Marked tubular and glomerular involvement, moderate interstitial scarring	+++	Lung: hemorrhage Heart: interstitial scarring	Spleen
9	46	78	0.9768	120.9	Moderate tubular and glomerular involvement	++	Lung: hemorrhage	None
10	43	54	0.9054	1242.0	Marked tubular and glomerular involvement, moderate interstitial scarring	+++	Lung: hemorrhage	None

and congestion, cloudy swelling, epithelial necrosis and casts in the tubules of the kidneys. In all three animals, there were small amounts of finely granular, brown pigment in the liver cells about the periphery of the lobules. This pigment neither stained with fuchsin nor gave a positive reaction in the test for iron.

The remaining seven rabbits of this group received injections over a period of from twenty-five to forty-eight weeks.

It is interesting to note that when the kidneys were severely damaged the daily output of albumin in the urine was high, in some cases amounting to over 1 Gm. a day. The normal excretion of albumin in the urine of rabbits varies from 20 to 80 mg. per twenty-four hours.

Rabbit 4 died in uremic coma. The blood urea just prior to the death of the animal was 258 mg. per hundred cubic centimeters of blood.

A general description of the gross and microscopic appearances of the organs of these rabbits will suffice, as the changes were identical, except for quantitative and minor differences. Of all the organs, the kidneys presented the most constant and severe lesions. The kidneys were pale and swollen, and when tested with iodine the glomeruli stood

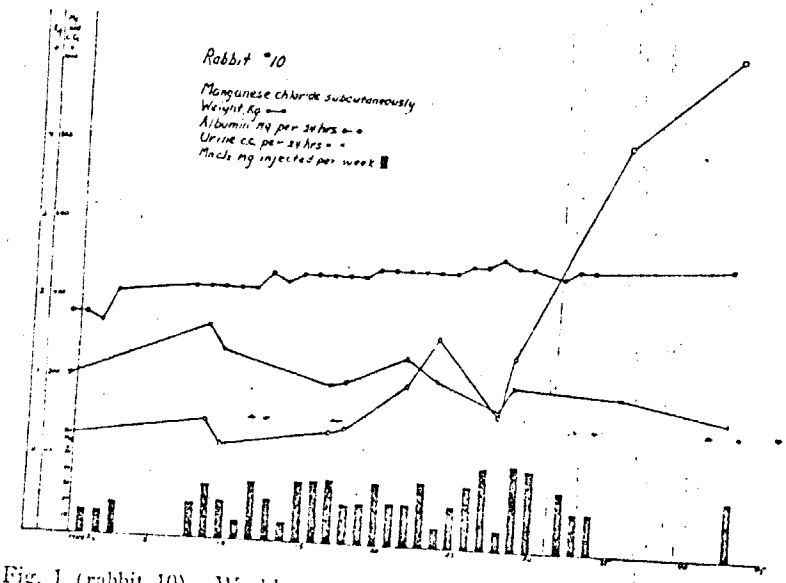

Fig. 1 (rabbit 10).—Weekly record of the weights and amounts of manganese chloride injected subcutaneously and the quantities of urine and albumin excreted in twenty-four hour periods.

out as small brown dots. Microscopically there were extreme changes in the tubules, particularly involving the first, second and third parts of the proximal convoluted portions and the medullary loops. These changes consisted of marked fatty degeneration and necrosis of the epithelial cells, accompanied by evidences of regeneration. Many of the proximal convoluted tubules were dilated to five and six times their normal size; some were completely denuded of epithelium, while others were irregularly lined with large, clear, oval cells, having pale-staining nuclei, or flat, fusiform epithelial cells with large nuclei. In a few places, the epithelial cells formed small masses projecting into the lumina. Some of the tubules contained casts; others granular

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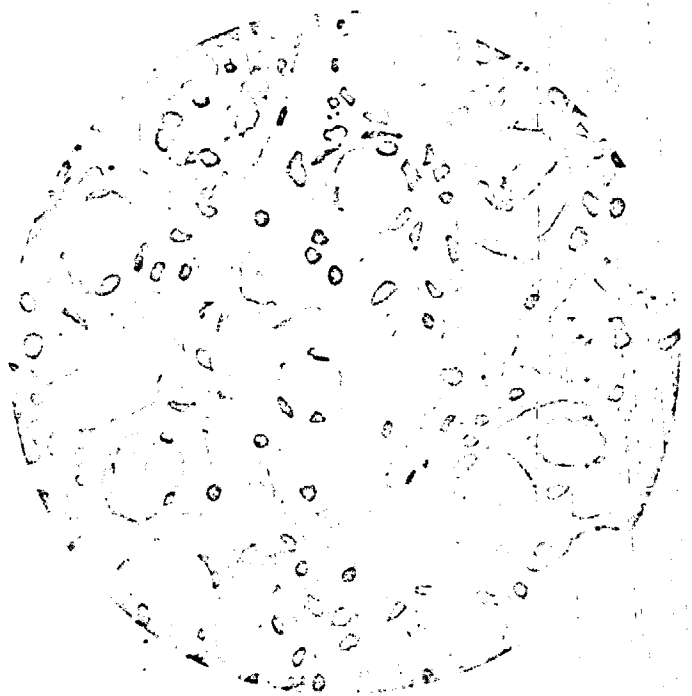

Fig. 2.—High power photomicrograph of kidney of rabbit 10, showing the amyloid infiltration about the tubular capillaries and the broad inner zone of the medulla.

Fig. 3 (rabbit 8).—A glomerulus infiltrated with amyloid, seen with high power objective.

The changes in the distal parts of the loops of Henle and in the distal convoluted and collecting tubules were negligible. There was a definite but moderate proliferation of interstitial fibrous tissue more marked in the kidneys having markedly dilated tubules. This was not accompanied by any signs of inflammatory reaction.

The glomerular changes were extremely interesting. Many of the capsular spaces were filled with granular material and in some places contained small amounts of blood. Throughout, the glomerular tufts were infiltrated with varying amounts of an amyloid-like substance that in places completely obliterated the glomeruli. This same substance was found about the tubular capillaries and was more in evidence about the capillaries of the medulla. This amyloid-like substance stained red with methyl violet and brown with iodine. The iodine brown color changed to a greenish blue when treated with sulphuric acid. Except for a moderate increase of perivascular fibrous tissue, the arteries were normal.

The renal changes in rabbits 6, 7 and 9 were less severe than those in rabbits 4, 5, 8 and 10, but in all there were varying amounts of amyloid.

The amyloid degeneration was limited entirely to the kidneys, except in rabbit 8, in which the spleen, in addition to the kidneys, was found to contain amyloid. This rabbit, by the way, was fed manganese chloride daily for a period of seventeen weeks, receiving a total of 1.05 Gm., and then later was given subcutaneous injections, receiving 0.154 Gm. of the metallic salt over a period of eight weeks.

The changes noted in the livers of these animals were variable and of little consequence. Grossly, the livers were normal, except for intense congestion. In three rabbits there were a few small areas of necrosis and slight increases of periportal fibrous tissue. In two rabbits there were slight amounts of finely granular, brown pigment in the liver cells about the peripheries of the lobules.

Only one rabbit of this group presented subcutaneous abscesses as a result of the injections. The sites of injections in the remainder of the rabbits presented no evidence of sloughing or of abscess formation.

RABBITS RECEIVING INTRAVENOUS INJECTIONS OF MANGANESE CHLORIDE

Three rabbits were given intravenous injections of manganese chloride (table 2). The kidneys of rabbit 11 were slightly swollen and microscopically presented lesions as follows: The tubular epithelium was swollen, granular and necrotic in places. The tubules and glomerular spaces were filled with granular material. Some of the

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glomerular tufts appeared shrunken; others were swollen, vacuolated and markedly congested. In a few places, the glomerular tufts were replaced by a mesh of fibrin in which there were red blood cells. About a few of the capillaries of the medulla of the kidney there were small amounts of amyloid. Lesions in the other organs consisted of areas of necrosis in the liver and hemorrhages in the lungs. The spleen was found to contain much iron-bearing pigment. In the liver there were small deposits of pigment confined to the liver cells about the lobular peripheries.

The remaining two rabbits received injections of smaller doses of the metal over a longer period of time. One rabbit showed large subcutaneous abscesses and was killed at the end of the thirty-fourth week.

TABLE 2.—*Rabbits Receiving Intravenous Injections of Manganese Chloride*

Rabbit	Duration of Experiment, Wk.	Number of Injections	Total Met'l Received, Mg.	Albumin in Urine, Mg. per 24 Hr. at Termination of Experiment	Lesions of Kidney	Amyloid in Kidney	Lesions of Other Organs
11	30	18	0.242	1101.6	Marked tubular and glomerular necrosis	+	Spleen: pigment Liver: necrosis, small amount of pigment
12	42	79	0.5104	130.4	Marked cloudy swelling; otherwise normal	None	Spleen: pigment Liver: moderate amount of pigment
13	34	58	0.2696	156.6	Moderate cloudy swelling; otherwise normal	None	Spleen: pigment Liver: small amount of brown granular pigment

The other animal was killed at the end of the forty-second week. Except for a moderate parenchymatous degeneration, the kidneys of both animals were normal. No amyloid was demonstrated. The livers and spleens contained moderate amounts of hematogenous pigment.

RABBITS FED WITH MANGANESE CHLORIDE

Five rabbits were given manganese chloride, daily, in their food for from eleven to forty-one weeks (table 3). The end-results were negative, and in none of the rabbits of this group were there evidences of damage of the kidneys or liver attributable to the action of the metal. Two of the animals (14 and 17) developed bronchopneumonia, accompanied by abscess formation. There were areas of necrosis in the liver of rabbit 14, and it seemed most likely that this lesion resulted from the severe pneumonic infection. There was a small amount of finely granular, brown pigment in the liver of rabbit 18, a lesion not demonstrated in the remaining four animals.

RABBITS RECEIVING SUBCUTANEOUS INJECTIONS OF (1) ZINC LACTATE, (2) MERCURIC CYANIDE

Two rabbits received injections of zinc lactate for thirty-eight weeks, each receiving a total of 1.65 mg. (table 4). Both rabbits developed large areas of sloughing at the sites of injection. Grossly and microscopically, the organs were normal.

TABLE 3.—*Rabbits Fed Manganese Chloride*

Rabbit	Duration of Experiment, Wk.	Number of Feedings	Total Met'l Received, Gm.	Albumin in Urine, Mg. per 24 Hr. at Termination of Experiment	Lesions of Kidney	Amyloid in Kidneys	Lesions of Other Organs
14	12	62	1.05	Test not done	None	None	Liver: necrosis
15	16	62	1.05	Test not done	None	None	Spleen: pigment
16	11	66	3.12	26.1	None	None	Liver: fatty infiltration
17	21	196	9.72	59.4	None	None	Liver: fatty infiltration Lung: bronchopneumonia
18	41	312	46.92	64.8	None	None	Liver: small amount pigment, fatty infiltration

TABLE 4.—*Rabbits Receiving Subcutaneous Injections of (1) Zinc Lactate and (2) Mercuric Cyanide*

Rabbit	Duration of Experiment, Wk.	Number of Injections	Total Metal Received	Albumin in Urine, Mg. per 24 Hr. at Termination of Experiment	Lesions of Kidney	Amyloid in Kidneys	Lesions of Other Organs
45	38	33	Zinc lactate, 1.65 mg.	46.0	Cloudy swelling	None	None
46	38	33	Zinc lactate, 1.65 mg.	57.2	Cloudy swelling, small amount tubular necrosis	None	None
27	44	56	Mercuric cyanide, 0.341 Gm.	594.0	Marked tubular necrosis, glomerular changes, interstitial fibrosis	None	Liver: small areas of necrosis
28	44	56	Mercuric cyanide, 0.278 Gm.	518.4	Same as in 27, except less marked	None	Spleen: pigment Liver: cloudy swelling

The remaining two rabbits received respectively, 0.341 and 0.278 Gm. of mercuric cyanide, over a period of fifty-six weeks. Both rabbits developed subacute lesions of the kidney, typical of the usual observations in mercury poisoning.

No amyloid was found in the organs of the four rabbits.

CONTROLS

Suffice it to say that an adequate number of control rabbits, twelve, maintained on the diet given above presented no gross or microscopic lesions.

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COMMENT

Local amyloid degeneration in kidneys of rabbits severely damaged by subcutaneous injections of manganese chloride is a constant observation, and moreover properly regulated doses of the same metal given intravenously give rise to the same lesion.

The production of the lesion of the kidney depends on giving manganese chloride over a long period of time, thus making it necessary to regulate the dose to the individual tolerance of the animal. This may be done by following the daily weights and the quantitative estimations of albumin in the urine.

At present, I am repeating and varying the experimental procedure with the hope of finding an explanation of the formation of amyloid in this lesion. I believe, however, that capillary damage, followed by the exudation and jelling of protein substance from the blood into the pericapillary spaces, is the most likely explanation. Nevertheless, I am mindful of the fact that the production of this amyloid substance may be a local phenomenon.

Local suppuration at the sites of injection of the metal may be definitely excluded as the cause of the amyloid degeneration, as only one rabbit of the group receiving subcutaneous injections developed abscesses, and the kidneys of this rabbit contained less amyloid than the kidneys of the remainder of the rabbits. This possibility was further controlled by subcutaneous injections of zinc lactate and mercury cyanide into rabbits, the former producing large areas of sloughing subcutaneously, and the latter giving rise to little or no effect locally but causing severe renal tubular damage typical of mercury poisoning, but in neither case resulting in local amyloid degeneration of the kidneys.

I am unable to explain why the damage of the liver that I observed was not so extensive as that which other authors have reported. It may be that higher doses of manganese chloride than I have employed are necessary to cause such damage.

Hurst and Hurst[4] reported the finding of pigment in the livers of their rabbits given manganese and concluded: "The pigment in manganese poisoning is like that in phenylhydrazine poisoning, a product of blood destruction, and manganese has in addition to the effect on the liver some slight hemolytic action; the pigment is not hemofuchsin." It will be seen in my tables that some pigment was demonstrated in the livers of the rabbits, a most constant observation in rabbits into which manganese chloride had been intravenously injected. The pigment was finely granular and did not stain with fuchsin or by Perl's prussian blue method. In fact, it does not resemble the type of pigmentation that copper produces, in quantity or in quality.

I was unable after thorough search to find pigment in the livers of the control rabbits. The question of the pigment will be considered in a later paper.

CONCLUSIONS

A method has been found to produce amyloid nephrosis in rabbits. The production of the lesion depends on the regulation of the doses of manganese chloride given subcutaneously so as to permit chronic poisoning. The amyloid degeneration is usually limited to the kidneys, except occasionally when the spleen in addition to the kidneys may be involved. The identification of this amyloid-like substance as amyloid is based on its reaction with the stains that are considered specific for amyloid, i. e., methyl violet and iodine.

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Effects of manganese on Q-T interval and distribution of calcium in rat heart[1]

Am J. Physiol
205: 1209
1963

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CONRAD, LOYAL L., AND DONALD J. BAXTER. *Effects of manganese on Q-T interval and distribution of calcium in rat heart.* Am. J. Physiol. 205(6): 1209-1212, 1963. Electrocardiographic observations were correlated with the 2- and 24-hr uptake of Ca^{45} by the myocardium of the normal rat and the rat injected subcutaneously with manganese chloride. The distribution of Ca^{45} in the heart cell was determined by differential centrifugation and the effects of manganese noted. Initially, about 90% of the Ca^{45} in the rat heart was found in the microsomal fraction, the remainder being distributed evenly in the other cell fractions at 2 hr. None was found in the supernatant material after centrifuging for 17 hr. After manganese injection the total uptake of Ca^{45} was doubled at 2 hr due to increases in the microsomal and 17-hr fractions. Control values were re-established by 24 hr. Electrocardiograms showed a significant prolongation of the Q-T interval at 2 and 24 hr after manganese injection. There was a significant increase in serum calcium concentration 24 hr after the injection of manganese.

Myocardial calcium electrocardiogram microsomes cell fractions

A SINGLE MANGANESE EXCESS in rats results in a prompt rise in serum calcium and iron concentrations together with a rise in serum magnesium, chloride, and phosphorus (Baxter and Smith, submitted for publication). Since decrease in serum calcium concentration is known to be associated with a prolonged Q-T interval, it was felt that a study of the rat electrocardiogram after manganese administration would be of interest. In addition, it was decided to determine the uptake and intracellular distribution of radioactive calcium in the myocardium because these observations have not been made in the intact animal in either normal or hypocalcemic states.

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This study was supported, in part, by National Heart Institute [illegible] HE 07787-PEF; and, in part, by a grant from the Oklahoma [illegible] Heart Association.

METHODS

Holtzman albino rats, weighing 224 ± 21 g were injected subcutaneously with 0.5 ml Ca^{45} solution. The isotope was furnished as the chloride and was diluted with demineralized water to a final concentration of 15 μc/ml. Approximately 4.7 μg nonisotopic Ca was present in the 0.5-ml injectate. Immediately following the injection of Ca^{45}, one-half of the rats were injected subcutaneously with 0.25 ml demineralized water containing 60 mg manganese chloride/ml. The remainder received an equal volume of demineralized water. Following the administration of manganese the animals were divided into two groups. One group was sacrificed 2 hr and the other group 24 hr following the administration of manganese. At sacrifice the rats were anesthetized with ether, the thorax opened, heart removed and placed in 0.25 M sucrose at 0° C. Cell fractionation was carried out according to the method described in detail by Schneider (4). Centrifugation was performed in a Spinco model L preparatory centrifuge equipped with an SW 39L or 30.2 rotor. An additional fraction was obtained by centrifugation at 123,000 *g* for 17 hr. After final washing pellets were dried at 50° C for 24 hr and weighed. Radioactivity was determined in each fraction using standard gas-flow counting procedures. Values were expressed as net counts per minute per gram dry weight. Serum calcium determinations were made using the method of Zak, Hindman, and Fisher (5).

Electrocardiographic studies were conducted at 2- and 24-hr intervals in the unanesthetized rat restrained in a prone position. Potential differences between the right forelimb and left hind limb (L II) were recorded from small needle electrodes inserted subcutaneously; an electrode in the tail served as the ground connection. The output of the preamplifier (designed and fabricated by R. L. Trendley) was fed unfiltered into a type D amplifier of a Tektronix type 535 oscilloscope. The trace was photographed with a Tektronix type C-12 camera at a sweep speed of 20 msec/cm. The frequency response of the system was ±1 db from 0.1 to 100,000 cycles/sec

TABLE 1. *Heart rate and Q-T interval in normal rats*

Heart Rate	Q-T Intervals, msec
449 or below	65.5±5.5 (6)
460-499	64.8±7.5 (15)
500-550	63.8±4.8 (12)
551 and above	58.2±4.4 (3)

Number of animals studied shown in parentheses.

when capacity coupled. All measurements were made on a Cambridge Universal measuring machine. Measurements of the QRS interval were reproducible to less than 0.1 msec. The P-Q and Q-T intervals were determined within ±0.3 msec.

The 2- and 4-hr time periods were selected on the basis of data obtained in a preliminary study on the uptake of Ca^{45} by cardiac muscle of the intact rat. After the administration of Ca^{45}, activity was measured in the heart muscle after sacrifice at time periods ranging from 1 min to 18 hr. In these experiments the content of radioactive calcium in the heart reached a nearly constant value within 15-30 min after intravenous injection. This value fell slightly for about 2 hr, and then declined slowly over the remaining 16 hr to a minimum.

RESULTS

A fall in serum calcium concentration from 5.34 ± 0.52 to 4.32 ± 0.43 mEq liter ($P < 0.01$) was noted within 24 hr in the manganese-treated rats and the Q-T interval was prolonged at 2 and 24 hr ($P < 0.001$). The heart rate was significantly lower in the treated animals at 24 hr ($P < 0.01$) but the prolongation of the Q-T interval was not due to slowing of the heart rate because, as shown in Table 1, the duration of the Q-T interval was much greater than that of untreated animals with similar heart rates. Complete electrocardiographic data are tabulated in Table 2. It is seen that there were no other significant electrocardiographic changes. Ventricular conduction defects, in particular, were absent. Representative electrocardiograms are shown in Fig. 1.

Data on the myocardial uptake of radioactive calcium at 2 and 24 hr in the control groups are shown in Table 3. In these animals about 40% of the Ca^{45} was found in the microsomal fraction 2 hr after injection. The remainder was distributed about equally in the other cell fractions. At 24 hr the total counts are less, but the distribution of Ca^{45} in the cell fractions is not significantly different except for the nuclear-cell membrane fraction which was lower ($P < 0.1$). The manganese-treated groups showed significant differences when compared to controls (Table 3): at 2 hr the total uptake of Ca^{45} was nearly doubled due to significant increases in the microsomal and 17-hr fractions; at 24 hr these differences were no longer evident. In all groups the number of counts found in the supernatant was only a fraction of 1% of the total counts. The complete data are summarized and the statistically significant changes are indicated in Fig. 2.

DISCUSSION

Several facets of the physiology and metabolism of calcium in the heart have been explored in these experiments. First, Ca^{45} has been shown to equilibrate rapidly in heart muscle when injected intravenously into the intact animal so that within 30 min after injection the concentration of the isotope in heart muscle has reached a plateau which is maintained nearly constant for about 2 hr. Second, the distribution of Ca^{45} within the myocardial cell itself has been determined for the first time. A significant proportion of the injected Ca^{45} was found in the microsomal fraction at 2 hr with lesser amounts equally distributed in the cell membrane-nuclear, mitochondrial, and 17-hr fractions. The data suggest that at 24 hr there has been a shift of the calcium from the cell membrane-nuclear fraction to the other cell fractions. The isotope did not diffuse into the supernatant after centrifugation. Third, changes in the total uptake as well as the intracellular distribution of Ca^{45} in the heart have been shown to occur with a specific hypocalcemic state that produced by the injection of manganese. These observations suggest that similar studies in other disorders of calcium metabolism would be fruitful. Four studies were performed simultaneously to relate, if possible, the changes in Ca^{45} uptake and distribution in the heart with alterations in the Q-T interval of the electrocardiogram. The data, however, suggest that no such direct relationship exists because a prolonged Q-T interval was found at 2 hr when Ca^{45} uptake was increased and at 24 hr when Ca^{45} uptake was normal.

The method of differential centrifugation of rat heart cells used in this study turned out to be essentially the same as that used for rabbit hearts reported by Dupande et al. (2) except that a higher rotor speed

TABLE 2. *Electrocardiographic observations and heart rate in manganese-treated rats*

	2-Hour		24-Hour	
	Control	Treated	Control	Treated
Rate, beats/min	513±29 (7)	482±58 (7)	492±36 (34)	456±55 (16)
P-Q interval, msec	35.4±5.8 (7)	35.9±7.4 (7)	39.8±3.9 (33)	37.5±3.9 (16)
QRS interval, msec	18.5±1.4 (7)	17.1±1.8 (7)	16.9±2.4 (34)	17.7±1.9 (16)
Q-T interval, msec	65.0±5.4 (7)	75.3±4.4 (7)	64.2±6.6 (34)	78.7±7.0 (16)

Number of animals studied shown in parentheses.

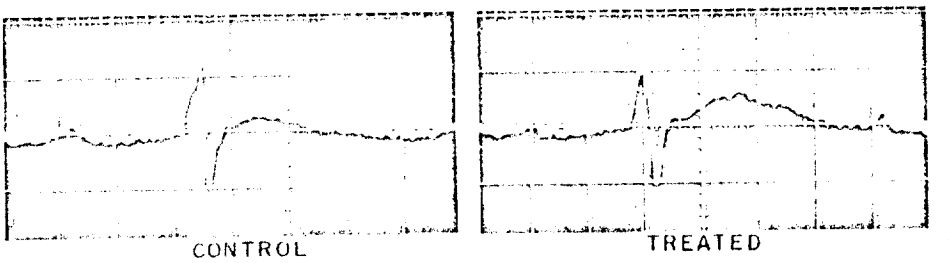

FIG. 1. Electrocardiogram of [illegible] before receiving 15 mg manganese subcutaneously (control) [illegible] 24 hr later (treated). In this [illegible] the heart rate increased [illegible] 426 to 495, the P-Q interval [illegible] from 44.6 to 34.0 msec, QRS interval remained unchanged at 17.3 msec, and the Q-T interval increased from 72.7 [illegible] 77.1 msec. Vertical lines are at [illegible] msec intervals. Standardization is 0.5 mv/cm.

TABLE 3. *Uptake of Ca^{45} (in net counts/min/g of tissue dry wt.) in cell fractions of rat at following manganese administration*

Cell Fraction	2 Hour		24 Hour	
	Control	Treated	Control	Treated
Nuclear	1,496±843 (8)	1,311±282 (5)	409±191 (3)	704±351 (5)
Mitochondrial	1,661±1,244 (7)	2,068±1,180 (6)	1,661±859 (3)	1,786±205 (3)
Microsomal	2,447±1,604 (8)	4,314±602 (5)	965±48 (3)	850±208 (5)
17-Hour	1,026±339 (5)	4,555±2,016 (5)	586±311 (3)	1,862±096 (4)
Supernatant material	33±34 (5)	9±4 (5)	167±92 (3)	3±2 (5)
Total	6,081±3,642 (8)	12,671±2,741 (5)	3,789±875 (3)	4,118±1,528 (5)

Number of animals studied indicated in parentheses.

[illegible] to obtain the "microsomal" fraction (57,000 g), [illegible] the final residuum was centrifuged for 17 hr at [illegible],000 g before the supernatant material was decanted. [illegible] centrifugal forces used to obtain the microsomal [illegible] yield a satisfactory pellet from tissues other than [illegible] and for this reason were used in this study (4). [illegible] methods for testing the homogeneity of this [illegible] were not available.

No attempt was made in this study to extract actomyosin preparatory to centrifugation, so it is possible that any calcium sedimenting in intimate association with this molecule might make the data less exact. The composition of the pellet after 17-hr centrifugation at a force of 123,000 g has not been determined as yet. The fact that the final supernatant material contained negligible amounts of Ca^{45} confirms experimentally the long-standing assumption that intracellular calcium, at least in the rat heart, exists in a nondiffusable state by virtue of being bound to various intracellular structures. It is not immobile in the cell, however.

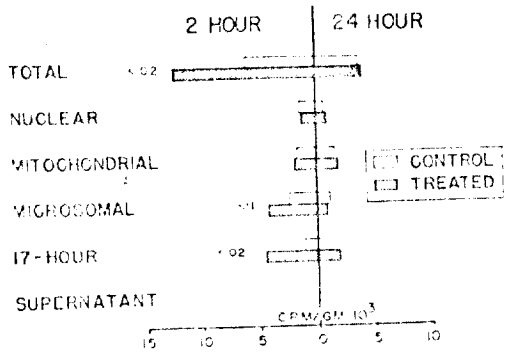

FIG. 2. Uptake of Ca^{45} by rat myocardium after manganese administration. Two-hour values are to the left and 24-hr values to the right of the vertical line. Total uptake and the uptake for each cell fraction are noted in the horizontal bars. Number of observations are shown inside each bar. *P* values were calculated using Student's *t* test. Values for *P* at ends of bars refer to differences between control and treated groups. Uptakes are expressed in thousands of counts per minute per gram tissue at dry weight.

The rate of turnover of Ca^{45} by the heart cell appeared to be increased by manganese injection: despite a greater total uptake and an increased uptake in the microsomal and 17-hr fractions at 2 hr after manganese, the net number of counts in these fractions as well as total counts returned to control values at 24 hr. Explanation is lacking but it seems obvious that the administered manganese has disrupted physiologic mechanisms governing the entry of calcium into the myocardial cell and its internal distribution as well. That the observed effects are not due to competition for binding sites by the two divalent ions is suggested by the fact that Ca^{45} uptake was increased, not decreased, in the presence of manganese.

The electrocardiographic effects of the injected manganese probably were not mediated by pH changes in serum affecting the amount of ionized calcium because manganese excess produces acidosis rather than alkalosis (Baxter and Smith, submitted for publication).

Finally, we have been able to obtain more precise measurements of the P-Q, QRS, and Q-T intervals than reported heretofore in the rat by using an amplifier-recording system with an extended frequency response, together with an instrument capable of measuring linear distances (time) to a high degree of accuracy. The normal values for these intervals obtained in this study differ slightly from those reported by Angelakos and Bernardini (1). The differences noted are attributed to the ages of the rats used in the two studies and to the use of ether anesthesia in the other study. The peculiar shape of the T wave previously described in the rat (3) is due [illegible] doubtedly to the use of electrocardiographic amplifiers with inadequate frequency response.

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Am. J. Physiol., 210(2): 357-359, 1966

Positive inotropic effect of manganese on dog myocardium[1]

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CONRAD, LOYAL L., ROBERT L. TRENDLEY, AND DONALD J. BAXTER. *Positive inotropic effect of manganese on dog myocardium.* Am. J. Physiol. 210(2): 357–359. 1966.—Intravenous manganese chloride (25–100 mg) produced an increase in myocardial contractile force in dogs subjected to thoracotomy, bilateral vagotomy, total spinal anesthesia, and pretreatment with reserpine. The positive inotropic effect of Mn was abolished by beta adrenergic blockade with pronethalol and by bilateral adrenalectomy. It appeared later than a similar effect produced by isoproterenol and was preceded by a fall in blood pressure possibly due to vasodilatation. An increase in heart rate and widening of the pulse pressure were observed during the peak inotropic effect. It is concluded that the positive inotropic effect of Mn is due to stimulation of the adrenal medulla by this cation and subsequent release of catecholamines from the adrenal gland.

heart chronotropic effect; blood pressure; adrenal medulla; catecholamines

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MANGANESE HAS BEEN OBSERVED to produce an increased uptake and altered intracellular distribution of Ca^{45} in the rat heart together with a fall in serum Ca concentration and an increased Q-T interval (1). From these observations it was supposed that other changes in cardiac function might occur. Consequently, the acute effects of Mn on cardiac contractility, heart rate, and blood pressure were investigated in dogs subjected to thoracotomy in a variety of experimental conditions including beta adrenergic blockade, ablation of pressor reflexes by total spinal anesthesia and vagotomy, adrenalectomy, and pretreatment with reserpine. From these studies it is concluded that the positive inotropic effect of Mn is due, at least in great measure, to the direct stimulatory effect of this cation on the adrenal medulla and liberation of catecholamines from the adrenal gland.

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[1] This study was supported, in part, by Public Health Service Research Grant HE 07787 (PET) from the National Heart Institute.

METHODS

Nineteen mongrel dogs, varying in weight from 10 to 30 kg (avg 15 kg), were anesthetized with sodium pentobarbital, 25 mg/kg. Respiration was maintained by using a National Cylinder Gas small-animal anesthetizer and 100% oxygen. After thoracotomy, the pericardium was opened, and a resistance-wire strain-gage arch of the Walton-Brodie type was secured to the right ventricle. Arterial blood pressure was measured from a cannula in the femoral artery using a Sanborn 267 B pressure transducer. Total spinal anesthesia was produced in eight animals by forcibly injecting Pontocaine, 100 mg in a volume of 6 ml, into the subarachnoid space through a spinal needle in the upper lumbar region. In these animals, the blood pressure was sustained by continuous infusion of angiotensin (Hypertensin, Ciba) by intravenous drip in a concentration of 5 μg/ml in normal saline solution. After initial stabilization of the blood pressure, the rate of angiotensin infusion was not changed for the remainder of the experiment. All other medications were administered by rapid injection into the contralateral femoral vein.

Two dogs were pretreated with reserpine (Serpasil, Ciba), 0.25 mg/kg intraperitoneally, 18 and 20 hr beforehand and received an additional amount of 1.5 mg intravenously at the time of experimentation. No increase in myocardial contractility resulted from this intravenous dose of reserpine.

In three dogs, the effects of manganese on myocardial contractility, heart rate, and arterial blood pressure were observed prior to performing bilateral adrenalectomy. As soon as the blood pressure had stabilized after the surgical procedure the injection of manganese was repeated and the effects noted.

Manganese was administered as the chloride in doses of 25–100 mg ($MnCl_2$) in 1 ml distilled water. Two animals received 1 ml 4.6% NaCl approximating the tonicity of the manganese solution containing 100 mg $MnCl_2$. Isoproterenol (Isuprel, Winthrop), in doses of 0.2 μg/kg body wt, failed to produce an increase in

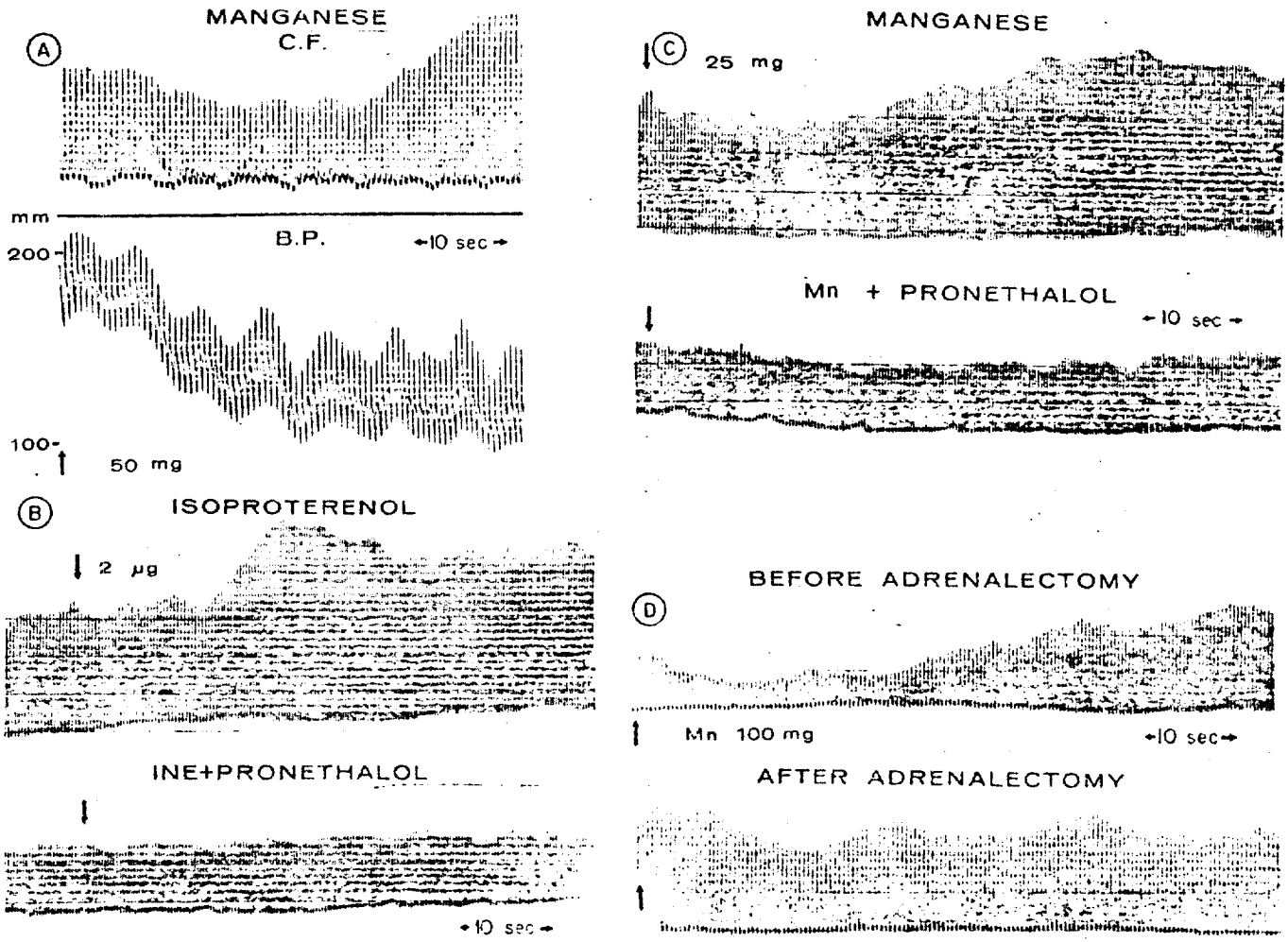

FIG. 1. *A:* changes in contractile force (C.F.) and arterial blood pressure (B.P.) after the intravenous injection of $MnCl_2$, 50 mg (arrow). There was an immediate decrease in C.F. and fall in B.P. C.F. increased above control 42 sec after injection. Experiment was terminated 10 sec later (end of recording) to obtain a myocardial biopsy. Spinal anesthesia, bilateral vagotomy, angiotensin infusion. *B:* positive inotropic effect of isoproterenol (INE), blocked by pronethalol. INE effect is earlier in appearance than that due to manganese. Reserpinized dog. *C:* increase in contractile force produced by manganese and its abolishment by pronethalol. Reserpinized dog. *D:* contractile force before and after adrenalectomy in response to manganese chloride. Sensitivity of the strain-gage arch recording was increased after adrenalectomy. Contractile force is changing cyclically with inflation and deflation of the lungs by the respirator. Spinal anesthesia, bilateral vagotomy, angiotensin infusion.

cardiac contractile force after beta adrenergic blockade was produced by the administration of pronethalol (Alderlin, Ayerst), 2.5 mg/kg. After blockade, responsiveness of the heart was determined by administering 1 ml 10% calcium gluconate solution intravenously at the end of the experiment. In addition, pronethalol was effective in abolishing the inotropic response to metaraminol (Aramine, Merck) in dosages of 0.025 and 0.25 mg (2 animals).

Blood pressure and cardiac contractility were recorded by using a Honeywell 1508 oscillograph at a paper speed of 2.5 mm/sec.

RESULTS

An increase in myocardial contractile force resulted from every injection of manganese except in animals subjected to either beta adrenergic blockade or bilateral adrenalectomy (Fig. 1, Table 1). The over-all magnitude of the positive inotropic response averaged 155% of the control values. It did not appear to differ quantitatively for doses in the range of 25–100 mg. For this reason the individual dose response has not been tabulated. The response was slower in its appearance than that produced by isoproterenol (Fig. 1*B*, Table 2). It was, however, always preceded by an immediate fall in blood pressure and a slight decrease in measured contractile force (Fig. 1*A*, Table 3). These effects were noted in all animals and were not abolished by beta adrenergic blockade or adrenalectomy. In the adrenalectomized animals the initial blood pressure fall persisted without correcting itself in contrast to the animals with intact adrenals in which the fall lasted for about 20 sec, after which the blood pressure rose to premanganese levels or even slightly above in some cases. In usual doses the duration of the manganese inotropic response averaged

TABLE 1. *Changes in cardiac contractile force produced by intravenous manganese chloride, 25-100 mg, in dogs*

Condition	No. of Obs.	Contractile Force, % Control
T, HS	2	100
T	1	132
T, R	3	121 (107-138)
T, R, P	2	100 (94-109)
T, S, V, H	11	158 (113-372)
T, S, V, NE, P	1	100
T, S, V, H, P	2	97 (97-100)
T, S, V, H, A	8	91 (65-107)

Values are means, with ranges given in parentheses. T = thoracotomy; HS = hypertonic saline; R = reserpine; S = spinal anesthesia; P = pronethalol; H = angiotensin infusion, V = bilateral vagotomy; NE = norepinephrine infusion; A = bilateral adrenalectomy.

TABLE 2. *Time of onset, peak action, and duration of manganese effect compared with isoproterenol (INE)*

	Onset		Peak Action	Duration	
	BP	CF	CF	BP	CF
Mn	19	40	53	20	53
INE		17	28		109

Time in seconds. BP = immediate blood pressure fall; CF = increase in contractile force.

TABLE 3. *Effect of iv MnCl, 25-100 mg, on heart rate and blood pressure in dogs with spinal anesthesia and vagotomy*

	Control	Treated	
		Immediate	Peak
Rate, beats/min	132		145
Blood pressure*	186/144	138/98	180/124

* Stabilized with angiotensin.

about 1 min. In one animal which received 650 mg $MnCl_2$ infused over a period of 6½ min, an appreciable inotropic effect (139% of control) was present 3 hr after stopping the infusion. The heart rate increased an average of 13 beats/min in all animals except those receiving beta adrenergic blockade or adrenalectomy.

DISCUSSION

Initially it was felt that the positive inotropic effect which occurred with the injection of manganese might be due to the direct effect of manganese on the myocardium because the uptake of radioactive calcium is increased in the rat heart by manganese administration (1). As a matter of fact, these experiments were initiated to see whether the increased uptake of calcium was associated with an increased myocardial contractility in this circumstance. However, it soon became apparent that it would be necessary to rule out a baroreceptor reflex and subsequent catecholamine discharge because of the initial fall in the arterial blood pressure which occurred a few seconds after the administration of manganese. This reflex was eliminated effectively by performing total spinal anesthesia in conjunction with bilateral vagotomy. The fact that the positive inotropic effect of manganese was elicited in animals subjected to spinal anesthesia and vagotomy eliminates a pressor reflex from consideration. That a catecholamine effect was involved in the mechanism, however, was still apparent because the manganese effect was abolished by beta adrenergic blockade with pronethalol. It was not abolished by reserpine, which suggested that the inotropic effect was not mediated by local discharge of catecholamine from stores in the heart itself. Bilateral adrenalectomy, however, not only abolished the positive inotropic effect, but resulted in a persistence of the blood pressure-lowering effect due to manganese. Thus, it is believed that the mechanism by which manganese produces a positive inotropic effect is indirect, i.e., by stimulating directly the adrenal medulla with subsequent release of catecholamines which increase cardiac contractility as well as restore the blood pressure toward normal. This hypothesis seems reasonable particularly in view of other experiments where manganese, together with other divalent cations, was capable of stimulating catecholamine secretion in the denervated, perfused adrenal glands of cats in vivo (2). That the initial fall in blood pressure alone was not capable of eliciting a catecholamine discharge from the adrenal was demonstrated in animals with spinal anesthesia and vagotomy in which increasing hypotension and decline in contractile force were observed until corrected by angiotensin infusion. In addition, the delay in onset of the inotropic effect of manganese as compared with isoproterenol, 40 sec vs. 17 sec, also suggests that the manganese effect is dependent on an intermediate rather than an immediate mechanism. Finally, the increase in heart rate and the widening of the pulse pressure during the period of maximum inotropic effect can be attributed as well to the discharge of catecholamines from the adrenal gland. It still seems possible the blood pressure fall observed immediately after the injection of manganese is due to a direct effect on vascular smooth muscle resulting in vasodilatation. Perhaps the transient decrease in cardiac contractile force seen at the same time is due to a direct depressant effect of manganese on cardiac muscle.

The Ayerst Laboratories supplied the pronethalol (Alderlin) used in these experiments.

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THE USE OF TETRAMETHYLDIAMINODIPHENYLMETHANE FOR THE DETERMINATION OF SMALL AMOUNTS OF MANGANESE IN PLANT MATERIAL AND SOIL EXTRACTS

By A. H. CORNFIELD and A. G. POLLARD

It is shown that the very sensitive reaction between manganese and 4 : 4′-tetramethyldiaminodiphenylmethane ('methane base') in the presence of periodate can be applied to the quantitative estimation of the element in the range 0·1–1 μg. The effect of possible interfering factors is studied and the method is shown to give satisfactory results for the determination of manganese in plant material and in the ammonium acetate 'leachate' of soils.

Introduction

The usual methods for the determination of small amounts of manganese involve oxidation to permanganate by such reagents as persulphate, bismuthate, or periodate[1, 2, 3] followed by colour or extinction measurements. The formaldoxime method,[4, 5] which is somewhat more sensitive, has also been used to a certain extent. Where only small amounts of material are available for analysis, e.g. plants grown in pot tests, these methods are not sensitive enough to give accurate results.

The formation of a blue colour when 4 : 4′-tetramethyldiaminodiphenylmethane ('methane base') and periodate are added to a solution containing manganese has been used for some years for detecting traces of the element. The test is very sensitive and Vogel[6] states that as little as 0·001 μg. of manganese can be detected in a concentration limit of 1 part in 50 million. It appears that little attention has been given to the use of this test for the quantitative determination of manganese. In 1914 Tillmans and Mildner[7] used the reaction for the approximate determination of manganese in water. The test sample was shaken with solid potassium periodate in the presence of acetic acid and the methane base was added in chloroform solution. The depth of the blue colour formed was taken as a measure of the amount of manganese present. In 1931 Harry[8, 9] measured the blue or violet colour formed when an alcoholic solution of the methane base was added to the test solution. Periodate was not used, which possibly explains why the sensitivity of the test was not very high. Nicholas[10] recently reported the use of the methane base in acetone solution and in the presence of periodate to give a very sensitive approximate determination of manganese in plant tissue extracts. He also made use of the initial blue colour formed.

Preliminary work

Preliminary tests showed that the initial blue colour was too fugitive to be of any use for accurate measurements. The proportions of methane base, periodate, acetate, and acetic acid were varied in attempts to stabilize the blue colour. It was not found possible to do this, but it was noticed that after about five minutes a relatively stable green colour, the intensity of which appeared to be proportional to the amount of manganese present, was formed, and it was decided to make use of this colour for measurements. These tests confirmed the high sensitivity of the method and showed that quantities of manganese of the order of 0·5 μg. in a final volume of 25 ml. (0·02 p.p.m. Mn) could be determined with ease.

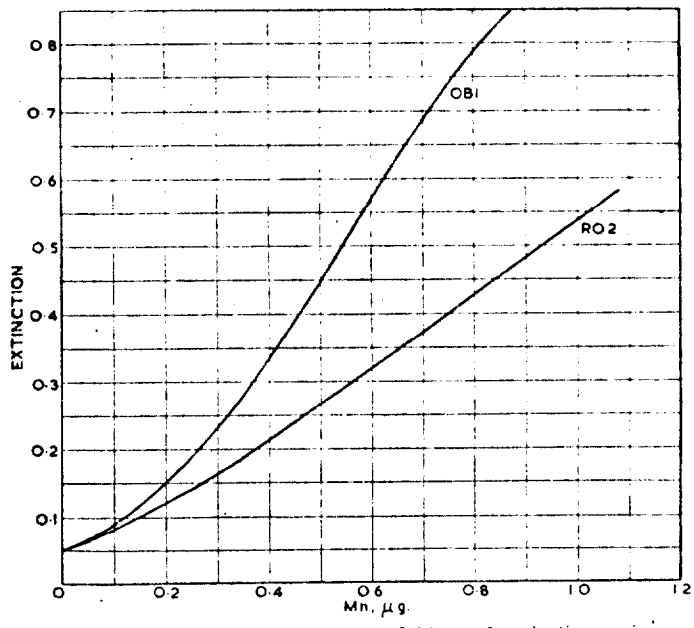

FIG. 1.—*Relation between amounts of Mn and extinction, using filters OB1 and RO2 (2-cm. cell)*

Extinction measurements were made with the Hilger Biochem absorptiometer, a direct-reading single-cell instrument capable of taking glass cells 4 cm. or less in effective length. Using the standard method indicated below the relation between extinction and amounts of manganese from 0 to 10 μg. for the five light filters supplied was examined (the ½-cm. cell was used for these measurements). The highest sensitivity was obtained with the dark blue filter (OB1, maximum transmission at 440 mμ) whilst the red filter (RO2, maximum transmission at 640 or more mμ) was the next in order of sensitivity. Beer's law is more closely followed in the case of the red filter than the blue, and the extinction values tail off less rapidly with the red filter.

It was decided that the range 0 to 1 μg. of manganese would be a suitable one for the determination. Fig. 1 shows the relation between extinction and amounts of manganese

in this range using both the red and dark blue filters (the 2-cm. cell was used). It was decided to use the red filter for subsequent measurements. The 2-cm. cell was the most suitable one for the range 0·1 to 1 μg. of manganese.

Method

The method finally adopted for the determination of total manganese in plant tissue is shown below.

Reagents

Acetate-acetic reagent.—15·0 g. Analar sodium hydroxide is dissolved in about 180 ml. water. 58·0 ml. glacial acetic acid is added, and the mixture is cooled and diluted to 250 ml. This solution is 1·5N. with respect to sodium acetate and 2·7N. with respect to free acetic acid.

Phosphate solution.—A solution containing 0·48 g. potassium dihydrogen phosphate per 250 ml.: this contains about 1 mg. P_2O_5, as phosphate, per ml.

Periodate solution.—2 g. Analar potassium periodate per l. A fresh solution should be prepared every 48 hr.

Methane base reagent.—0·5 g. tetramethyldiaminodiphenylmethane is dissolved in 6 ml. 2N-hydrochloric acid and the solution is diluted to 100 ml. This solution is stable for 2 days.

The temperature of the last two reagents should be adjusted to approximately 20° C. before use.

Standard manganese solutions

(*a*) Concentrated standard (containing 20 μg. manganese per ml.): 0·0575 g. potassium permanganate is dissolved in about 50 ml. water. 2 ml. 2N-sulphuric acid is added and sulphur dioxide is passed through the solution till the colour is discharged. The solution is boiled vigorously for a few minutes to remove excess sulphur dioxide, cooled, and made up to 1 l.

(*b*) Dilute (working) standard (containing 0·1 μg. manganese per ml.): 5 ml. of the concentrated standard is diluted to 1 l.

Preparation of the standard curve

Quantities of the dilute manganese solution containing from 0 to 1 μg. are placed in 6 in. × ¾ in. test tubes calibrated at the 21-ml. mark. 2 ml. of the acetate-acetic solution and 1 ml. of the phosphate solution are added and the mixture diluted to 21 ml., shaken, and placed in a water bath at 20° C. (± 0·5° C.) for 15 min. 3 ml. periodate solution is then added and the mixture is shaken, immediately after which 1 ml. of the methane base reagent is added, shaken with the solution, and the extinction is measured after 10 min. (± 1 min.) in the Biochem absorptiometer using the 2-cm. cell and the red light filter, RO2.

Treatment of plant material

The ash from 0·05 to 0·1 g. of oven-dried plant material is treated with concentrated hydrochloric acid, excess of which is evaporated off; the residue is baked for some hours to dehydrate the silica. More hydrochloric acid is added and the mixture is evaporated to dryness on a water bath. After extraction with water and removal of the insoluble residue, the filtrate is diluted to 25 ml. and an aliquot, containing from 0·1 to 1 μg. of manganese, is placed in the reaction test tube. 2 ml. acetate-acetic solution and 1 ml. phosphate solution are added and the procedure described under the preparation of the standard curve is then followed.

Discussion

The blank value obtained was relatively small and it was not considered worth while purifying the reagents with a view to eliminating or reducing it.

The presence of a certain amount of acetate is essential to obtain high sensitivity, and free acid is also necessary to prevent the methane base from being precipitated. The free acetic acid present in the acetate-acetic solution and a slight excess of hydrochloric acid present in the methane base reagent allow for this. The acetate-acetic solution is prepared from sodium hydroxide and acetic acid, since this yielded a smaller blank than when the acetate was furnished by Analar sodium acetate. Since the extinction value decreases with increasing amount of free acid it is essential that the test solution be neutral or have an acidity equivalent to not more than 0·25 ml. 0·1N-sodium hydroxide. The acetate-acetic solution is not in any sense a buffer reagent.

The presence of phosphate in the test solution is essential to prevent the interference of ferric iron. This point is discussed further under 'interfering factors.'

The extinction values increase slowly with time, but providing readings are taken 10 min. after adding the methane base reagent reproducible results can be obtained. A variation in the time of ± 1 min. is permissible. The extinction values decrease with increasing temperature, e.g. at 5° C. the test is about twice as sensitive as at 20° C. The temperature of the test solution should be maintained at 20° C. ± 0·5° C. in order to obtain reproducible results.

Duplicate analyses of solutions containing varying amounts of manganese showed that results were reproducible to within 10%.

Interfering factors

Table I shows the maximum amounts of various ions, likely to be present in the test solution, that are tolerable without interference. Values are given in actual amounts and also in p.p.m. of the final test solution.

Table I

P_2O_5 (as phosphate)	5 mg.	200 p.p.m.	Ferric iron	25 μg.	1 p.p.m.
Chloride ..	10 mg.	400 p.p.m.	Aluminium	50 μg.	2 p.p.m.
Sulphate ..	5 mg.	200 p.p.m.	Copper ..	100 μg.	4 p.p.m.
Nitrate ..	5 mg.	200 p.p.m.	Lead ..	25 μg.	1 p.p.m.
Calcium ..	5 mg.	200 p.p.m.	Arsenate ..	50 μg.	2 p.p.m.
Magnesium ..	2 mg.	80 p.p.m.	Arsenite ..	100 μg.	4 p.p.m.
			Fluoride ..	400 μg.	16 p.p.m.

Sodium, potassium, or ammonium: in amounts equivalent to 10 mg. chloride.

The interference of copper, lead, arsenite, arsenate, and fluoride are given in order to allow for the possible presence of insecticide or fungicide spray residues containing these elements.

In the absence of phosphate, as little as 2 μg. ferric iron was found to interfere by reducing the extinction. When phosphate was added to the test solution it was noticed that much larger amounts of ferric iron could be present without interference; the presence of at least 1 mg. P_2O_5, as phosphate, eliminated the interference of up to 25 μg. ferric iron. This phosphate is incorporated in the standard method being added as the 'phosphate solution.'

The results on interference show that the test is highly selective for manganese and also that the amounts of ions generally present in plant material are not likely to interfere with the determination.

Spectral absorption curves

Towards the end of the work on this method a Unicam spectrophotometer became available for use. The spectral absorption curves for amounts of manganese ranging from 0 to 0·8 μg., as determined by the standard procedure, over the wave-length range 400 to 700 mμ. are shown in Fig.

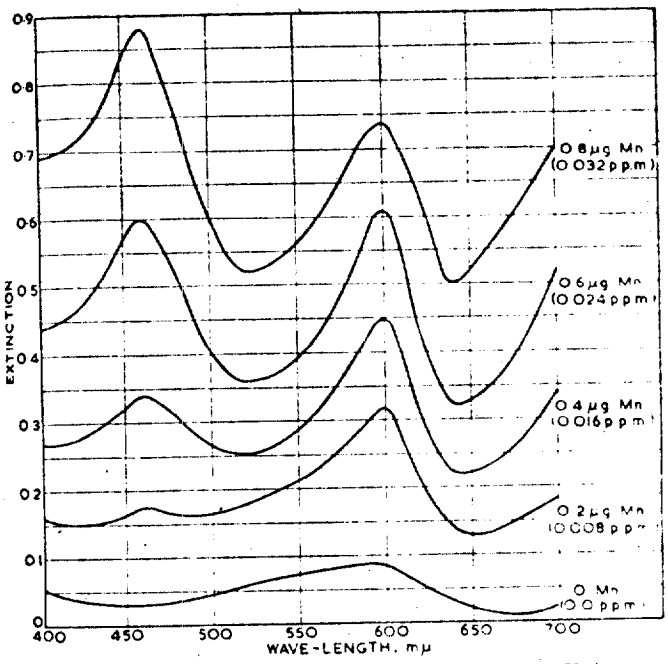

FIG. 2.—*Spectral absorption curves obtained with the Unicam spectrophotometer*

It is seen that there are three extinction maxima, viz. at 460, 600, and 700 mμ, which, with the exception of one of the blank values, occur at the same wave-length for all quantities of manganese.

These results more or less confirm those obtained by using the different filters on the Biochem absorptiometer, i.e. maximum sensitivity is given in the blue, region but in the red region there is a more nearly linear relation between extinction and amount of manganese present.

Recovery of known amounts of manganese

Table II shows the recovery from plant ash extracts of a known added amount of manganese (0·20 μg.).

Table II

	Mn originally present, μg.	Total Mn, μg.	Mn added, μg.	Mn recovered, μg.
Plant 1	0·55	0·74	0·20	0·19
" 2	0·50	0·69	0·20	0·19
" 3	0·40	0·61	0·20	0·21
" 4	0·42	0·62	0·20	0·20

These results show that recovery is complete within the limits of experimental error.

Comparison with the permanganate method

Table III compares the results obtained by the methane base method with those given by the permanganate method (in which manganese is determined absorptiometrically after oxidation to permanganate by means of potassium periodate in the presence of phosphoric acid).

Table III

Plant material	Mn, p.p.m. Permanganate method	Mn, p.p.m. Methane base method
Barley straw	101	107
Mixed feeding stuff	100	106
Grass	53	59
Potato leaves	39	42
Beans	22	20
Parsnips	10	7·8
Hay	3·0	3·7

These figures indicate that for most practical purposes both methods yield very similar results.

By using the Biochem absorptiometer with the appropriate light filter and the 2-cm. cell the permanganate method covers the range 30 to 300 μg. of manganese. The methane base method is therefore about 300 times more sensitive than the permanganate method.

Exchangeable manganese in soils

Table IV gives values, as obtained by the two methods, for the neutral N-ammonium acetate-extractable manganese (exchangeable manganese) of soils. The ammonium acetate extract is evaporated to dryness, organic matter is destroyed with aqua regia, and the residue is then treated in the same way as the plant ash material.

Table IV

Soil no.	Mn, p.p.m. Permanganate method	Mn, p.p.m. Methane base method
1	58	64
2	45	44
3	11	13
4	3·3	3·5
5	2·4	2·8
6	1·2	1·5
7	0·34	0·21

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Manganese in Health and Disease[1]

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MANGANIA is the Greek word for magic or, in modern parlance, for voodooism. If this is at the root of the metal's name, it reflects some reality in the biology of manganese, which is rich in phenomena and lacking in adequate guiding principles. The phenomena have been discovered empirically and to a very large extent by accident. On the other hand, several apparently sound theoretical concepts do not reflect themselves too obviously in the behavior of this metal in the body. Therefore, this review has gathered as many topics as possible, in order to show that this area is wide open for fruitful investigation. Being addressed to the nonspecialist, it trespasses the boundaries of several biological disciplines, but it does not cover any one aspect exhaustively. As a rule, the experience which has been gained exclusively *in vitro* is not discussed here because of its enormity and because of the uncertainty involved in extrapolating from the test tube to the intact animal. Furthermore, the existence of several specialized articles (88, 114, 123, 129, 175, 176), treatises (8, 89, 122, 126, 153, 194) and reviews (39, 55, 56, 135-140, 159, 165, 198) has made the exhaustive citation of literature unnecessary, while it has placed a premium on the integration of the principal information. Notable among these excellent articles is the one by Von Oettingen in *Physiological Reviews* for 1955 (198). Not only is the subject covered extensively, but the perceptive conclusions have withstood the passage of one generation. Von Oettingen's treatment of manganese as a specific biological entity has been followed here with the hope that it will again facilitate the unification of diverse information.

Determination of Maganese in Biological Materials

The large number of methods when compared with the relatively small yield of quantitative information bears witness as to the basic dissatisfaction with methodology in this area. As is the case for other trace elements, if excessive handling is imposed by the method, the sample has a high probability of becoming contaminated with extrinsic manganese. Ashing on the other hand, might easily cause losses. Superimposed on these limitations are the problems created by the requirements for sample size and by the degree of specificity of the end-point. As a rule, it appears that the better the technique, the lower the values for the manganese content of a given tissue have been (25). These considerations explain the emphasis placed on neutron activation analysis (175), at the end of this section.

A flame photometric procedure for manganese has been developed (58) but here flame photometry has not had the impact it has shown elsewhere, probably because a large amount (100 μg/gm) is necessary for optimal precision. Spectrophotometric techniques have fared much better. As a rule, spectrophotometric determinations of manganese appear to have been coincidental to the study of a number of other elements suitable to assay by these techniques. Therefore a standard other than manganese itself has been ordinarily used. The reproducibility of the

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determinations was probably not affected by this, but there might have arisen some uncertainty as to accuracy. Furthermore, observance of all the precautions inherent to classical trace metal work, as well as preliminary ashing of the sample, has been necessary. The sensitivity with the copper spark technique is of the order of .2 μg/ml, and that of the graphite direct-current arc is .02 μg/ml (129). Useful information has been gathered with these techniques by Vallee and his colleagues (195), by Tipton and hers (191), by Butt and his group (32, 33, 83) and by others (56, 165, 187).

The periodate methods have used the oxidation of manganese into permanganate as a colorimetric test for the metal. Among them, that of Reiman and Minot (156, 157) has a lower limit of sensitivity of 2 μg. Mehlig (128) has developed a very reliable periodate method which is presumably optimal for the .07-3 μg range.

The fact that permanganate can act catalytically on some organic compounds and form a more intense color than that of permanganate alone was explored by Gates and Ellis (77). They used sodium periodate to oxidize the metal and develop the color with 4,4,tetramethyldiaminotriphenylmethane. The color fades when exposed to light, changes with time even in the dark, and its development is generally temperamental. The lower limit of sensitivity is about .1 μg/ml. A similar method developed by Fore and Morton (69) obtains the color from the reaction between, N,N diethylaniline and permanganate in buffered solutions of the ashed samples. When the authors' precautions are skillfully observed, the method is reliable at the .005 μg level. Single (178) recommends the catalytic reaction between permanganate and tetramethyldiaminophenylmethane (Tetrabase). He suggests several precautions for stabilizing the color. He considers the method as useful for the 0.01-0.1 μg range. Wiese and Johnson (205) have used the oxidation of benzidine by permanganate in strongly acidic solutions for the development of the color. If the interfering substances are removed and the oxidation to permanganate is achieved with sodium bismuthate, the method, although laborious, seems to be reliable for the 0.1-10 μg range.

The procedure of Bentley, Snell and Phillips (14) uses the growth of a *Lactobacillus arabinosus* strain in a synthetic medium first as a means of reducing the manganese concentration in the medium and, after the addition of the ashed sample, as a means of determining the concentration of manganese. The end-point is the electrometric titration of the lactic acid produced by the bacilli from glucose and the method is sensitive at the 0.05-0.5 μg level.

Clearly, a methodological 'package deal' which provides high specificity, precision, high sensitivity, rapidity and ease of operation, would be highly desirable in this field. It has yet to become available, but a step in that direction has been taken by H. J. M. Bowen (25) with his elegant thermal neutron activation analysis. He activates the natural Mn^{55} in the sample into the highly radioactive Mn^{56} ($T/2 = 2.6$ hr.) by inserting the sample in a nuclear reactor. He recommends a number of easy precautions in obtaining the sample, including the use of nonmetallic instruments. If the sample remains as untouched as possible up until its entrance into the nuclear reactor, it is safe thereafter. It can be dissolved in acid and precipitated with carrier manganese. 'Hold back' carriers are used to remove the interfering elements. The precipitate is counted in a suitable radiation detector and compared to identically treated standards.

As Bowen himself has suggested, his published approach constitutes merely a beginning. The effort at Brookhaven (21) is designed to go some steps further. By a suitable electronic circuit it was found possible to discriminate in favor of the Mn^{56}

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radiations and against the ones which are contributed by activation of elements other than manganese in the sample. By judicious use of shielding during activation and by sorting out specific radiations for counting, this approach is well on the way to implementing the 'package deal' which was alluded to above. The method is capable of 10 per cent precision at the 3×10^{-5} μg/ml level (129) and guarantees against both contamination and loss. The sample is preserved and can be used again for this or other similar analyses, if desired.

The efforts discussed above were designed to make possible the quantitative estimation of manganese in various tissues. Cohn and Townsend (43) have pointed out that the kind of complex in which manganese participates can be determined by the paramagnetic resonance absorption method. This method has not been applied as yet to manganese in tissues of animals. It is being mentioned only because it appears to constitute a very promising approach for future work.

Absorption and Excretion of Manganese

Von Oettingen's review (198) documents the fact that manganese given in an inorganic form is slowly and incompletely absorbed in the gut. Further observations will be discussed here. It is necessary, however, to point out at the outset that there is no necessary correlation between the rate of absorption of large amounts of inorganic manganese salts and that of the trace amounts occurring in foods. The latter most probably exist in the form of organic chelates. (For discussions of such coordination compounds, the reader is referred to the writings of Martel and Calvin (122), Williams (212), Stern (186) and Christensen *et al.* (42).) In view of the fact that the properties of the individual complex determine the rate of transport across any cell walls, the observations with inorganic salts are of more specific significance than was hitherto presumed. The behavioral differences between inorganic and coordinated manganese would be important, even relative to a possible specific transport mechanism for manganese, such as the one for iron. The same reasoning leads to similar reservations relative to studies of the metal's elimination. If large amounts of inorganic compounds are fed, there is little wonder that they should be recovered rapidly and almost completely in the feces. Furthermore, the trace amounts which are excreted as complexes with constituents of bile, pancreatic and possibly intestinal juices might be reabsorbed to be again excreted.

The isotopic techniques, if properly applied, are ideally suited to the study of this type of problem. Such studies were done early by Greenberg and associates. In a paper published in 1940, Greenberg and Campbell (81) gave data on the absorption excretion and distribution of Mn^{54} in the rat. The isotope's specific activity was 0.1 μc/mg of Mn. One milligram of stable manganese was given as the chloride to each animal, which is indeed a high dose. At the end of 75 hours, 97.2% of the orally given manganese was recovered in the feces. Following intraperitoneal injection, 90% had appeared in the feces at the end of the same time. On dissection of the orally-tagged animal, 2.8% of the dose was found distributed among the various tissues. The authors have given a detailed list of concentrations in each organ.

In a later publication, Greenberg, Copp and Cuthbertson (82) gave again Mn^{54}-tagged manganous chloride to their animals. Now, however, the doses given ranged from 10 to 100 μg of stable metal, a marked difference from the previous loads. They estimated that about 3 to 4 per cent of the orally administered dose was absorbed. By creating bile fistulae, they found that manganese quickly appeared in the bile, and that this might constitute the main excretion route. They found little or no radioactivity in the urine of their animals. Injections of the radioisotope preparation

into a lactating female resulted in tagging of the young with isotope. Among the various organs of the adult rat, the liver seemed to accumulate the highest concentrations of the labeled manganese.

Bertinchamps and Cotzias (18) have found that the early equilibrium of carrier-free Mn^{54} between blood and bile was exceedingly rapid. This was revealed by the fact that the change in concentration of the isotope in bile as a function of time had been almost identical with that seen in the blood stream. The isotope was shown electrophoretically to exist in the bile in the form of three different kinds of compounds.

Mohamed and Greenberg (130), in a study of perotic and normal chicks, observed that Mn^{56} was slowly absorbed from the gastrointestinal tract in both groups. The deficient chicks might have absorbed slightly more manganese than the controls. The short half life of their isotope (2.6 hr.) did not permit sufficiently prolonged observations.

In the work of Burnett *et al.* (31), use was made of both Mn^{56} and Mn^{54}. One dog with pancreatic fistula received one intravenous injection of 7 mg of manganese as the chloride, labeled with 541 μc of Mn^{54}. The juice was collected for 3 days thereafter. Concomitant fluctuations of radioactivity and nitrogen concentration were observed in the juice.

Another dog was similarly handled, but the observations were continued for 12 days. One hundred and ten collections were made. The data pertaining to nitrogen and Mn^{54} concentration were subjected to a statistical analysis which showed a high probability of positive correlation. Spectrographic analyses of pancreatic juice collected prior to the readministration of manganese showed it to contain 2–20 μg of stable manganese per 100 ml. Following the administration of the isotope preparation, these figures rose to 5–100 μg per 100 ml. The authors interpret this as meaning that no excess of metal had been given, and one might argue this point. The feeding periods were accompanied by increases of Mn^{54} in the pancreatic juice. Secretin injections produced little change in concentration of Mn^{54}, in spite of marked increase in the volume of juice. Eserine and pilocarpine had little effect. Mecholyl increased the concentration of Mn^{54} while it decreased the flow of juice. Dialysis of the juice or precipitation of its proteins with trichloroacetic separated the radioactivity from the protein fraction entirely. Large amounts of radioactivity were found in the feces of both dogs, while the urine contained only traces. The total excretion of radiomanganese via the pancreatic juice is not stated; a very rough calculation from a figure illustrating data on their first dog showed that about 1.5 per cent of the injected radioactivity appeared in the pancreatic juice during the first 70 hours. In view of the load of carrier given to that dog, one might assume that pancreatic juice is normally a minor route of excretion.

Cotzias and Maynard (47) have shown that the early distribution of Mn^{56} in the tissues of mice (and, therefore, presumably its excretion) was markedly affected by various chelating agents.

Maynard and Fink (125) have presented data collected on patients, one of whom had a bile fistula. They used both Mn^{56} and Mn^{52} with stable manganese carrier (<1 mg per test). They observed that the excretion of manganese was primarily fecal. The isotopes could be briefly diverted into the urine by means of pre-chelation with versene. They observed a striking correlation between the bilirubin content of bile specimens and the concentration of Mn^{56}. Thereafter they injected mice with Mn^{52} and studied the total body retention of the isotope. This was accomplished by means of determining the total body radioactivity daily in these mice. They found that manganese was lost from the body at two rates, which had respectively a half

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time of 13 hours and 168 hours when 1.0 mg of stable manganese had been given the animals concomitantly with the injection of the isotope. When 10 mg were given instead, these values fell to T/2 = 9.4 and 130 hours respectively. These figures show that the rate of elimination of radiomanganese is a function of the degree of the body's stable manganese load.

All these observations, with the exception of the work of Burnett *et al.* (31), make exclusive use of tracers without reference to analyses for stable metal. The behavior of the radioisotopes provides only an indirect means of making assumptions relative to the behavior of stable manganese in the body. Direct measurements on the rate of excretion of stable metal have been reported by Kosai and Boyle (108). They observed that rats given injections of $MnSO_4$ in very large doses excreted manganese primarily, if not exclusively, in the feces. Following administration of calcium ethylenediaminetetraacetate, the urine contained considerable amounts of metal. These observations, when viewed with that of Penalver (145), suggest that stable manganese is available to some extent for chelation in the body and diversion into the urine.

Cotzias and Greenough (48) were primarily interested in studying the specificity of the pathway of manganese through the body of the mouse. However, in pursuing this study, they made some observations on the rate of elimination of radiomanganese from the body. The fact that they injected carrier-free Mn^{54} permitted them to see an exquisite sensitivity of the isotope's rate of elimination towards the amounts of stable manganese given to the animals. This was the case irrespective of whether these compounds were fed or injected. Acceleration of the rate of elimination was seen irrespective of how early or late after the tagging of the animals they gave the stable compounds. Furthermore the kind of manganese compound seemed immaterial, since qualitatively similar results were seen following injections of manganese metal powder or manganous, manganic and permanganic compounds. Obstruction of the gastrointestinal tract at the anus, or starvation, abolished the elimination of radiomanganese, while changes in acid-base balance had no effect. In marked contradistinction to the ease with which manganese compounds eluted radiomanganese from the bodies of mice was the inability of metals other than manganese to bring on any change in the rate of elimination of the isotope. This is discussed elsewhere in this review.

Cotzias, Curtis and Greenough (51) have obtained evidence that manganese which is excreted with the bile into the gut is partially reabsorbed. This observation tends to explain, in part, the exceedingly rapid rate of elimination of injected radiomanganese in mice eating diets of high manganese content: unabsorbed amounts of carrier manganese in the gut would carry with them some of the bile-bound radiomanganese out of the body. Furthermore, it is known that manganese deficiency can be aggravated in chicks and rats by feeding the animals high phosphorus or high calcium diets. It is, therefore, conceivable that a number of inorganic compounds might act as carriers of manganese out of the gut or might dissociate the metal from absorbable chelates. It would be interesting to know, in that connection, what would be the risk of inducing trace metal deficiency nowadays, when 'minerals,' including iron, are being fed promiscuously to humans.

Manganese as a Nutrient

Food (127), feed (13) and fertilizer (133) alike have been studied in order to show that manganese is essential in nutrition. The next step, namely how much manganese is essential (and for what) constitutes a difficult task. Here, one does

not enjoy the comfort of a clear-cut end-point (e.g., hemoglobin [illegible] in iron deficiency). Only too often maximal responses are taken as meaning optimal ones, irrespective of whether an end-point is sought in an enzyme's average reaction velocity, in the weight of a bone, or in the mating of animals. Furthermore, the fact that severely deficient animals benefit from the addition to their diet of a precise amount of inorganic manganese, does not imply that the specified amount constitutes the daily requirement of a normal animal. The diets which produce deficiency might not be normalized merely by the addition of an inorganic salt. The manganese which normally occurs in animals or plants (in their tissues or their secretions) has a high probability of forming one or more specific organic coordinates. This can hardly be expected to be reproduced when arbitrarily selected diets are supplemented with an arbitrarily selected manganese salt. This issue is important not merely to the eventual understanding of manganese metabolism, but also to the study of the metabolism of any metal capable of forming chelates. It is, therefore, stressed repeatedly throughout this review. Furthermore, the addition of one inorganic metal salt in large amounts to a given diet can be expected to induce a series of changes in the association of a number of other metals with their own organic receptors. Thus the results might be far reaching indeed. This is illustrated by the large additions of calcium or iron to diets of chicks: perosis might be produced in the presence of adequate manganese concentrations. Conversely, similar 'chain reactions' might readily occur with the addition of organic coordinating agents into diets. This is reflected in the effects of choline, inositol, aureomycin, and thiamine which are being discussed elsewhere in this review. Therefore, the transport of manganese through the gut, its utilization and its excretion are indeed subject to the peculiarities of the individual chelates formed during each step. The experience with hydralazine indicates that the agent diminishes abnormal urinary excretion of manganese while it concomitantly creates what seems to be a manganese depletion (46). Christensen (41) has discovered coordinate manganese complexes with Schiff's bases of pyridoxal and amino acids, which seem to enter the tissues with great speed (42). Conversely, chelation with versene sequesters the metal in the blood and finally diverts it into the urine (125). These findings suggest that the concept of 'available' manganese is important not simply while the metal is outside the body, but particularly after its entrance has been consummated.

If all these points were to be conceded, there is little that the literature can offer relative to requirement of animals for 'normal' dietary manganese. One might even doubt the accepted notion that birds need more manganese than do mammals (194), since it is based primarily on manganese levels in the diets and not necessarily on amounts in the portions consumed.

The work of Smith and Ellis (182) indicates that rabbits require about 300 μg of inorganic manganese per animal per day for satisfactory growth. Holtkamp and Hill (97) found that 0.2 mg per rat per day was optimal for growth, while Shils and McCollum (177) found .3 mg per kilogram diet to be suboptimal. The results of Smith, Medlicott and Ellis (183), as well as those of Wachtel, Elvehjem and Hart (200), are similar to those of Shils and McCollum. Bentley and Phillips (15) found mild symptoms of manganese deficiency in chicks receiving 7–10 mg of manganese per kilogram of diet. Plumlee and associates (152) could not see signs of manganese deficiency in weanling pigs receiving 3.5 mg of manganese per kilogram of diet. Johnson (99), again in weanling pigs, found the required level to lie nearer to 0.5 mg of manganese per kilogram diet. Grummer, Phillips and Bohstedt (85) observed

effects on reproduction but not on bone development in pigs reared on 12 mg of manganese per kilogram of diet.

Gallup and Norris (74) found that perosis in chickens was prevented by diets containing 30–50 μg of manganese per kilogram. That this level had to be materially raised under some circumstances, and that the picture is similar in turkeys, swans and ducks, is discussed under the heading of PEROSIS.

Man's requirements for manganese can only be roughly estimated, since, until the appearance of Comens' work (46), even the essentiality of manganese in human nutrition was mooted. Kent and McCance (107) have estimated their patients' daily intake as ranging between 3 and 9 mg of manganese per day, depending upon the selection of the ingredients in the diets. This estimate is important, since no supplements were used. In children, Everson and Daniels (65) believe that 200–300 μg of manganese are required for each kilogram of body weight—indeed a substantial estimate. However, Plumlee *et al.* (152) have also found that suckling pigs require more manganese than do even weanling pigs. Basu and Malaekar (12) have considered 5 mg per day as adequate for manganese balance in the adult human. Monier-Williams (131) has estimated the daily adult intake as 7 mg of manganese per person per day, while the data of Kehoe, Cholak and Storey (104) suggest 4 mg per day as an average figure.

Manganese concentrations in feeds of chickens have been investigated quite extensively. Schaible, Bandemer and Davidson (168) have published analyses of several wheat products. They found that their samples of wheat germ contained 100 mg of manganese per kilogram sample, while low grade flour contained only 5 mg per kilogram. Bandemer, Davidson and Schaible (10) have investigated the availability of manganese to the hen when the salts of the metals were varied. Caskey and Norris (36) have also studied the manganese contents of various cereals. Heller and Penquite (91) have analyzed rations of chicks, in relation to their work on the effects of high calcium and phosphorus intakes in perosis.

Peterson and Skinner (151), as well as Hodges and Peterson (95), have analyzed a considerable number of foods for their manganese content. Their lists of foodstuffs show that manganese concentrations were highest in samples of nuts and lowest in sea foods. Seeds of various kinds were also high in manganese, while dried milk and milk products were almost as low as sea food. Thus it appears that diets consisting largely of nuts, cereals and dry legume seeds would provide 20–23 mg per kilogram diet. Menus using only vegetables and meat would provide 1–3 mg per kilogram diet. Exclusive consumption of fish, meat and poultry would lower this figure to 0.25–0.5 mg of manganese per kilogram diet. The earlier figures of Lindow and Peterson (110) are roughly comparable with the ones given here, and so are those of Monier-Williams (131).

Archibald and Lindquist (6) have observed an increase in the manganese content of milk from cows receiving high manganese intakes. Sato and Murataki (166) have found 50 μg of manganese per liter of mare's milk, which indicates that not only cow's milk but probably milk in general is a low manganese food. This is indeed interesting in view of the truism that mother's milk is good for the young. Lorenzen and Smith (118) have shown very limited reserve stores in their newborn animals, which showed an increase between birth and weaning, indicating a high degree of availability of manganese in milk.

Twyman's work (193) has pointed out the fact that plant tissues show much larger variance in manganese concentrations than do animal tissues. This fact was

fully recognized at the time Von Oettingen's review (198) was written. The relative stability of manganese concentration in mammalian tissues, as well as the lack of important species differences, has been pointed out by Fore and Morton (70). Thus, until such time as manganese analyses become easier, these data can help in obtaining rough estimates of dietary manganese intakes by humans.

Coleman and Gilbert (44) have studied the caffeine and manganese content in many samples of teas and coffees. In teas the manganese concentration varied from 80 to 140 mg per kilogram of dry leaf. As a rule, the higher the manganese concentration, the lower was the caffeine content. This indicates to this reviewer that manganese in tea might be chelated with a constituent, (possibly an alkaloid) other than caffeine. The coffees examined contained 14–32 mg of manganese per kilogram of raw coffee beans. The variance was larger than that of the caffeine concentration in the same beans, and supports the notion relative to the manganese chelates in these foods. In any event, these two beverages constitute rich sources of dietary manganese.

BEHAVIOR OF MANGANESE IN THE TISSUES

Kinetic Observations. In this section, some of the evidence is discussed which has been obtained primarily by studying living animals. Reference is made to exclusively *in vitro* work only if it is expected to supplement the discussion at hand. The reasons for avoiding the strictly biochemical experiences are two-fold: the literature is enormous, and the reviewer does not quite know what it all means relative to integrated animal life. This reservation is due to the fact that the actions of manganese *in vitro* can be duplicated with metals other than manganese, as shown by enzyme activation (126, 188), transport systems (163), and even by the preservation of isolated mitochondria (68). However, if our review of manganese deficiency and chronic manganese poisoning shows anything, it certainly shows the existence of some degree of specificity of their manifestations. To illustrate this point further: manganese has been known to activate arginase *in vitro* (7, 59, 60). Some (194) even believe that this might be the metal's main function, but birds have apparently high requirements for manganese and their tissues do not have any arginase. Thus, it was considered best to avoid integration of purely biochemical observations into this discussion.

In their early observations in the rat, Greenberg and Campbell (81) found that after injection of $Mn^{54}Cl_2$, the tissues were enriched with radioactivity in the following order of decreasing concentration: skin, bone, liver, muscle and small intestine. Such a partition of an injected manganese isotope is now known to be characteristic of a large load of stable manganese, of a large time period of equilibration, or of both. Indeed, Greenberg and Campbell had waited 75.5 hours before killing their animals, and had given 1 mg of Mn with the tag. The time element and the specific activity of the preparations used have been found subsequently to be the two variables which have caused the largest discrepancies between various studies of partition of manganese in tissues. The same paper contains evidence of the fact that another important variable is the route of administration of the isotope. They found a different ranking of the organs after injection than after feeding the isotope preparation. In a subsequent paper, Greenberg, Copp and Cuthbertson (82) found the liver to be a preferential site of accumulation of the isotope. This time the amount of stable manganese administered was lower, and the equilibration time was shorter.

Born, Timofeeff-Resowski and Wolf (24) have studied the partition of Mn^{56} among the organs of the rat as a function of time. They have found a different partition after ½ hour than they did 6 hours after injection. These observations clearly demonstrate that the rate of turnover of manganese differs from one organ to the other. A definitive study of such rates has not been done as yet, in part due to the vagaries of the analyses of stable manganese.

Deysach and Ray (57) were impressed by the fact that the thyroid of the guinea pig tends to accumulate stable manganese following injection. The accumulation reached its peak at about 24 hours, and a decline set in thereafter.

Lang *et al.* (113) became interested in the intracellular behavior of manganese in relation to arginase. They isolated pig liver nuclei by a centrifugal method of fractionation with 10% sucrose as the medium. They found an equal partition of arginase between nuclei and cytoplasm. Then they injected a Mn^{56} preparation intravenously into the pigs. They found that 1.5 hours later the liver contained 13% of the injected dose, while the isolated liver nuclei contained 0.3%. In terms of the radioactivity of the liver, the liver nuclei contained 2.1%. Three hours after injection the content of the whole liver had fallen to 7% of the injected dose. The liver nuclei contained 2.5% of the organ's radioactivity. The reviewer has assigned to the nitrogen content of the nuclei the value of 5% of the total liver cell nitrogen. In that case it is evident that even as early as 3 hours after injection of Mn^{56} into the pigs, about twice the concentration of isotope was localized in the cytoplasm as compared to the nuclei. This point will be discussed further below.

Burnett *et al.* (31) have studied the localization of Mn^{56} in the liver and pancreas of rats and mice. In both species they found evidence suggesting that the liver becomes enriched faster than the pancreas, following injection of the tag.

In a series of four papers, Fore and Morton (69–72) have studied various aspects of manganese metabolism. These are referred to repeatedly in other sections of this review. In their study of ox bone, they found that most of the manganese was associated with the inorganic matrix and that little was bound to organic matrix, including the water-soluble alkaline phosphatase. They vigorously dialyzed kidney and liver homogenates and found that there exists a non-dialysable fraction of manganese in those organs, which amounts to 500 μg per kilogram of tissue (or 20% of the total manganese content). In their experiments with retinal tissue, they found not only that retina is very rich in manganese, but also that the latter is nondialysable.

Maynard and Cotzias (124) observed that intravenously injected Mn^{56} disappeared very rapidly from the blood stream of man and rat. They were impressed by the early accumulation of the isotope in organs which are known to be rich in mitochondria (liver, pancreas, kidney) and by the relative lack of avidity of mitochondria-poor tissues (muscle). They thought that the mitochondrium might constitute a site of early and preferential accumulation. They therefore fractionated centrifugally the liver homogenate following injection of Mn^{56} into their animals. They found that ½ hour after the injection, the mitochondria contained (on the basis of nitrogen) twice the amount of radioactivity that was found in the whole homogenate. They presented evidence suggesting that this was a minimal figure, since the washings inherent in the technique tended to extract more radiomanganese than nitrogen from the isolated organelles. In later experiments, Cotzias and Greenough (50) have extended these observations to mouse and cat liver as well as brain, with essentially concordant results. The early localization of radiomanganese in brain mitochondria is interesting also in view of the slowness with which the isotope enters that tissue.

The kinetics of the disappearance of Mn^{52} from the blood of humans were studied further by Maynard and Fink (125). They found that prechelation of the tag with ethylenediaminetetraacetate tended to cause some degree of retention of the isotope in the blood.

Thiers and Vallee (190) conducted spectrographic analyses of stable metals, including manganese, in fractions of rat liver. The latter were identical to the ones reported by Maynard and Cotzias (124). Their study of metals other than manganese is interesting to this reviewer because it shows the individuality which each of them displayed. The fact that a complementary reciprocity was seen between the manganese and the iron content of the various fractions makes the differences of the patterns of the other metals even more striking. The reciprocity between iron and manganese content manifested itself when the fractions were ranked on the basis of the percentage of concentration of these metals per cent of nitrogen. Nuclei, mitochondria, microsomes and supernatant fractions showed a decreasing number of 'units' for manganese and an increasing one for iron. (Note that the ranking of stable manganese is different from that found with manganese tag ½ hour after injection.) Washing the mitochondria with sucrose solution had little effect on their stable manganese concentration, while it will be recalled that the same maneuver had markedly affected the radioisotope content of these organelles.

Now, a comparison of the experiments of Maynard and Cotzias (124) and Cotzias and Greenough (50) on the one hand with those of Thiers and Vallee (190) on the other leads to the conclusion that the intracellular organelles of the hepatic cell have different rates of turnover of manganese. The mitochondrial turnover is very high and the nuclear one low. This means, in common parlance, that there exists a highly organized intracellular traffic for manganese. Whether the entire pathway is intracellular or whether individual manganese moieties exit the cell on their way from one organelle to another, is presently a semantic problem. The definition of the turnover for each one of the organelles is not. They should be considered as individual pools and subjected to the type of analysis recently discussed by Robertson (159). The fact that radioactive (= new) manganese was easily removed from isolated mitochondria while stable (= old) manganese was not, shows the existence of variable degrees of association of manganese with mitochondria. A similar thesis was reached by Fore and Morton (71, 72) relative to kidney and liver homogenates. Although their experiments are not comparable to these, one wonders whether their drastic dialysis did not preferentially remove cytoplasmic manganese.

These considerations have led Borg and Cotzias (23a) to study the possibility of developing a bed-side method for the measurement of total mitochondrial mass. They found that the blood disappearance of Mn^{56} (injected intravenously) showed a consistent pattern from patient to patient. The curves had 3 components, indicating 4 participating body compartments. They observed a striking correlation between the second of their components and the uptake of tag by the liver when the latter was being viewed by a radiation detector. As can be seen in figure 1, the rates involved were exceedingly fast. On the basis of related evidence published by others, Borg and Cotzias have concluded that the second of the components in question reflects the entrance of Mn^{56} in all the mitochondrial pools and not only those of the liver.

In view of the known displacement out of the body of such elements as lithium

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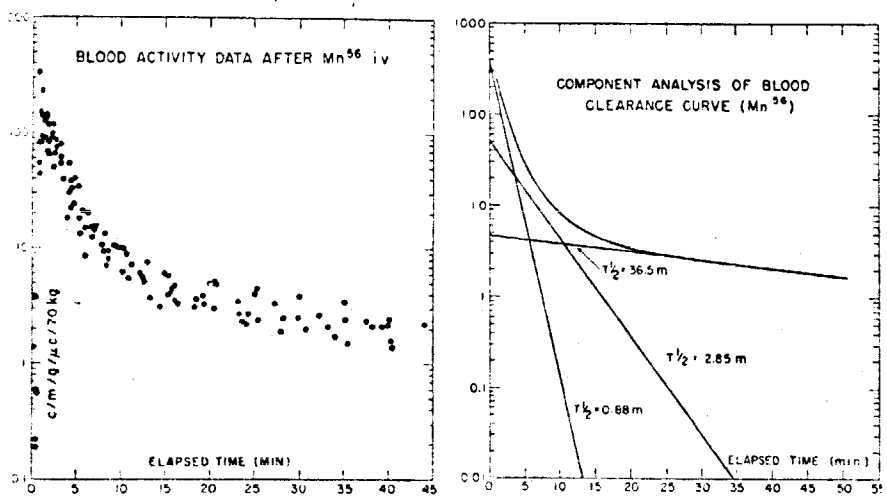

FIG. 1. Blood radioactivity and clearance of manganese.
Left, disappearance of radioactivity from blood following intravenous injection of Mn^{56} (humans). *Right*, exponential component analysis from same data. From further development of these data along with liver area radioactivity measurements, the second component ($T/2 = 2.85$ min.) appears to reflect the movement of manganese into and from mitochondria (23).

by sodium, strontium by calcium, molybdenum by tungsten, and others, Cotzias and Greenough (48) have studied manganese in that regard. They did this because manganese is excreted in the feces, primarily via the bile, while these other elements are excreted primarily in the urine. They found that Mn^{54} could be eluted out of the bodies of mice by injections of several manganese compounds, including manganese metal powder. Injections of a considerable number of other stable metal compounds failed to cause the slightest change in the normal rate of excretion of radiomanganese. Concordantly, injections of stable manganese compounds markedly changed the distribution of radiomanganese in the body while other metal compounds did not. From this and some other evidence they argued that there exists a specific step in the pathway of manganese through the body and that that step is located intracellularly.

Concentration. The concentration of manganese in individual tissues seems to be constant in spite of the rapidity which characterizes some phases of its transport. The stability of the metal's concentration impressed the early investigators (194, 198). More recently, Brückmann and Zondek (30) have studied the concentration of manganese in human livers and have pointed out its constancy from one specimen to the next. This constancy, however, seems to hold even when comparisons of organs from different species are made. Fore and Morton (70) have averaged the data in the literature and have compared that average with their own analyses of rabbit tissues. A striking similarity between the concentrations in various tissues became apparent. This has strengthened the notion that, unlike plant tissues, each animal tissue contains a concentration of manganese which is almost characteristic of the individual organ and independent of the species of the animal with which the tissue has originated. This indicates that manganese metabolism is homeostatically controlled, as has been also suggested by the dependence of its turnover upon the total body burden of metal.

A further comparison, wherever applicable, between the data of d Morton (70) on the one hand and those of Butt *et al.* (32, 33, 83), of Thiers a allee (190) and of Bowen (25) on the other, showed them all to be roughly comparable. The values of Tipton *et al.* (191) were considerably lower. Inspection of the concordant data showed that bone contained the highest concentration of manganese with an average of 3.5 mg per kilogram of wet tissue. The pituitary, the pineal body and the liver were next, with concentrations of roughly 2.5 to 2.0 mg. per kilogram. The component parts of the gastrointestinal tract, the pancreas, the salivary gland and the kidney contained 1.7–0.7 mg per kilogram. The other organs contained lower concentrations. Among the lowest were the bone marrow (0.04 mg/kg), the blood (0.03 mg/kg) and the lung (0.01 mg/kg).

In other sections of this review evidence is discussed which indicates that the concentration of manganese in some tissues may be substantially decreased by the creation of manganese deficiency, or that it may be increased by means of feeding of inorganic manganese salts. In relation to the latter situation, it would be of interest to know whether administration of natural foodstuffs of high manganese concentration is capable of bringing on the increase in tissue concentration which has followed the administration of inorganic manganese salts.

Excessive Manganese Intake

The great difficulties which have characterized the efforts to induce brain damage in animals with the aid of manganese compounds (196, 198) are reflected also in the experiences involving feeding of large amounts of manganese. Richards (158) has seen no toxic effects after he had fed his pigs 3.5 gm of manganous citrate daily for 9 months. Lindow, Peterson and Steenbock (117) have reported merely high tissue concentrations after feeding large amounts of manganese to their animals. Gallup, Walter and McOsker (76) found a similar situation in lambs. Skinner (179) gave diets containing 2.0 gm of manganese per kilogram diet to rats, without seeing any ill effects. On the other hand, Chornock, Guerrant and Dutcher (40) did observe retardation of growth when their rats were given massive doses of manganese (1.73% of dry diet). There was loss of calcium in the feces of those animals and the phosphorus balance seemed to be negative. Severe rickets developed in some animals. These observations seem to indicate that massive manganese intake interfered with the absorption of calcium and phosphorus. (It should be recalled that massive intakes of these elements interfere also with manganese absorption.) That this effect is not reflected only in bone is shown by the study of Wessinger and Weinman (204) who observed hypoplasia of the rat's dental enamel following the institution of high manganese body burdens.

Hobbs and Hansard (94) have seen retardation of growth in pigs the diet of which contained 500 mg of manganese per kilogram of diet. They report that at the end of the trial the animals became 'stiff.' Reid *et al.* have similarly observed a negative calcium and phosphorus balance in lactating cows given large oral doses of manganese salts (155).

Hartman, Matrone and Wise (87) have studied the effect of high manganese intake on hemoglobin formation in lambs. The animals were depleted of iron either by feeding them a milk diet or by phlebotomy. High manganese intakes caused reduction of hemoglobin formation and low iron concentration in the liver, spleen and kidney. These experiments indicate that massive amounts of manganese can carry the dietary iron right through the gastrointestinal canal. That the opposite

is also true is discussed in relation to perosis of chickens. The experience of Gubler *et al.* (86) relative to the interplay of dietary manganese with copper is discussed, together with the evidence which relates manganese to some disease states.

Paterni (143) has reviewed the relation between blood formation and abnormal metabolism of metals. He stated that Zolezzi has seen a stimulation of hematopoiesis in animals following injections of manganous sulfate. Sabatini is credited with the observation that permanganate injections cause hemolysis and Penati with the observation of polycythemia following administration of manganese to animals.

In a very extensive communication, Ravera and Bellotti (153) have given a painstaking account of experiences related to the pathological physiology of manganese. This publication is notable for its discussion of work which was published in journals not readily available to American scientific workers.

Blakemore, Nicholson and Stewart (19) have reported that lactation tetany of cows occurs in pastures which are high in manganese, and that the disease is accompanied by a very marked elevation of the manganese level in the blood. Fain, Dennis and Harbough (66) have suggested that there might exist a correlation between the level of dietary manganese and the development of low blood magnesium levels in cows. It seems that when the supplement amounted to 100 mg per kilogram diet, the magnesium level fell, but above and below this value it did not seem to do so. Carlstrom and Hjarre (35) have correlated the development of infectious anemia in horses with the animals' grazing habits. This disease is presumably of virus etiology, but it was prominent in horses grazing in pastures which contained excessive amounts of manganese.

While Gallup and Norris (74) found that chickens tolerated diets containing 1000 mg of manganese per kilogram of diet, Heller and Penquite (91) have reported toxic effects at the massive intake of 4.8 gm per kilogram of diet.

Manganese Deficiency

Induction of manganese deficiency has proven its worth as an experimental tool in proving the essentiality of manganese in the body (177, 208, 209), in demonstrating some of its physiological functions (198), in suggesting nutritional requirements (131, 194) and possible interrelationships with other nutrients (101, 102, 200, 211).

The fact that the cardinal manifestations of the deficiency are similar in birds and in mammals seems to argue against this metal being primarily involved in urea synthesis, as was thought to be the case (7, 59, 60), since birds do not synthesize urea and their tissues contain little or no arginase. Furthermore, birds appear to require more manganese than do mammals (194). This, when viewed in the light of the higher body temperatures and oxygen consumption of birds, lends support to the notion that manganese is primarily involved in oxidation-reduction processes (115, 124, 213).

The most reproducible manifestations of the deficiency have included deceleration of the growth process, induction of bone abnormalities, dysfunction of reproduction and central nervous system pathology.

As noted by Underwood (194), manganese requirements and utilization appear to be much higher in birds than in mammals. Because of this, as well as because of the many other metabolic differences between these two groups, they will be considered separately. It has to be emphasized, however, that the manifestations of manganese deficiency in various species are more impressive in their similarities than

in their differences. This consideration might justify some extra as to man, about whom very little direct evidence is available in this regard. variance in the extent of the reported deficiency, and the occasional coexistence of obvious deficiency other than that of manganese, tend to confuse the picture only relative to the less striking manifestations.

Manganese Deficiency in Mammals. Bertrand and his colleagues (198) were among the first to comment on the omnipresence of manganese in all living matter, and on the constancy of its concentration in various tissues. They have postulated an essential role for this micronutrient. The several efforts designed to prove the latter thesis are summarized by Von Oettingen (198) and, more recently, by Underwood (194). In 1931 Kemmerer, Elvehjem and Hart (100) had produced conclusive evidence as to the role of manganese, by showing that fortified milk diets caused unsatisfactory growth and ovarian dysfunction in mice—both ameliorated with 0.01 mg of Mn^{++} per mouse per day. Waddell, Steenbock and Hart (201) made similar observations in the rat. At the same time, Orent and McCollum (141) discovered that poor lactation, high mortality of the young and testicular degeneration accompanied manganese deficiency. They observed, however, no significant differences in ovarian function or in the growth of the animals. Skinner, Peterson and Steenbock (181) observed that the total body manganese increased continuously after birth but that, concentrationwise, it had two peaks, one at birth and one at weaning. Addition of manganese to the mother's ration during gestation increased the concentration in the newborn. Addition of manganese to the diet of deficient mothers during lactation did not increase the metal's concentration in the sucklings. The interesting observation was made that, following weaning (at the beginning of consumption of manganese-rich rations) the percentile concentration of manganese decreased. A similar experience may be found also in the work of Smith, Medlicott and Ellis (183).

Daniels and Everson (54) found a high mortality among the offspring of their manganese-deficient rats. In order to differentiate between debility of the young and poor lactation of the mothers as a cause of the mortality, they switched the young from deficient to normal mothers; this failed to restore normal development. Boyer, Shaw and Stewart (26) observed that manganese-deficient rats showed lower food consumption, lower food utilization, delayed opening of the vaginal orifices and degeneration of the testes, affecting primarily the exocrine apparatus.

Working with pigs, Miller *et al.* (132) as well as Keith *et al.* (105) discovered the appearance of lameness and stiffness of the legs preventable, but not curable, by manganese supplements. Although this is the first allusion to a neurological disorder associated with manganese deficiency in extrauterine life, it will be discussed together with the later observations of Plumlee *et al.* (152) for the sake of brevity. Johnson (99, 100) working also with pigs, observed a tendency of their tissues to reflect the manganese concentration in the food. Amdur, Norris and Heuser (3) showed that manganese deficiency in the rat was associated with bone changes, including low breaking strength and density as well as decreased bone length. Rudra (162) observed that manganese-deficient animals displayed the phenomenon of manganese hunger. The animals plucked one another's fur and ate it, attacked wood, cork and thread, and licked their metal cages. Control animals fed manganese did not show this vicarious appetite. Hill, Holtkamp, Buchanan and Rutledge (93) raised four generations of rats on a diet calculated to offer .03 mg of manganese per day per adult rat. Ataxia, loss of equilibrium, backward gait and serious postural reaction to stimuli (even of their own making) appeared in subsequent generations of defi-

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cient animals. The brains were examined histologically with serial sections stained with hematoxylin-eosin. No morphological abnormalities could be seen. Barness, Sperling and Maynard (11) found changes in bone structure similar to the ones discussed above.

While the previous investigators have relied primarily on fortified or pure milk diets for the development of manganese deficiency, Shils and McCollum (177) introduced a solid diet containing 20 per cent powdered milk. This diet provided on the average 3 μg of Mn per day per rat. Growth appeared normal when 0.18 gm $MnCl_2$ was added to 100 gm of diet. The overwhelming majority of offspring of deficient mothers were not weaned until manganese was given to these mothers during subsequent pregnancies. The deficient mothers, however, behaved normally and supported the growth of normal newborn foster offspring. (These findings are at odds with the earlier ones of Boyer *et al.* (26) who had used milk diets to induce the deficiency.) Shils and McCollum (177) further induced the deficiency in adult females, which proceeded to give birth to successively less viable offspring. The young of all deficient animals showed abnormal growth and skeletal deformities similar to the ones to be described below in connection with the rabbit. Frequently ataxia, incoordination, and loss of balance were seen. (Note that the pigs referred to above, as well as the chickens to be referred to later, also developed nervous system manifestations. So do men with chronic manganese poisoning.)

Determinations of liver arginase activity in deficient and control rats showed that the former was lower but could be raised to the level of the latter with manganese. Shils and McCollum (177) extended their observations to mice, with essentially concordant results.

Wachtel, Elvehjem and Hart (200) used a diet offering about 5 μg per rat per day. This created impairment of growth, corrected by the addition of manganese and aggravated by high Ca/P ratios in the diet. These results indicate that manganese deficiencies of even more pronounced degree than the ones hitherto encountered might become evident if even lower manganese concentrations are offered the animals or if interference with absorption of the element becomes more pronounced. The bone changes encountered by Wachtel *et al.* were similar to the ones already described. A progressive anemia was seen and the deficient animals had markedly elevated alkaline phosphatase activities in their blood. Anterior pituitary extracts initially helped the condition, and in view of the entire picture, the authors postulate that the pituitary participates in manganese deficiency.

In an exceedingly interesting paper, Smith, Medlicott and Ellis (183) essentially extend some of the above findings to the rabbit. Their rabbits were kept on a milk powder diet from weaning to the eighteenth week. Control animals receiving manganese supplements grew better than the deficient animals but still at a slower rate than normally-fed animals. The deficient animals showed striking bowing of the front legs. Careful measurements of weight, density, length, breaking-strength and ash content of their humeri showed them all to be significantly diminished as compared to the controls. The humeri were short, thick and had swollen heads. X-rays showed regular epiphyseal lines in the otherwise decalcified and malformed bones, which differentiated this condition from rickets. Similarly, the marked reduction of cartilaginous spicules and the delayed osteogenesis speak in favor of this differentiation. The bone marrows were abnormally over cellular, in spite of the existence of a moderate anemia. One male rabbit showed marked degeneration of the exocrine part of its testes. The manganese content of the livers of deficient animals was lower than

that of the controls. The syndrome was prevented with 300 μg of Mn^{++} per rabbit per day, but maximum growth occurred with 1.4 mg per rabbit per day. Ellis, Smith and Gates (61) have extended the observations of Smith *et al.* (183). By using a paired feeding technique, they were able to show that liver arginase and bone alkaline phosphatase became considerably diminished as a result of manganese deficiency. They gave data on several other tissues which they did not consider significant. The bone phosphatase determination showed 9 units per gram in the deficient animals versus 32 units in animals receiving 1 milligram of manganese daily. These analyses were done on the ends of ulnae.

Smith and Ellis (182), in an effort to fix the manganese requirement for the rabbit, gave some interesting data on the interrelationships between tissue and dietary manganese levels. On plotting all their data, it appeared to this reviewer that both liver and bone manganese concentrations were, in the early part of the curves, in almost linear relation to the manganese intake. For both values, the maximum was reached with 4 mg Mn^{++} per rabbit per day. Although there was only one observation at 8 mg per rabbit per day, the data represent 6 animals. This reviewer became convinced that at 8 mg Mn^{++} intake, the Mn^{++} concentrations in these tissues fell significantly below those of the 4-mg intake level. He furthermore wishes to draw attention to table 3 of that paper, as he sees some interesting observations which the authors chose not to stress.

Plumlee *et al.* (152) have studied manganese deficiency in pigs, with special attention to growth, fattening and reproduction. Weanlings weighing 30-43 lb were fed low manganese diets without any effect on either growth or reproduction. When female suckling pigs (9 lb) were fed a dry skim milk diet, deficiency did develop. The test diet provided .5 mg of manganese per kilogram of diet, while the controls received the same diet with manganese added (40 mg/kg diet). The animals of the two groups had similar weights, but in the case of the test animals, this was contributed to a large extent by fat. The back fat was much thicker in this group. They further had muscular weakness, decelerated skeletal growth and shortening of the legs. Estrous cycles were irregular. Pregnancies did occur, but the fetuses were usually resorbed. If they came to term, the young were weak, small, ataxic, and displayed bad locomotion and balance. Milk production, milk secretion and udder development of the sows were poor at best. (Note that Amdur, Norris and Heuser (4), Schroeder (171, 172), and Curran (52, 53) have observed biochemical links between the metabolism of manganese and that of fats and lipids.) On the other hand, Speer *et al.* (185) have also reported increases in normally deposited fat in swine fed normal diets fortified with trace minerals, including manganese.

Bentley and Phillips (15) have studied manganese deficiency in cattle. They found that 10 mg of manganese per kilogram diet were inadequate for proper nutrition, and that the animals' ovaries showed low manganese concentrations, while other tissues apparently did not. The cows showed delayed onset of estrus, and their calves tended to have weak legs. Manganese supplements had a preventive effect. These observations were extended by Gessert *et al.* (78). Hawkins *et al.* (90) fed a milk diet to cattle which caused a deficiency state. That state was not reversed by manganese supplement unless the diet that had produced it also contained large amounts of calcium phosphate. Underwood (194) quotes Grashuis, Lehr, Benvery and Benvery-Asman as having encountered poor growth, leg deformities, poor fertility and frequent abortions in cows which had been grazing in manganese-poor (peat) pastures. The administration of 2 mg of manganese per day per cow was preventive.

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The work of Comens (46), who showed indirectly that manganese deficiency may occur in man, will be discussed under a different heading.

Manganese Deficiency in Birds. The deficiency manifests itself in birds in a manner similar to mammals. Here, however, it has been the spontaneously occurring clinical picture which was first recognized, before its relationship to manganese metabolism was discovered. The condition may manifest itself in two manners. In the postnatal birds, the deficiency gives rise to the disease known as perosis, while if it sets in during the embryonic life of the bird, it results in the development of chondrodystrophy. Perosis is characterized by bone deformities and dislocation of the Achilles tendon (slipped tendon). Perotic birds give birth to young suffering from ataxia. Chondrodystrophy is characterized by abnormalities of the bones and of the beaks of the birds, together with edema, protrusion of the abdomen and retardation of growth. Both of these conditions are prevented by adequate supplements of manganese in the diet.

Perosis was first linked to manganese deficiency by the far reaching investigations of Wilgus, Norris and Heuser (208). As seems often to be the case with important discoveries, these investigators came to recognize their lead as a result of an experimental incongruity which they followed up. While studying the aggravation of perosis of chickens with high calcium and high phosphorus diets, they encountered a technical grade calcium monophosphate preparation which improved the condition instead of aggravating it. They performed spectrophotometric analyses on this preparation and tested its metal impurities for their therapeutic effects. Later (209) they found that manganese possessed the capacity to fully prevent the appearance of perosis when added in amounts of 25 mg per kilogram to perotogenic diets already containing 10 mg per kilogram. Zinc and aluminum were far less effective, if at all.

Subsequent investigations have further defined the pathogenesis, species susceptibility and prevention of perosis. Bandemer, Davidson and Schaible (10) investigated the availability of various forms of manganese added to feeds in order to prevent perosis. Schaible and Bandemer (169) created manganese deficiency in chicks reared on wheat products. They found that high amounts of calcium and phosphorus in the diet aggravated the condition or produced perosis in spite of adequate amounts of manganese in the diet. Omission of bone meal without additional manganese prevented the appearance of perosis. When bone meal was added, addition of high supplements of manganese (62 or 124 mg/kg diet) markedly reduced the incidence of the disease. Caskey and Norris (36) determined the concentration of manganese in various cereals fed to hens, and found that the breaking strength of the egg was higher when diets of higher manganese content were offered. Gallup and Norris (73) found that the normal chick bones contained 2.0 μg per gram while the deficient ones went as low as .6 μg per gram. These figures are for the fat-free dry bones.

Insko, Lyons and Martin (98), as well as Wilgus, Norris and Heuser (210), have studied the relative effectiveness of manganese and other minerals in the prevention of perosis. Manganese, among the metals, was quite specific in that regard. The latter group also observed the development of ataxia in their manganese-deficient chicks.

Wiese, Johnson, Elvehjem and Hart (206) have presented evidence suggesting that addition of inositol to perotogenic diets tended to protect from perosis when 'borderline' diets were used. Later, it was shown by Hogan *et al.* (96) that choline also influenced the development of perosis.

Wilgus and Patton (211) studied the relationship between the phosphorus and calcium content in the diet of chicks. They found that high concentrations of these

elements in low manganese diets aggravate the disease, while very concentrations can make a normal diet cause perosis. The effect of calcium and phosphorus is probably exerted on the availability of manganese for intestinal absorption. This view is supported by their finding that ferrous citrate supplements also tend to increase the severity of perosis in chicks.

Gallup and Norris (74) found that 80 per cent of their chicks would develop perosis if placed on a diet containing 10 mg of manganese per kilogram diet. At 50 mg per kilogram diet of manganese as $MnCl_2$, $MnSO_4 \cdot 4\,H_2O$, $KMnO_4$, $MnCO_3$ or MnO_2, the incidence fell precipitously to 4 per cent. The value of 50 mg of manganese per kilogram diet proved minimal also in a quantitative study of the requirements of the chicks. When perosis developed early, however, it could not be prevented even with manganese at the level of 1000 mg per kilogram diet, thus suggesting that the prerequisite pathology had started in the embryo. Furthermore, a strain of susceptible chicks developed perosis at the rate of 18 per cent while on a diet which provided 50 mg of Mn per kilogram diet. Another strain was completely protected with 30 mg per kilogram, indicating a strain susceptibility to the disease. The low manganese intake levels prevented optimum growth of chicks and pullets while the high intakes mentioned were not toxic.

Gallup and Norris (75), in a further study of the diet of the hens, have adjusted the manganese concentration at levels ranging from 13 to 1000 mg per kilogram. The manganese content of the eggs was proportionately increased from 45 to 342 μg per 100 gm of dry yolk. The deficient birds produced a low number of eggs and the embryos were subject to high mortality. The rate of hatching was concordantly low. The minimum required concentration in the embryo was found to be 5 μg, or 70 per cent of the total amount of manganese normally present in the egg, indicating a rather low safety margin. Surprisingly enough, chicks hatched from eggs low in manganese were not more susceptible to perosis than those hatched from high manganese eggs. Probably the total amount of manganese taken by the chick after hatching was more important than the manganese content of the egg at the time of hatching.

Caskey, Gallup and Norris (37) have done an extensive study of the disease in chicks. They have described in detail the shortening and thickening of the bones of these animals. They found no differences in body composition between their manganese-deficient and control animals (other than the already noted low manganese concentration). Golding, Schaible and Davidson (80) showed that perosis may occur at slightly different levels of manganese intake for different breeds of hens.

Wiese *et al.* (207) have discovered that prior to the actual development of recognizable perosis, the alkaline phosphatase levels of blood and bone fell below the control levels. Manganese added *in vitro* failed to bring the level of enzymatic activity up to the control level. This probably indicates that the protein moiety itself, rather than its metal activator, was deficient. Combs, Norris and Heuser (45) found that both bone and serum alkaline phosphatases were low in perosis, while Nielsen and Madsen (134) have found elevated blood phosphatase in that disease.

Mohamed and Greenberg (130) made an effort to study perosis with the aid of the isotope Mn^{56} (half life 2.6 hr.). They produced the disease in chicks and fed the isotope by mouth. Both the diseased birds and the controls absorbed only a small amount of the radioactivity given. The perotic birds concentrated more in the liver than did the controls. The isotope had too short a half life for the job it was supposed to do, and it is probable that longer observations with Mn^{52} ($T/2$ = 140 hr.) or Mn^{54} ($T/2$ = 310 days) the results might have been more striking.

Evans, Robertson and Wiebelm (63) have extended the observations of perosis to turkey poults. Evans, Rhian and Draper (64) have shown that concomitant choline deficiency aggravates the disease. Emmel (62) has extended the observations of the manifestations of perosis to swans and Bernard and Demers (17) to ducklings. The latter investigators have further confirmed that in ducks also there appears retardation of growth and decrease of the manganese content of bones when the deficiency is established. Low alkaline phosphatase activities were found in the serum of these animals. High phosphorus and high calcium intakes aggravated the disease here also. Van Reen and Pearson (197), also working with ducks, found that normal growth occurred with 40 mg of manganese per kilogram of diet. The alkaline phosphatase was reduced in the liver, kidney, heart and plasma of their deficient animals. These changes were not due to the presence of an inhibitor or to a shift in the pH optimum of the enzymes. No differences between deficient and control animals were found in an impressive series of enzymatic assays and vitamin tests. Caskey, Norris and Heuser (38) have done a comparative study of the body composition of perotic versus normal birds.

Comens (40) has produced perosis in chicks as part of his argument that hydralazine disease of man is a manganese deficiency. The drug hydralazine alone caused the disease, and the latter was prevented with manganese supplements.

Chondrodystrophy was known for a long time to affect chicken embryos. Its sporadic form had been described in 1926 by Landauer and Dunn (112) and was thought to be genetically propagated. In 1935 Byerly, Titus and Ellis (34) discovered a nutritional chondrodystrophy, and noted that green vegetables in the maternal diet, as well as exposure of hens to sunlight, had a preventive effect. The disease is characterized by retarded growth of the embryo, edema, large protruding abdomen, perosis affecting legs and other bones, shortening of the lower mandible and rounding of the heads which makes these chickens look like parrots. In 1937 Lyons and Insko (120) showed that manganese deficiency in the maternal diet would cause the disease and that manganese (40 mg/kg diet) would prevent it. Similarly, injection of .03 mg of manganese as the sulfate directly into the eggs prevented the disease. Chondrodystrophic eggs contained 2.5 μg of manganese as compared to normals, which contained 7.0 μg. This shows that the manifestations of manganese deficiency are a function of both the degree of the deficiency as well as the biological timing of its occurrence. The value of 2.5 μg of manganese is one-half that found in the experiments of Gallup and Norris referred to above.

Chronic Manganese Poisoning

This syndrome is now a classical one, being described in standard textbooks of both pharmacology (184) and neurology (84). The literature up to 1935 was covered by Von Oettingen's review (198). In the article at hand a description of the syndrome will be given only to facilitate some rough comparisons between the chronic poisoning and the deficiency of manganese.

Pathogenesis. The utilization of manganese compounds by industry (steel, batteries, ceramics, dyes, etc.) has markedly increased human exploitation of the earth's manganese deposits, the most popular of which seem to be the pyrolucites. Among the various workers exposed to manganese compounds, the ones who run the risk of inhaling manganic oxide dusts have been developing the disease almost exclusively. As a matter of fact, only the report of Kawamura, *et al.* (103), as far as I am informed, suggests that chronic manganese poisoning can appear in man by means other than inhalation of dust. They claim that manganism had developed

among people whose water supply was being contaminated by battery elements which had been buried nearby. Otherwise, the general experience has been that patients with manganese poisoning are to be found exclusively among underground miners, ore crushers and men operating sieves (27, 160). Furthermore, inhalation of manganese compounds seems to have provided the most effective means of causing toxic manifestations of a chronic nature in animals (196) and will be so discussed below.

The exact amount of manganese in the inhaled air necessary to produce poisoning is not of great importance. In their exhaustive study, Flinn *et al.* (67) report air concentrations as massive as 173 mg of manganese per cubic meter of air. They found no cases among men breathing air containing .30 mg or less of manganese per cubic meter. Justly, they have not considered this as a threshold concentration. Perusal of Rodier's (160) table also shows similar massive exposures prior to the development of manganism. The fact that relatively simple preventive measures such as those recommended by Rodier (160), Boyer and Rodier (27), and by Flinn *et al.* (67) tend to eradicate the disease, also argues for massive inhalation as a prerequisite in the development of this disease. Furthermore, Rodier (160) has pointed out that dusts of small average particle size ($< 5\ \mu$) were more deleterious than coarser varieties. Although he did not seem impressed by variance of chemical composition as a cause of variance in incidence of the disease, he made one interesting observation: that newly drilled dusts are poisonous, while old dusts are 'dead.' He has stated that braunite (a mixture of Mn_2O_3 and $MnSiO_3$) is much more poisonous than pyrolusite (MnO_2). From this he has assumed that "the less oxidized the compound the higher its toxicity." The above facts might partially explain some of the many failures to produce experimental manganism in animals (196, 198).

The fact that, even under conditions highly favorable to the development of manganism, a relatively small percentage of men has developed the disease, points out the existence of variance in individual susceptibility. This is discussed by Rodier (160) and some explanations are given (27). Penalver (144) has observed that affliction of brothers is not uncommon. He stresses, as does Rodier (160), the possible importance of alcoholism and syphilis as predisposing factors in manganism.

The time of exposure necessary for the appearance of the disease has varied from a few months to several years (27, 67, 160, 198), being apparently most common after 2 years of almost continuous exposure.

Manifestations. In view of the fact that the present article is not a clinical one, the nosological manifestations will be merely enumerated. The reports by Flinn *et al.* (67), Boyer and Rodier (27), Rodier (160) and Penalver (144, 145), who, among them, have studied a few hundred cases, have formed the basis for this enumeration.

The onset is very insidious, with asthenia, anorexia, apathy and loss of desire to talk. Manganese psychosis may set in, characterized by impulsiveness, or, according to Penalver (144), even by stupid crimes. Impotence is very common, sometimes following sexual excitement. Euphoria eventually sets in. Uncalled for laughter is characteristic. It is of interest that insomnia is followed by severe somnolence, the latter being present in the overwhelming majority of cases. (This throws severe doubt on the validity of somnolence as a sole criterion for virus infection in the evaluation of extrapyramidal illness of humans.) Headache is often present during the predromal phase, which, together with the often occurring leg cramps, confuses the differential diagnosis between manganism and infection.

Following these manifestations, or overlapping with them, there appear speech disturbances in the form of loss of modulation, slowness and difficulty in articulation.

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These symptoms might develop to complete muteness. Concurrently, expressionless, mask-like facies sets in, which is very similar to that of other, 'naturally occurring' extrapyramidal disorders. That these are manifestations of general clumsiness is evident from the fact that later difficulties set in with skilled movements, with ascending or descending, with maintenance of balance, and with gait. The latter is particularly prone to become a major problem with severe propulsion, retropulsion and, eventual, *pes striatum* (*Hahnetritt* = hen's gait) developing.

As the disease progresses, rigidity becomes marked, 'cog-wheel' becomes evident, frequent falls occur, all with little loss in muscular power. Finally tremor becomes noticeable, which, as in the other extrapyramidal diseases, is exaggerated by emotion, fatigue or stress. Similarly, micrographia also occurs here. Complete indifference, coupled with spasmodic laughter, is striking. As in the other extrapyramidal diseases, excessive sweating and salivation occur while pyramidal, cerebellar, general and special sensory symptoms and signs are, as a rule absent. Also, as a rule, the disease is not life limiting, although it has been severely incapacitating.

Accompanying the extrapyramidal manifestations, or independently, there occurs a pulmonary disease kindred to the pneumoconioses, characterized by a chronically underlying granulomatous process with acute febrile episodes setting in. Penalver (144) has seen little or no pulmonary disease in Cuba, while Rodier (160) has witnessed it in epidemic proportions in Morocco.

In terms of laboratory examinations, the presence of manganese in the urine, as revealed with periodate methods, is pathognomonic of the disease. Leucopenia with an otherwise normal differential count has been reported. The anemia which might accompany the disease is hypochromic (27, 67, 160).

The case autopsied by Flinn *et al.* (67) showed a very high concentration of manganese in both lungs. The other organs, when compared to the data collected by Fore and Morton (71), show a normal concentration of the metal in these organs with no abnormal distribution of manganese among them. This interesting finding is being discussed below.

Treatment and Course. The untreated syndrome of manganese posioning, if mild, can reverse itself as indicated by the evidence summarized by Van Bogaert (196). More advanced cases might become stationary after removal from exposure, while severe cases might become progressively worse at a relatively slow rate for long periods of time. If Penalver's case (as there is every reason to believe) had manganism, then at least one very severe case has been reversed following prolonged administration of di-calcium, disodium ethelenediaminetetraacetate. This important finding is also discussed below.

The pathological neurohistology of this disease is a controversial subject. Van Bogaert (196) has discussed the merit of various investigations, the findings of which have ranged from absence of morphological changes, through the specific basal ganglia lesions, into generalized pathology of both central and peripheral nervous systems (199). In view of the fact that this is strictly a 'biochemical' disease, the extent of the structural changes might be of secondary importance to the experimentalist, at least for the present.

The Mechanism responsible for the extrapyramidal manifestations of manganism is obscure and, therefore, invites speculation. To summarize the evidence: Inhalation of dust is a necessary step. It results in huge pulmonary depots of manganese. The latter floods the blood stream (110, 111) to the point of abnormal appearance in the urine (67, 144). In spite of this, there does not seem to exist well documented evidence

of an increase of this metal's concentration in the brains of victims of the disease. On the contrary, there exists evidence in such a case (67) which suggests that both the concentration as well as the partition of the metal in tissues other than the lung is normal. If the evidence is summarized correctly, it suggests two speculations in seeking the pathogenesis of the disease; *1)* that a manganese compound is formed in the lung which is toxic to the brain, *2)* that continuous, rapid perfusion of the brain with a manganese compound is *per se* injurious to the brain.

Both these suggestions are amenable to experimental test. Of the two, the latter is more appealing since, if it were valid, it would introduce a novel element in the study of the pathogenesis of not only this disease. The studies with radiomanganese show that this metal exists in a highly dynamic state in the body. The transport is rapid and exquisitely sensitive to the body burden with this metal. This fact is reflected in the stability of manganese concentrations in various tissues (69, 70) almost irrespective of the species from which the sample has been taken. The metal's anatomical pathway through the body seems to be specific and to carry it through the various intracellular organelles (48, 49, 124). Chelation is of cardinal importance in this process (41, 180). Thus the possibility of an adverse effect accompanying high turnover rates of manganese suggests itself: a local exhaustion of an organic coordination compound might take place within an intracellular organelle. A second possibility lies with the various valence species of this metal: it is fair to assume that various valence forms of manganese may occur, as dictated by the pH and redox potentials of various brain particulates. Rapid perfusion of the brain with one species of manganese might be expected to disturb the equilibria of the other species.

The postulate that abnormally rapid turnover of manganese might be *per se* injurious to the brain is compatible with the great difficulties encountered in attempts to reproduce the disease in animals. Furthermore, Penalver's success (145) in the treatment of a severe case with versene suggests that sequestration of the metal in the blood was sufficient for the reversal of the disease process. Thus, the 'lesion' could not have been a structural one, since structural regeneration of the brain is notoriously poor.

The appearance of extrapyramidal disease following jaundice in childhood, and following poisoning with cyanide or carbon monoxide, suggests that an abnormality in the metabolism of manganese or of some other transition group element might be instrumental in the development of that disease. A diligent search for such an abnormality might be fruitful relative to human Parkinsonism. This view receives encouragement from the observations of Borg and Cotzias (23) that some of the drugs employed in the treatment of Parkinsonism form coordination compounds with manganese and are thus capable of removing the latter from brain preparations. A study designed to test the thesis that Parkinsonism might be a form of manganism is now under way at Brookhaven.

Experimental Manganism

Some of the unsuccessful attempts to produce the syndrome in animals are summarized by Van Bogaert (196), who has been able to produce convincing manganism in one monkey. Kozai and Boyle (108), working with rats which were given large body loads of manganous sulfate, observed that EDTA diverts some of the manganese into the urine, a fact which had been observed by Maynard and Fink (125) in man.

Lloyd Davis (119) has made an extensive study of the pulmonary disease in

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man and mouse. In relation to the latter, he has devised an ingenious method for administering manganese salts by inhalation of their dusts. He was primarily interested in duplicating the lung lesions which characterize the disease in man. He discovered a remarkable capacity of the mouse lung epithelium to absorb material inhaled in the form of dust.

Makarchenko (121) has studied the pathology of chronic manganese poisoning, but the reviewer has not been able to secure a translation from the Russian in time for discussion here. Rodier, Mallet and Rodi (161) have studied the effect of calcium versenate on the lethal dose of manganese in mice. They have recommended this chelating agent for treatment of human manganism.

Manganese in Some Disease States

There have appeared over the last years some observations which suggest the existence of some relationships between the metabolism of manganese and various aspects of disease and of therapy. These are more subtle than the recognized specific deficiency or the poisoning with known compounds of the metal, which are reviewed in preceding sections.

The metabolism of choline, both normal and abnormal, seems to be intimately linked with the metabolism of manganese. The structure of choline should probably permit the ready formation of coordination compounds with manganese. It is surprising that there does not seem to exist published stability constants for such chelates, in spite of the fact that there is ample evidence which shows that deficient choline intake markedly aggravates perosis in birds (64, 96, 101, 102). Furthermore, it has been known from the work of Amdur, Norris and Heuser (4) that the metabolic correlation between these two nutrients does not merely reflect itself in the bone disease of chickens, but is actually manifested also in the metabolism of fat by the liver of intact rats. These investigators have shown *1*) that manganese supplements decrease the liver fat, *2*) that choline supplements do likewise, and *3*) that there is a highly significant interaction between the two factors. This correlation received additional support from the observations of Jukes (101) and Jukes and Welch (102), who conclude that for total protection from perosis, manganese supplements are not sufficient but that high choline intakes are necessary. It is, therefore, obvious that the role of manganese in choline metabolism, and vice versa, should be further investigated. In view of the fact that choline is excreted in the bile, there exists the possibility that manganese might be excreted as a choline complex and not only as a bilirubinate (125). The question as to whether the action of acetylcholine is in any way related to the metabolism of manganese is equally important, since this compound seems also to be capable of forming manganese chelates. The roles of both manganese and acetylcholine in the function of nervous tissues need not be stressed again.

The experiments of Wiese, *et al.* (206) which pertain to the role of inositol in manganese metabolism are discussed in relation to perosis. The manganese chelates of inositol might be well worth studying. Skinner and McHargue (180) were unable to confirm earlier observations that manganese is instrumental in ascorbic acid synthesis from mannose in the guinea pig.

Hill and Holtkamp (92) have demonstrated that storage of manganese in the liver was dependent upon the amount of thiamine in the diet. Conversely, it appeared that storage of thiamine was dependent upon the level of dietary manganese. In a similar vein, Perla (147) has shown that toxic doses of thiamine could be rendered innocuous by manganese supplements. This finding was further defined by Perla and Sandberg (148). They observed that daily administration of thiamine in amounts of 30 40 times the daily requirement resulted, among other things, in inability of the rats to rear their young. Manganese supplements (2 mg./day/rat) reversed this condition. Following some additional experiments, Sandberg, Perla and Holly (164) were of the opinion that the depletion period for the establishment of thiamine deficiency in rats was shortened by manganese supplements (as well as by copper and iron). Anderson and Parker (5) have expressed reservations relative to these latter results.

The role of manganese in hematopoiesis (normal and abnormal) has been a debatable question in the past. Paterni (143) has claimed that small doses of manganese have a stimulatory effect on erythropoiesis. Large amounts were presumed to cause depression of both erythropoiesis and granulocyte formation. Although the latter has been frequently encountered in chronic manganese poisoning, it is of doubtful value in the treatment of leukemia. The council of the American Medical Association (2) has made an unfavorable report on the usefulness of manganese preparations as remedies in human blood disease. Sullivan (189) has questioned earlier reports of the effectiveness of manganese therapy in human skin diseases. The anemia which accompanies prolonged intake of milk alone, was also not combatted by manganese supplements (109). However, Carlstrom and Hjarre (35) have studied the infectious anemia of cattle, which is presumably due to a virus, and found that it tends to occur in pastures which offer the animals a high level of dietary manganese On the other hand, Orten *et al.* (142) found that polycythemia of rats which was caused by high cobalt intakes was aggravated by dietary supplements of manganese.

An interesting series of observations has been made on the relation of antibiotics to the metabolism of manganese. Albert and Rees (1) have found that tetracycline possesses a marked avidity for manganese and some other metals. Working with aureomycin, Pepper, Slinger and Motzok (146) found that the antibiotic could prevent perosis from appearing with diets which were low enough in manganese to produce normally a high incidence of the disease. Saz and Slie (167) have been able to reverse the aureomycin-induced inhibition of nitroreductase with manganese additives to their *in vitro* system. These observations indicate a definite relationship between manganese and some antibiotics. The possibility of explaining drug action on the basis of metal chelate formation has been extensively and critically discussed by Chenoweth (39).

The fact that hormonal imbalances might affect the metabolism of manganese has been shown by Bolton (20). He found that the blood level of manganese was increased in pullets by the administration of estrogens. Ray and Deysach (154) have observed a thyroidal accumulation of injected manganese salts and a concomitant diminution of the basal metabolic rate of the guinea pig.

Underwood (194) raises the question of the relationship between slipped epiphysis (= perosis) in birds and slipped epiphysis in children. There is no evidence known to this reviewer that a study of such a proposed relationship has been made. However, Ghormley and Fairchild (79) have discussed the possible metabolic or hormonal etiology of this disease. Everson and Daniels (65) have shown that children need high amounts of manganese and that they tend to retain the metal in proportion to the dietary level. Their estimate of the daily requirement is about 0.1 mg. of manganese per pound of body weight. There is a good chance that this estimate was too high. If not, it shows that there exists ample opportunity for the develop-

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ment of manganese deficiency in childhood, since so high an intake is hard to achieve with baby foods only.

A possible relationship of manganese to carcinogenesis was suggested by the work of Balo and Banga (9). In a study of the role of transition metal chelates in experimental cancers, they found that manganese maleate inhibited new growths. The inhibition was striking with both Ehrlich's ascites tumor (mouse) and Guerin's cancer (rat). It was evidenced in some cases by *1*) the disappearance of the tumor which normally affected almost all the animals; in other cases by *2*) the marked impairment of growth of the tumor; *3*) by prolongation of survival in all treated animals. Evidence was also presented which indicates that the manganese maleate chelates tend to change the metabolism of these cancers from anaerobic to aerobic.

Lindberg and Ernster (115) have discussed the possibility of manganese being a cofactor in oxidative phosphorylation. This was also investigated by Bronk and Kielley (28, 29). Zotterström and Ernster (213) further found that bilirubin is a complexing agent for manganese. The addition of bilirubin to *in vitro* systems interfered with oxidative phosphorylation. They have shrewdly suggested that kernicterus might well be due to such an interference. This suggestion is attractive because it ties in well together with what is known about manganism and another disease which affects the basal ganglia, namely hepatolenticular degeneration. This latter disease has interested Gubler *et al.* (86) from the viewpoint of its similarity with chronic manganese poisoning of humans. In their studies with rats, they produced no central nervous system lesions. They found that copper assimilation and storage are affected by the level of dietary manganese. High copper and high manganese intake, operating together, caused significant changes in the body storage of copper. High manganese intake alone changed the normal partition of copper among the various organs but it did not affect the total body copper content.

Butt *et al.* (32, 33) as well as Griffith, Butt and Walker (83) have studied various human tissues obtained at autopsy for their content of trace metals. The figures for manganese are of a similar order of magnitude as those reported by Fore and Morton (71) and by Tipton *et al.* (191). The concentration of trace metals in iron storage diseases was studied by Butt *et al.* (33). The livers of patients suffering from hemochromatosis showed essentially normal manganese concentrations. In cases classified as 'refractory anemia' the manganese concentration of the liver might have been decreased.

In a study of the effect of orally given manganous glycerophosphate in human rheumatoid arthritis, Bepler and Rogers (16) were unable to detect any change in the symptoms or signs of the disease.

Schroeder (171, 172) has brought forward extensive evidence bearing on the problem of the relationship of trace metals (including manganese) to chronic diseases. Prominent among these chronic diseases are atherosclerosis and hypertension. Although manganese figures extensively in the various data, this interesting study does not implicate manganese in a specific enough fashion for it to be summarized here. However, Schroeder *et al.* (173) have shown that manganese and chelating agents may inactivate phercatasin, which is a factor instrumental in hypertension. Furthermore, Perry, Schroeder and Fredrickson (150) have shown that hypertensive patients excreted a high amount of trace metals in their urine. In one case manganese was excreted at the level of 60 μg per liter, indeed a large amount. The administration of complexing materials (hydralazine) has restored the excretory levels of these metals to normal, together with the establishment of a normotensive state. Schroeder and

Perry (174) have used chelating agents in the treatment of the experimental hypertension of rats. When coordinate compounds of manganese and some other metals were injected intravenously, an antihypertensive effect was seen. This was correlated with the dissociation constants of the coordinates in question. Curran (52, 53) has reported that cholesterol synthesis in rats is increased by manganese (and chromium) supplements. Tietz (192) found similar results with the synthesis of fatty acids *in vitro*, as did Wakil, Porter and Gibson (202). The work of Amdur *et al.* (4) has been referred to above. An analysis of all these findings has led Schroeder (170–172) to the development of a regime for the treatment of arteriosclerosis and of hypertension.

Comens (46) has reported some important findings relative to the 'apresoline disease.' This condition resembles spontaneously occurring human lupus erythematosus. (It was first observed by Schroeder in humans whose hypertension was being treated with hydralazine (= apresoline)). The disease was induced also in dogs. In these animals, it was found to duplicate the clinical, pathological and hematological picture of human lupus erythematosus disseminatus. However, when hydralazine was given to cockerels, the animals developed classical perosis, preventable by manganous citrate supplements. The formation of lupus erythematosus cells (LE phenomenon) was specifically inhibited by manganous ions in 10^{-2} M concentrations. Iron, zinc, copper and cobalt were ineffective in that regard. When rats were injected with hydralazine, they developed convulsions which could be prevented by injections of manganous glycerophosphate or of pantothenic acid. Five other trace metals and pyridoxine did not prevent the convulsions. Three patients with 'hydralazine disease' and two with lupus erythematosus disseminatus apparently showed symptomatic improvement when manganous salts were administered. Comens' brilliant evidence suggests that hydralazine disease has the earmarks of a manganese depletion, and that in lupus erythematosus disseminatus manganese might also be playing a hitherto undetermined role. Walburn's findings (203) relative to the stimulation of antibody synthesis by injected manganous salts have apparently not been followed up since Von Oettingen's review (198) was written. They might be relevant to the apresoline story summarized above.

Concluding Remarks

This review has suggested some thoughts which are amenable to experimental evaluation.

Currently, the determination of manganese in biological samples is cumbersome at best. Neutron activation analysis promises to become a remedy for this situation.

Several parameters of manganese metabolism have been explored by means of the use of inorganic manganese compounds. It is probable that a great portion of the dietary manganese is coordinated with organic compounds. Absorption, excretion and daily requirements might have to be redefined.

The pathway of manganese through the body seems to be specific for this metal and seems to pass through the intracellular organelles. Application of the techniques of the pool problem to the gross and microscopic anatomical constituents of the body might well yield bedside methods for the determination of the total mass of such organelles.

Human manganese poisoning is indeed a challenging problem. The disease is brought on by massive inhalation of oxide dusts. Surprisingly, only the lungs and the blood seem to constitute sites of high manganese concentration. The distribution as well as the concentration in the other organs seems to remain normal. Assuming this

to be correct, an interesting question becomes valid: whether rapid turnover of a homeostatically controlled substance is not *per se* adequate cause of degenerative disease. High intakes of sugar have caused diabetes and high intakes of sodium have caused hypertension. In both cases the period of 'normalcy' is characterized by a high turnover of the respective substances.

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G.C. Cotzias, Mineral Metabolism. 1962

CHAPTER 33

Manganese

GEORGE C. COTZIAS

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I. Introduction

Long before its isolation by Scheele (1742–86), manganese was recognized by the Romans as possessing "female" magnetic properties, and its ores were considered important in the manufacture of glass. However, the term *magnesia* (magnetic substance) was applied interchangeably to the ores of both magnesium and manganese. In the Byzantine era, black magic was referred to as *mangania*, while the generic term for machines of both war and peace was *manganon*.

These primordial notions survive in modern science: The magnetic properties of manganese are viewed today with increasing interest in physics and biology alike. In the latter discipline one is sometimes more impressed by the similarities between magnesium and manganese than by their differences. A great deal of evidence indicates that the metal might be essential to life (Von Oettingen, 1935; Cotzias, 1958). Paradoxically, however, one is forced to assign a growing number of functions to this metal, while the trace amounts of manganese found in biological samples tend to be smaller with each analytical improvement—a situation kindred to *mangania*.

The biology of manganese suffers from the lack of unifying principles. There exists, therefore, a widespread accumulation of information and experience, the integration of which is a task of the future. As of the present, no single function can be ascribed to this metal *in vivo*. *In vitro* experiments have shown a wide range of biochemical activities which cannot be satisfied by the trace concentrations characteristic of tissues, even though this element is extremely mobile in the living animal. It seems safer, therefore, at least for the present, to stress experiments done in living animals rather than those made with isolated systems. The present chapter emphasizes the former and tends to discuss results obtained *in vitro* only when they have some bearing on the article's primary orientation.

II. Analyses of Manganese in Biological Materials

As in medicine, where a large number of treatments is characteristic of an incurable disease, so in analytical chemistry a large number of methods is characteristic of a substance which is hard to analyze. In the analyses for manganese, the basic difficulty lies with the handling of the sample, during which losses of and contamination with manganese are equally likely to occur. Superimposed on this is the questionable specificity of some methods. Therefore, neutron activation analysis will be considered here especially, since it virtually eliminates handling while offering high specificity and accuracy with small samples.

A. Chemical Methods

1. *Periodate Methods*

In the periodate methods the manganese in solutions of ashes is oxidized to the blue permanganate. The procedure of Reiman and Minot (1920a,b) has a lower limit of sensitivity of 2 μg. Mehlig's method (1939) is optimal for the 0.07–3μg range.

2. *Catalytic Methods*

In the presence of several organic compounds, permanganate develops a more intense color than in their absence. The development of this second color requires much skill because it depends on the interaction of catalytic quantities of permanganate with the organic compound. Like other catalytic reactions, the ones involving permanganate are sensitive to numerous variables.

Gates and Ellis (1947) have used 4,4-tetramethyldiaminotriphenylmethane for the catalytic development of color; this is an exasperating procedure, but it is sensitive at the 0.1-μg/ml level. Fore and Morton (1952a) have done impressive work with a reaction between permanganate and *N*,*N*-diethylaniline. With skill, this method is reliable at levels as low as 0.005 μg. Single (1957) has proposed tetramethyldiaminophenylmethane (tetrabase) for the 0.01–0.1-μg range, and Wiese and Johnson (1939) have used the oxidation of benzidine by permanganate in acid, after removal of interfering matter with sodium bismuthate. The reviewer's laboratory has had no significant experience with the last two methods.

B. Bacteriological Methods

These are exemplified by the method of Bentley, Snell, and Phillips (1947). A *Lactobacillus arabinosus* strain is grown first to deplete the medium of manganese, and its growth in the medium following addition of the ashed unknown is then quantitated by means of an electrotitration of the lactic acid produced from glucose. The assay corresponds to a range of 0.05 to 0.5 μg of manganese. The author has no experience with this method.

C. Physical Methods

1. *Flame Photometry*

Diamond (1956) has shown that flame photometry can be used for manganese analysis, but it requires quantities which are enormous by biological standards (100 μg/gm).

2. *Spectrophotometry*

Most of the spectrophotometric data have been obtained collectively for a number of elements. Manganese analyses were usually incidental, and therefore a standard other than manganese was used. While the precision of the measurements has not suffered by this maneuver, their accuracy is often questionable. With the copper spark, the method is capable of a sensitivity of 0.2 μg/ml. With the graphite direct current arc, the sensitivity is one order of magnitude lower (Meinke, 1955). Butt *et al.* (1954, 1956), Griffith *et al.* (1954), Vallee (1955), and Tipton *et al.* (1956) have obtained much useful knowledge with these methods.

3. *Neutron Activation Analysis*

Discussion of this approach presupposes some familiarity with reactor physics, a topic which is outside the scope of this treatise.

H. J. M. Bowen (1956) has developed and used an ultramicro-method based on the activation of the natural, stable Mn^{55} into the intensely radioactive Mn^{56} by means of thermal neutron bombardment in a nuclear reactor. A blood sample is obtained with nonmetallic instruments, thus minimizing contamination during the only phase of the procedure in which contamination matters. The sample is then introduced into the nuclear reactor where it is subjected to neutron bombardment for a period of time at least equal to the half-life of Mn^{56} (2.6 hr). During this phase of the procedure, manganese, but also some other elements, become radioactive. In order to determine the radioactivity contributed by manganese only, this element is chemically separated from the other tissue constituents. The separation is accomplished by wet digestion of the sample followed by (*a*) precipitation of manganese as one of the oxides, and (*b*) solubilization of the other elements. Both of these aims are helped by adding carriers of the elements in question. The manganese carrier is of known amount, and the weight of the precipitate gives a gravimetric measure of manganese (meaning the carrier and presuming it to be a true carrier for the radioactive species). The addition of carriers ("hold-back" carriers) for elements which become coactivated serves in the elimination of contaminating isotopes. Of these, sodium, potassium, chlorine, and bromine are the most bothersome. The last two steps are repeated several times in order to obtain as pure a sample of manganese as possible. Known traces of manganese, handled identically, constitute the standard.

By the use of monochromatic neutrons it should be possible theoretically to favor the activation of manganese and to discriminate against activation of other elements. Furthermore, multichannel pulse-height analyzers permit the observation of small segments of the γ-ray energy spectrum emitted by an activated sample. Coincidence counting techniques permit

selection of γ-rays which are emitted concomitantly wi[illegible] adiations of specified energies. Borg (1959) has attempted to apply these principles in order to develop a nondestructive method for the determination of manganese. This method would eliminate all handling except that involved in obtaining a sample of tissue and may lead to eventual activation analysis *in vivo*. Up to the present, however, this procedure is not practical. Papavasiliou and Cotzias (1960) have therefore developed a technique which constitutes essentially a marked simplification of the method of Bowen.

The reactor is utilized in the usual fashion, without specific shielding of the sample. The activated sample is flash-burned in a closed vessel filled with O_2. The ashes are transferred to acid solution containing known amounts of manganese carrier. The solution is oxidized with persulfate to permanganate. The permanganate is precipitated with tetraphenylarsonium chloride. The precipitate is washed, the radioactivity is measured, and the "specific activity" is computed by comparing both radioactivity and the blue permanganate color to known standards. The procedure is simple and fast. It seems to be accurate within 5% for samples containing 2.5×10^{-3} μg manganese. Experience with this technique is limited.

It should be noted that neutron activation analysis is a novel approach. The present discussion will probably be outdated at the time of publication.

4. *Magnetic Techniques*

The techniques discussed above were designed to measure the total amount of manganese in biological samples, but they yield no information as to either the valence of the metal or its state of binding with organic receptors. This latter type of information can be obtained with the aid of magnetic techniques. Theoretically, these techniques are applicable to intact tissues and are often capable of yielding information about the natural state of the element.

Among the magnetic techniques, nuclear magnetic resonance and electron paramagnetic resonance have found applications in studies relevant to the biology of manganese.

a. Nuclear Magnetic Resonance. Nuclear magnetic resonance is studied as follows: A sample is subjected to a powerful, steady magnetic field and, at the same time, to a low power radio beam of a few megacycles in frequency. The intensity of the radio signal *emerging* from the sample is recorded as the endpoint of the measurement.

The atomic nuclei in the sample have characteristic "spin" angular moments, meaning that they rotate in a characteristic quantized fashion. The magnetic moment created by the spinning electrical charge of the nucleus interacts with the magnetic field supplied by the magnet in the

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apparatus. In these circumstances some nuclei tend to become oriented in a "north-south" direction with respect to the poles of the magnet. In so doing, they undergo a precession analogous to a gyroscope which might be spinning in the earth's gravitational field (Richards, 1957). This precession has a frequency (the Larmor frequency) that varies with the strength of the applied magnetic field and with the characteristic gyromagnetic ratio of the nucleus (nuclear magnetic moment divided by nuclear spin). A radio beam will then excite the nuclei to flip back and forth between adjacent energy levels permitted by quantum rules, only when its frequency is exactly equal to the (Larmor) frequency of nuclear precession; and the radio beam them "resonates" with these nuclei. In doing this, the beam loses a measurable amount of energy and the recorded intensity of the emergent radio signal will vary. The electromagnetic fields of atoms in the immediate environment of the resonating nucleus will couple weakly with it. This produces small shifts in gyromagnetic ratio resulting in the production of "fine structure" in the recording of the resonance signal. Thus this technique constitutes in essence a "microscope" by which binding forces and the configuration of atomic groupings within molecules can be viewed.

b. Electron Paramagnetic Resonance (EPR). Electron spin resonance uses the same fundamental principles in order to allow detection of the magnetic moment created by the spin of unpaired electrons. Unpaired electrons occur in the transition group elements (including manganese), in free radicals, in semiconductors, and in triplet excited states (although certain quantum considerations prevent their alignment for EPR measurement in this latter case). Electron paramagnetic resonance measurements are made with a fixed radio frequency in the microwave region (several thousands of megacycles, usually supplied by a Klystron tube and wave guide) and variable strength of the magnetic field. With this method, a transition group metal itself may be detected by means of a resonance spectrum representing changes in microwave power reflected from the sample cavity as a function of the changing magnetic field. This spectrum may show broadening, splitting, or "fine structure" because of the magnetic coupling of the unpaired electron spin to the nucleus or to the *orbital* angular momentum of the electron itself. This latter coupling provides a means by which nearby electric and magnetic centers that alter the charge distribution of the electron about its atom or molecule can influence the measured electron resonance. Thus, changes in spectral shape and/or diminution of signal strength that occur as parallel electrons become paired in covalent bonds often can provide information regarding the extent *and* chemical character of transition metal bonding with various ligands.

Faber and Rogers (1959) have studied the resonance spectra of manganese absorbed on fixed phases. Using EPR measurements, Malmström *et al.* (1958) have found that the free enzyme enolase (and not the enzyme-substrate complex) was able to bind manganese. This indicated that enolase bound its substrate with the aid of manganese.

When manganous ions in solution are bound, as by the addition of EDTA, their hyperfine structure as detected by paramagnetic resonance studies will disappear (Cohn and Townsend, 1954). When Mn^{2+} is bound by phosphate esters, the intensity of the peaks of the hyperfine structure diminished in proportion to the amount of metal bound (Cohn and Townsend, 1954). With this method, dissociation constants for manganese complexes of these and other ligands could be calculated. In addition, it was possible to define the type of bond in the complex.

Articles of general interest in this area have been written by Odeblad (1956), R. E. Richards (1957), Commoner *et al.* (1957), Blyumenfeld and Kalmanson (1958), and Balazs *et al.* (1959).

III. Metabolism of Manganese in Vitro

This section summarizes some *in vitro* experiments to illustrate the wide range of biological reactions in which manganese may take part. Some attempt is made to correlate these experiments with what is known about manganese metabolism. For more detailed discussion of any one aspect, reference should be made to treatises on pharmacology and enzymology (Sumner and Myrbäck, 1950; Chenoweth, 1956).

A. Bacterial Genetics

When applied to bacterial colonies for a few hours in concentrations of 0.01%, manganous chloride has mutagenic properties (Steinman *et al.* 1958) which are potentiated appreciably by some, but not by other, mutagens. At present, no rational explanation can be given for these effects.

Sarachek (1959) has reported mutagenic effects of manganous chloride on yeast which were nullified by magnesium ions.

B. Transport

Saltman *et al.* (1956) found that manganese competitively inhibited iron uptake and release by liver slices. This finding indicates that both ions have a common pathway. The authors thought that simple diffusion constitutes the driving force for the transport of both iron and manganese. Rothstein *et al.* (1958) working with yeast, presented evidence for active Mn^{2+} and Mg^{2+} transport. They were able to differentiate between surface binding and actual absorption, as absorption yielded a nonexchangeable product and seemed to involve a protein and a phosphate constituent. Jennings *et al.* (1958) have studied this system further and have concluded

that absorption of Mg^{2+} and Mn^{2+} involves a phosphorylation step closely coupled with the absorption of phosphate. Christensen (1957) and his colleagues (Christensen *et al.*, 1956; Pal and Christensen, 1959), on the other hand, have discovered anil derivatives ("Schiff bases") of pyridoxal with amino acids which strongly chelate with bivalent metals, including manganese. By following the complex both *in vivo* and *in vitro*, these workers have observed much more vigorous transport of the complex than of the amino acid alone; this shows that the complex functions *in vivo*.

Cotzias and Greenough (1958) have questioned whether some of these observations apply to the living animal. Their results suggest a highly specific pathway for manganese through the integrated mammalian body which is highly specific for manganese alone. The author's group has evidence, hitherto unpublished, indicating that a portion of manganese might be in the trivalent state in the body. This state is unstable when the metal is unbound and therefore inaccessible to investigators who wish to duplicate natural processes.

C. Enzyme Systems

1. *Arginase*

Arginase has been used as a prototype of metal-activated enzymes. Mohamed and Greenberg (1945) have shown that arginase preparations are markedly stimulated by divalent metals, the extent of activation depending upon the kind of metal, its concentration, the period and temperature of incubation and similar factors. Dialyzed yeast arginase (Edlebacher and Pinosch, 1937) was shown by Edlebacher and Baur (1938) to lose its activity, but to become reactivated by divalent metal additives. In both of these instances, divalent manganese seems to be merely one of the several metals which accomplish arginase activation. However, Boyer *et al.* (1942), as well as Shils and McCollum (1943), have shown that arginase activity was lower in livers obtained from manganese-deficient animals than in livers of normal rats. Boyer *et al.* were unable to raise the arginase activity to control levels by manganese additives *in vitro*, whereas Shils and McCollum succeeded in doing so. Other metals, the intake of which had not been knowingly restricted, were not tested for their activating capacity *in vitro*; consequently, it is uncertain how specific the use of manganese is in these experiments. Furthermore, it is most doubtful that these experiments have shown the exclusive site of manganese action, as birds, which lack urea-synthesizing systems, develop a manganese deficiency similar to that of mammals.

2. *Cysteine Desulfhydrase*

Cysteine desulfhydrase of yeast and bacteria (Binkley, 1943) and *thiaminase* (Reddy *et al.*, 1948) are inactivated by dialysis and reactivated

by metal additives, including Mn^{2+}. Manganese acti carnosinase (Hanson and Smith, 1949), as does zinc, though less so.

Magnesium and manganese activate *deoxyribonuclease* (McCarty, 1946).

Enolase (Malmström *et al.*, 1958), intestinal *prolinase* (E. L. Smith and Bergmann, 1944), *glycyl-L-leucine dipeptidase* (Smith 1948b, 1948c) and *prolidase* (Smith, 1948a) are all activated by manganese, but the activation is said to be specific only for prolidase. Horseradish *peroxidase* (Kenten, 1955) requires manganese of a valence higher than 2+.

3. *Oxidative Phosphorylation*

Oxidative phosphorylation is thought by several authors to require manganous ions. The discovery by Maynard and Cotzias (1955) of the early accumulation of injected radiomanganese in mitochondria lends support to the possibility that body manganese plays a major role in oxidative phosphorylation. Lindberg and Ernster (1954) consider this ion as a necessary cofactor. Zetterström and Ernster (1956) have discovered that bilirubin interferes with oxidative phosphorylation by binding manganese. Bronk and Kielley (1957a,b) have studied a mitochondrial enzyme complex which is capable of carrying on vigorous oxidative phosphorylation. They found that a number of metals activate this system but that manganese is the most effective among them.

The work of Bentley and Phillips (1951b) is perhaps significant in this context. They showed that liver homogenates from manganese-deficient chicks had a lower capacity to esterify phosphates than did appropriate controls. That this was not a mere reflection of a general metabolic breakdown was shown by the virtual identity of easily hydrolyzable phosphates synthesized by the two sets of homogenates. Manganese (2+) additives *in vitro* failed to restore this metabolic defect. However, this is not necessarily an important drawback, since the valence state of the manganese fraction which might have participated in oxidative phosphorylation has not been determined as yet.

4. *Fatty Acid Synthesis*

Stern (1956) has studied the metal chelates of acetoacetyl-*S*-coenzyme A. The manganese chelates were among the most stable, while the zinc and copper ones were unstable. Porter *et al.* (1957) have found that one of the necessary cofactors for their soluble pigeon liver enzyme was manganese. The others are ATP, CoA, DPNA, TPN isocitrate, and either glutathione or cysteine, all of which are substances capable of metal binding (Porter and Tietz, 1957). In her study of a similar system from chicken liver which synthesized long-chain fatty acids, Tietz (1957) has found manganese to be more effective than magnesium as a metal cofactor. Curran (1954, 1955)

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has shown that the surviving rat liver's incorporation of acetate into fatty acids is markedly stimulated by Mn^{2+}. Similarly, M. O. Amdur *et al.* (1946) have found a lipotropic action of manganese on the liver of the intact rat. Manganese can be considered therefore as playing a major role in the metabolism of fatty acids in the intact organism.

5. *Cholesterol Synthesis*

Here also, manganese has been found to act as a cofactor. Curran and Chute (1953) and Curran (1954, 1955) have shown that addition of Mn^{2+} or Co^{2+} stimulates acetate incorporation into cholesterol by surviving rat liver. B. H. Amdur *et al.* (1957) have demonstrated the essentiality of manganese in the enzymatic conversion of mevalonic acid to squalene, a cholesterol precursor. Since ATP was also necessary in this synthesis, while no coenzyme A was required, the authors predicted the discovery of a phosphorylation without carboxyl group activation. Tchen (1957) later isolated a phosphorylated derivative of mevalonic acid, the synthesis of which also required manganese. The assumption that manganese and other trace metals are also instrumental in abnormal cholesterol synthesis has led to the development of a therapeutic regimen for atherosclerosis by Schroeder and his colleagues (Schroeder, 1956a,b,c; Schroeder *et al.*, 1955; Perry *et al.*, 1954) that will be touched on later in this chapter.

The studies summarized in this section point to the importance of manganese in mitochondrial *function*. They are strengthened by the studies of Fonnesu and Davies (1956), who found that the *structure* of isolated mitochondria is preserved by the addition of manganese in trace amounts.

IV. Manganese Requirements in Normal Nutrition

Manganese is considered as essential in nutrition for the following reasons. (*1*) It is omnipresent in foodstuffs. (*2*) Its concentration in mammalian tissues is steady, fairly characteristic of each organ, and not species-linked. (*3*) It shows numerous impressive biochemical functions *in vitro*. (*4*) When diets deficient in it are eaten, specific symptoms result. (*5*) When given to severely manganese-deficient animals, it specifically and reproducibly relieves a major part of their deficiency. (*6*) Given as a dietary supplement, it prevents symptoms of deficiency from appearing (Underwood, 1956; Cotzias, 1958).

In spite of these considerations, the actual dietary requirements for manganese can only be surmised at present. Nutritional experiments have been conducted so far with supplements of inorganic manganese. Therefore, they cannot be considered yet as duplicating the natural state of manganese in foods: probably manganese in food is chelated, and therefore its nutritional availability differs from that of inorganic forms. This statement is based on considerations of the coordination chemistry of manganese which

leave little doubt that in the lumen of the gut most of the metal is likely to be in the form of metalloorganic complexes (Martel and Calvin, 1952; Williams, 1953). Bound forms of manganese have been discovered by, among others, Fore and Morton (1952c,d), Borg and Cotzias (1958b), Bertinchamps and Cotzias (1959), Maynard and Cotzias (1955), and Cotzias *et al.* (1960). Their observations were made on tissues which are representative of foodstuffs.

It is well known that heavy dietary loads with compounds of one element are capable of interfering with the utilization of other elements. This is illustrated by the creation of manganese deficiency (perosis) in birds in the presence of ample manganese when the diets are heavily enriched with either iron or calcium compounds. The converse may also occur. However, the notion of "available manganese" has received limited attention in spite of ample evidence (Christensen *et al.*, 1956; Pal and Christensen, 1959) that in a number of instances the chelate of which manganese is a part and the activities of ambient competing metals (Combs *et al.*, 1942; Hawkins *et al.*, 1955) determine, to a large extent, the behavior of manganese as a nutrient.

The established view that *birds* require more manganese than do mammals (Underwood, 1956) is based on the required concentration of inorganic manganese per unit weight of diet and not on the actual amount of metal consumed by each bird. Mild deficiency appears in birds when they receive 7–10 mg manganese/kg diet. Generally, deficiency is prevented by an intake of roughly 50 mg manganese/kg diet but there are exceptions to this rule (cf. Section X, B, 2).

In *mammals* manganese deficiency is prevented by variable supplements of inorganic manganese to deficient diets. In the *rabbit* (S. E. Smith and Ellis, 1947) 300 μg/animal/day was found satisfactory for growth. The same amount was found to be suboptimal for the *rat* (Shils and McCollum, 1943). In one study weanling *pigs* showed no evidence of deficiency when given 3.5 mg of manganese/kg diet (Plumlee *et al.* 1956), while in another study their requirement was closer to 0.5 mg/kg diet (Johnson, 1943). In adult pigs defective reproduction occurred with a diet offering 12 mg manganese/kg diet (Grummer *et al.*, 1950). This was prevented by larger supplements.

In *man*, obvious manganese deficiency has not yet been established, although Comens (1956) has presented indirect evidence on this point, as discussed elsewhere in this chapter (Section XI, K).

In *children* the daily requirement was estimated by Everson and Daniels (1934) as lying between 200 and 300 μg/kg body weight. If this figure should represent a minimum requirement, many children must run the risk of manganese deficiency, since such dietary levels are not easy to come by.

In *adults* Kent and McCance (1941) have found a variable manganese intake (3.9 mg/person/day) depending upon the menus selected. Basu and Mahakar (1940) consider 5 mg/person/day as adequate intake. North, Leichsenring and Norris (1960), in a study of nine college women found a mean daily intake of 3.70 mg manganese of which 1.54 mg (41%) were retained and the balance was excreted primarily in the feces. The retention of manganese seemed proportional to the intake. These observations are difficult to interpret as far as this reviewer is concerned, since it is hardly conceivable that growth, menstruation, or previous deficiency can account for such a discrepancy between intake and output of manganese.

V. The Concentration of Manganese in Feeds and Foods

A. Manganese Concentration in Feeds

A larger amount of information exists in this area than exists for foods. Some wheat germs have been found to contain 160 mg Mn/kg, while the low-grade flour contained only 5 mg/kg (Schaible *et al.*, 1938). Several investigators have given estimates for other feeds (Bandemer *et al.*, 1940; Heller and Penquite, 1942; Underwood, 1956). Feeds of vegetable origin show marked variability in manganese concentration; they reflect the ambient manganese concentration during plant growth. This contrasts with the constant levels found in fresh products of animal origin.

B. Manganese Concentration in Human Foods

Table I offers an illustration of the variability of the manganese content of various foodstuffs (Hodges and Peterson, 1931; Peterson and Skinner, 1931). It is of interest that "genetic materials" such as seed and nuts are high in manganese, while sea food and milk products are low. The concentration of manganese in milk varies from 20 to 50 μg/liter.

A high manganese intake for humans (20-25 mg/kg dry diet) can be provided by large intakes of nuts, cereals, and legume seeds, tea, and coffee. Exclusive use of vegetables and meat would yield 1-3 mg/kg diet. Consumption of fish, cheese, and poultry would give 0.25-0.5 mg manganese/kg diet. The consumption of coffee and tea (Coleman and Gilbert, 1939) results in large manganese intakes.

VI. The Concentration of Manganese in Tissues

As is the case with other cardinal tissue constituents, manganese occurs in constant concentrations which are characteristic of individual tissues and almost independent of the species from which the samples were derived. Early work summarized by Von Oettingen (1942), Underwood (1956), and Cotzias (1958) had already suggested this constancy. Brückmann and

TABLE I

Manganese Content of Foodstuffs[a]

Foodstuffs	Mn (mg/100 gm)	Foodstuffs	Mn (mg/100 gm)
Grains		Vegetables	
Barley, whole	2.2	Banana	1.9
Barley, ground	1.9	Beets	2.2
Beans	2.4	Cabbage	1.5
Cotton seed meal	2.7	Carrots	1.7
Linseed, ground	4.2	Lettuce	13.3
Maize	1.1	Onion	0.7-1.44
Millet	2.4	Peas	1.1-2.7
Oats, whole	6.6	Potato, peeled	0.4-2.8
Rice, polished	1.4	Spinach	8.9
Rice, unpolished	1.8	Turnip	0.4
Soya bean, ground	5.9	Yam	0.2
Sunflower seed, decorticated	7.2	Miscellaneous	
Tapioca	0.8	Eggs	0.02-0.05
Wheat, whole	3.1	Meat	1.36
Wheat, ground	4.9	Milk	0.001*
Wheat, embryo	39.0	Yeast	0.95+
Wheat, bran	13.8		

[a] Alphabetically compiled as mg/100 gm dry weight; * indicates mg/100 ml and + indicates mg/100 gm fresh. From B. M. Richards (1930) by kind permission of the Editors of the *Biochemical Journal*. These analyses were performed in Scotland prior to the universal fortification of fertilizers with manganese. They are presented only to illustrate possible orders of magnitude.

Zondek (1939) performed analyses of human organs which showed them to contain almost identical concentrations of this element. The work of Fore and Morton (1952c) established the constancy in manganese concentration for a large number of organs of several species. This conclusion is further strengthened by the work of others (Kehoe *et al.*, 1940; Butt *et al.*, 1954, 1956; Griffith *et al.*, 1954). However, some data (Tipton *et al.*, 1956) seem systematically lower than the values alluded to above, probably due to the differences in the standards used.

Table II was compiled from the work of Fore and Morton (1952c). Bone seems to be the site of highest manganese concentration. In view of its role as a storehouse for calcium, bone might also function similarly for manganese, but there is no experimental basis for this statement. The glandular tissues are also rich in manganese. Among the latter, the liver has been considered as a possible site of storage for manganese. The evidence is summarized by Cotzias (1958). Lung, blood, and blood-forming tissue

TABLE II

MANGANESE CONTENT OF TISSUES[a]

Tissue	Mn (μg/gm)	Tissue	Mn (μg/gm)
Bone	3.5	Ovaries	0.60
Pituitary	2.5	Bile	0.48
Pineal	2.3	Thymus	0.45
Lactating mammary gland	2.2	Spinal cord	0.39
Liver	2.1	Hide	0.38
Ileum	1.7	Testes	0.36
Duodenal mucosa	1.6	Brain	0.36
Pancreas	1.6	Uterus	0.33
Submaxillary gland	1.4	Heart	0.28
Kidney	1.2	Nonlactating mammary gland	0.25
Duodenum	1.1	Thyroid	0.24
Stomach	1.0	Spleen	0.22
Colon	1.0	Muscle	0.13
Hair	0.99	Milk	0.045
Gall bladder	0.91	Bone marrow	0.045
Cecum	0.82	Blood	0.03
Parotid	0.70	Fascia	0.024
Small intestine	0.70	Lung	0.011
Adrenal	0.67	Urine	0.000
Lymph nodes	0.65		

[a] In descending order as μg/gm fresh tissue. By kind permission of Dr. Fore and and the Editors of the *Biochemical Journal*. Note the tenfold increase in manganese content of the mammary gland following onset of lactation. Compiled from Fore and Morton (1952c). At present these figures are recommended for use in compiling diets of animal origin.

are low. The increase of manganese concentration in the mammary gland brought upon by lactation is noteworthy.

In other sections, it is noted that dietary restriction or oversupply can bring on relatively minor concordant changes in tissue manganese concentration. It is evident, therefore, that the concentration of manganese in the body must be homeostatically controlled, but the specific hormonal mechanisms have not as yet been defined.

The apparent incongruity of chronic manganese poisoning (where tissue concentration is said to remain almost normal) is discussed under that heading.

The author is unaware of experiments in which normally occurring, dietary manganese was varied by suitable choice of natural sources.

VII. Absorption and Excretion

The precise gastrointestinal loci which are concerned with the absorption of manganese are unknown. The evidence summarized in relation to

manganese deficiency is compatible with observation (Emmel, 1944; Gallup *et al.*, 1951; Gessert *et al.*, 1952; Hobbs and Hansard, 1952) indicating that the amount of metal absorbed is proportional to that presented for absorption. This pertains again mainly to inorganic manganese supplements.

As will be seen below, manganese is excreted in the feces, primarily via the bile. The gastrointestinal contents are both the source of and the sewer for manganese. It is difficult indeed to differentiate between metal moieties on their way to absorption and those headed for elimination. Unpublished data of the author's group indicate that bile-bound manganese is probably reabsorbed, so that each metal atom finally eliminated may have entered a given tissue several times. In practice, this means that sometimes absorption can be confused with excretion. It is therefore permissible at present to consider the gastrointestinal contents as a manganese pool which is in more rapid equilibrium with the tissues than it is with the outside world.

Working with an isotope of low specific activity, Greenberg and Campbell (1940) found that the rat excreted 97% of the orally given Mn^{54}, while 90% was excreted when the isotope was given parenterally; the differences were recovered in the tissues. When Mn^{54} of a higher specific activity was used (Greenberg *et al.*, 1943), 3–4% of oral manganese was absorbed. The excretion was primarily biliary. Previously induced manganese deficiency seemed to result in a higher than normal capacity of the gut to absorb manganese (Mohamed and Greenberg, 1943), indicating a possible negative feedback mechanism for the control of manganese absorption.

Greenberg and Campbell's (1940) experiment showing the transmission of the radioactivity from the mother to the young via the milk was duplicated in this author's laboratory. There can be little doubt that the radiomanganese incorporated in the mother's milk exists in some "natural" form with regard to its states of valence and of coordination. Therefore, milk tagged *in vivo* is an excellent tool with which to study the natural history of manganese in the body of the suckling animal. The importance of such a study was emphasized in the preceding section.

The biliary excretion of manganese has been further investigated by Bertinchamps and Cotzias (1958). They found that early after administration of tracers, the concentration in bile parallels that of blood, with a ratio of 10:1. At the end of an hour, however, these workers observed a second wave of radioactivity, occurring only in the bile of rats receiving luxuriant amounts of manganese in their diets (0.002%). These workers have tentatively postulated that each wave indicates an individual excretory pathway, possibly one each for two predominant valence states of the metal. The first is in use normally and the second under conditions of dietary or parenteral loading with manganous salts.

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The marked change in distribution of manganese in the body following artificial chelation (Cotzias and Maynard, 1955) has been taken as presumptive evidence of change in the excretory pattern induced by chelation. This stresses again the metabolic differences to be expected between naturally chelated dietary manganese and the commonly investigated inorganic compounds. Indeed, in the human (Maynard and Fink, 1956), manganese radioisotopes are normally excreted in the feces via the bile. Analyses of bile have shown an impressive correlation between its bilirubin content and its enrichment with radiomanganese. (These observations correlate well with the *in vitro* experiences of Zetterström and Ernster (1956) discussed in relation to oxidative phosphorylation.) Chelation with Versene tended to sequester the element in the blood and to divert significant amounts into the urine. Corresponding observations have been made in man and animals (Rodier *et al.*, 1954; Kosai and Boyle, 1956; Penalver, 1957).

Among the extrabiliary routes which contribute to the fecal excretion of manganese, the pancreatic juice (Burnett *et al.*, 1952) has been found to be a less effective one than the bile. Here, radiomanganese excretion parallels closely that of protein. It is sensitive to food intake and to some drugs which affect pancreatic secretion (mecholyl), but not to others (secretin, pilocarpin, eserine).

The intestinal secretions have not yet been studied with regard to manganese excretion, as far as this author knows.

The rate of excretion of Mn^{54} is exquisitely sensitive to the body's load with stable manganese, regardless of the valence state of the administered metal (Cotzias and Greenough, 1958). In contradistinction to the *in vitro* experience, however, no other metal was found to affect the rate of elimination of manganese. Marked changes in acid-base balance are similarly without effect on manganese excretion.

VIII. The Dynamic State of Tissue Manganese

In this section, information pertaining to the fate of manganese in the body tissues will be discussed. Because most of this information was obtained by isotopic techniques without concomitant analysis for total manganese, compartmental analysis cannot yet be applied (Robertson, 1957). However, rough estimates are possible by using known tissue concentrations which are normally quite steady (Table II).

A. Partition of Radiomanganese

1. *Partition among Organs*

The early investigators were probably not aware of the rapidity with which manganese travels within the body. Some of the supposedly conflicting information (at least relative to the partition of manganese tracers among tissues) may now be reconciled, albeit in a descriptive sense. If one agrees that manganese is a substance rigorously controlled by homeostatic mechanisms, one may discern several factors which will affect the observed partition of its radioisotopes among various tissues of intact animals. (*1*) The level of dietary manganese controls the turnover rates of the element. (*2*) The total amount of metal administered with the tracer might constitute a body load; this might expand some body pools and distort the normal partition pattern of the isotope. (*3*) The route of administration might favor the filling of some body pools over others. (*4*) The time elapsed until the observation is completed will determine whether "fast" or "slow" pools are observed as having become enriched with isotope.

From the practical point of view, this means that a partition pattern normally characteristic of a long equilibration period might be brought about earlier if the body load and turnover rate of manganese are both high. Thus, two different reports from the same laboratory indicated that skin (Greenberg and Campbell, 1940) or liver (Greenberg *et al.* 1943) might be the organs of preferential uptake. In the former case, an isotope of lower specific activity and a longer period of equilibration were used than in the latter. Similarly, the first report indicated that the partition might have varied with the route of administration. It was shown by Born *et al.* (1943) that the partition of radiomanganese among the tissues varies greatly with the time of equilibration. This shows that the various organs exhibit markedly different turnover rates for this element.

2. *Partition among Tissue Constituents*

Lang *et al.* (1951) were interested in whether the enzyme arginase (which may be activated by manganese *in vitro*) constitutes a site of preferential radiomanganese uptake. While arginase was found to be partitioned almost equally between nuclei and cytoplasm in hog liver, Mn^{56} accumulated in the cytoplasm in roughly twice the concentration found in the nuclei. Also, Fore and Morton (1952b) have brought forth evidence to suggest that alkaline phosphatase of bone (which is activated by Mn^{2+} *in vitro*) does not exist as a complex with stable manganese. They found that a large part of stable manganese exists in a dialyzable form, with roughly one-fifth remaining bound to liver or kidney homogenates. Retinal tissue, on the other hand, which is rich in stable manganese, contains all of the element in a nondialyzable form. A similarly nondialyzable manganese compound (hemoglobin heme) was discovered by Borg and Cotzias (1958b) in erythrocytes of patients injected with $Mn^{54}Cl_2$.

Burnett *et al.* (1952) and Maynard and Cotzias (1955) found that, early after injection, manganous isotopes tend to localize preferentially in the liver and pancreatic tissues of rats and mice.

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Maynard and Cotzias (1955) further found that the curve of disappearance of intravenously injected $Mn^{56(2+)}$ from the blood could be analyzed to suggest three distinct rates. They were impressed by the early and preferential accumulation of the isotope in the mitochondria-rich tissues and suspected that the mitochondria might constitute a site of sojourn of manganese early in its transport through the tissues. Their hypothesis was confirmed by centrifugal fractionation of livers from rats injected with $Mn^{56(2+)}$. The washed mitochondria were found to contain twice as much Mn^{56} per unit of nitrogen as the whole homogenate. Since the washings removed substantially more radiomanganese than nitrogen, the true mitochondrial concentration was probably greater than reported by these authors. Cotzias and Greenough (unpublished) have confirmed these observations on liver and brain obtained from mice and cats. The brain was chosen because of the blood-brain barrier and the liver because of the speed with which Mn^{56} makes its appearance there.

B. Turnover, Transport, and Their Specificity

Thiers and Vallee (1957) have reported analyses of stable metals (including manganese) in fractions of perfused rat liver obtained by fractional centrifugation. Fractions rich in iron tended to be poor in manganese and *vice versa*, the one containing the highest concentration of stable manganese being the one rich in nuclear fragments. A comparison of the intracellular localization of stable (old) with that of radioactive (new) manganese by this reviewer suggests that the turnover of manganese might vary among the different intracellular organelles, being fast in mitochondria and slow in the nucleus. This statement presupposes that perfused liver contains as much manganese as the fresh tissue.

These observations led Borg and Cotzias (1958a) to study the possibility of a bedside method for the measurement of total body mitochondrial mass. As was the case with the rat, a study of humans showed that intravenously injected $Mn^{56(2+)}$ disappeared from the bloodstream at three distinct rates. The second term of the blood disappearance curve exhibited a consistent and striking correlation with the uptake of the tracer by the liver, as determined by an external scanning device. Since it was known from animal studies that the early liver uptake was principally mitochondrial, and since the blood clearance of injected $Mn^{56(2+)}$ was similar in man and rat, the second component of the blood disappearance curve was proposed as a measure of the total mitochondrial pool of the body.

The reciprocity between manganese and iron concentrations in various tissue fractions (Thiers and Vallee, 1957) and the fact that other elements can often substitute for manganese *in vitro*, caused Cotzias and Greenough (1958) to assume that manganese might be replaceable *in vivo* also. By

employing a total body counting technique they observed a marked acceleration of the excretion of injected $Mn^{52(2+)}$ or $Mn^{54(2+)}$ from mice that had received manganese loads. The valence state of the manganese in the load seemed to be immaterial, as compounds ranging from metal powder to permanganate (valences 0 and 7+, respectively) were effective. Surprisingly, the exquisitely sensitive rate of excretion of radiomanganese proved totally insensitive to challenge with elements other than manganese. Neither injections of magnesium, nor of members of the transition group, nor of Group VII caused the slightest change in the total body turnover or the partition of manganese. It was concluded that there exists a specific pathway in the body for manganese alone. However, the authors were unable to study the effects of technetium on manganese turnover, as they were unable to secure an adequate supply of sufficiently stable technetium. There is every reason to believe that technetium (in view of its similarities to manganese) might replace manganese at least partially. However, technetium is not a biologically important element (Cotzias, 1960).

Since the body is capable of differentiating between manganese (element 25) and iron (element 26), the question of transport of manganese by a plasma protein other than transferrin, the iron-carrying protein, became pertinent. Bertinchamps and Cotzias (1959; also unpublished work) concluded on the basis of electrophoretic, ultrafiltration, and metal competition studies that the carrier protein of manganese in human plasma is a β_1-globulin other than transferrin. This protein seems to be specific for manganese, since this element could not be eluted from the protein with iron or magnesium in concentrations of 500 μg (ml of plasma). This protein was named "transmanganin" in contradistinction to "transferrin" since, like the latter, it seems to bind the trivalent form of the metal.

As of the present writing, it is still unknown how much the plasma clearance of radiomanganese will be changed from that described here if the element is bound to this protein prior to injection. Similarly, the distribution and partition of transmanganin-bound metal have not been studied as yet. The importance of such studies lies in the definition of pathways for various manganese valence states in the body.

Bertinchamps and Cotzias (1959 and unpublished data) have studied the behavior of plasma-bound manganese with fractional ultrafiltration, by which is meant the sequential "milking" of the same plasma sample. Analysis of ultrafiltrate fractions for a large number of ions had shown in the past a steady concentration of the element in question in sequential ultrafiltrates. In the case of manganese, however, the concentration fell rapidly and progressively. This type of behavior has been observed only with plasma-bound manganese, plasma, or transferrin-bound iron and insulin-bound zinc, while several other ligand-metal systems displayed the

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classic behavior. On the basis of the mass law, this "aberrant" behavior of manganese has been interpreted as indicating that more than one protein moiety participates in the formation of the manganese-protein complex. This is purely speculative since some alternative reactions resulting in this progressively more evident binding had not been fully ruled out as of the present writing.

IX. Manganese Excesses

A. Chronic Manganese Poisoning in Man

This is a syndrome of insidious onset, characterized by progressive extrapyramidal manifestations, which follow chronic inhalation of manganese dusts. An unrelated pneumonitis due to dust inhalation sometimes accompanies this syndrome (Davies, 1946).

1. *Pathogenesis*

Miners who chronically inhale manganese dusts constitute the almost exclusive victims of this disease. Those exposed to manganic oxide dusts with particle size $<5\ \mu$ are more prone to develop the disease than miners who inhale coarser dusts or different compounds (Rodier, 1955). Braunite (a mixture of Mn_2O_3 and $MnSiO_3$) is reported to be much more poisonous than pyrolusite (MnO_2). This observation, together with the fact that ores allowed to age lose toxicity along their surface, has led Rodier to assume that "the less oxidized the compound, the higher its toxicity." The same writer also discusses the rather striking variations in individual susceptibility to this disease. On the average, it takes two years of exposure for the disease to supervene, but exposures may vary from as little as a few months to as long as several years. The amounts of manganese inhaled have to be massive. Control of dust (Flinn *et al.*, 1940; Boyer and Rodin, 1954; Rodier, 1955) is quite effective in preventing the disease, which now appears to be extinct in the United States, while in other countries (Morocco, Sudan, Cuba) it is not yet controlled.

2. *Clinical Picture*

This description of the disease is based on the reports of Flinn *et al.* (1940), Boyer and Rodin (1954), Rodier (1955), and Penalver (1955, 1957) and thus represents experiences in the United States, in Morocco, and in Cuba.

a. Prodromal Phase. The disease begins insidiously with anorexia, asthenia, psychotic behavior, and occasional criminal acts. Severe somnolence (sleeping sickness) followed by insomnia is often found early in the disease. Headache and leucopenia may further confuse the differential diagnosis between manganism and viral encephalitis.

b. Onset of the Extrapyramidal Disease. Overlapping with these encephalitic manifestations there appears clumsy articulation, often resulting in muteness. A masklike facies and general clumsiness with loss of skilled movement are characteristic. The *pes striatum* seen in postencephalitic conditions is seen here also.

c. Established Manganism. The patient with fully developed manganism suffers from severe rigidity and his extremities show the "cogwheel" phenomenon. He might have a tremor which becomes exaggerated by emotion, stress, fatigue, or trauma. Indifference, interrupted by spasmodic laughter or by crying spells, occurs here as it does in other extrapyramidal illnesses. Similarly, an autonomic disturbance manifested by excessive salivation and sweating may become apparent. Evidence of lesions other than extrapyramidal and autonomic is lacking. The disease is incapacitating but not life-limiting.

d. Manganese Pneumonopathy. This is a chronic, granulomatous pulmonary disease, similar both to beryllium poisoning and to the pneumoconioses. In some regions (Morocco) it occurs in epidemic proportions, while it is almost unknown elsewhere (Cuba).

e. Laboratory Examinations. The presence of manganese in the urine as revealed by a periodate method is pathognomonic of the disease. Leucopenia with otherwise normal white cells and hypochromic anemia are present.

f. Autopsy Findings. This author was unable to find any agreement as to positive anatomical or histological findings in this disease. For a discussion of various findings, the reader is referred elsewhere (Flinn *et al.*, 1940; Voss, 1942; Van Bogaert and Dallemagne, 1945). The case autopsied by Flinn *et al.* (1940) is important, as these authors report careful histological and skillful chemical procedures done to demonstrate the presence of manganese. *The concentration of manganese in tissues other than lung was normal and comparable to that found by Fore and Morton* (1952c). This interesting finding may provide an important clue for further work and is discussed below.

g. Treatment. Withdrawal from exposure may reverse mild, untreated manganism. Penalver (1957) was successful in reversing an advanced case after persistent treatment with dicalcium disodium ethylenediaminetetraacetate. This important finding is also discussed below.

h. Experimental Manganism. While acute manganese poisoning can be produced readily, manganism similar to the human syndrome is indeed most difficult to duplicate in the laboratory. For a detailed account of the many failures and the controversial successes, the reader is referred elsewhere (Van Bogaert and Dallemagne, 1945; Makarchenko, 1956).

i. Biological Importance. Manganism seems to be characterized by an active depot of manganese in the lungs. As a result, the tissues (including

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the brain) are continuously perfused with some form of the metal, to the point where manganese may be excreted in the urine. The homeostatic control in the body seems efficient enough to keep the actual tissue concentration of manganese within normal limits. This can be accomplished only by a marked increase in the turnover rate of the metal. While these events take place, extrapyramidal disease appears. If the metal is stopped from flooding the body, either by exhaustion of the lung depots or by chemical sequestration of the metal in transit, the disease is reversed. Thus, it appears that the process which itself is responsible for rapid turnover of this homeostatically controlled substance can produce the degenerative disease. For a discussion of possible mechanisms and further implications, the reader is referred to Cotzias (1958).

B. Excess Manganese Intake

The one constant finding which follows protracted ingestion of large amounts of manganous salts is the development of elevated concentrations of manganese in the liver (Gallup *et al.*, 1951). No other ill effects, specifically no extrapyramidal disease, were observed under conditions of high or massive intake of manganese. Gubler *et al.* (1954) have reported a change in the partition of copper among the organs of rats fed high manganese diets. They observed no disease or changes in the total body concentration of copper.

Massive feedings of manganese to rats (1.73 % of dry diet) have caused loss of calcium in the feces, a negative phosphorous balance, and severe rickets (Chornock *et al.*, 1942). Under similar conditions there has been observed hypoplasia of the dental enamel in rats (Wessinger and Weinmann, 1943). Lactating cows (Reid *et al.*, 1947) as well as pigs (Hobbs and Hansard, 1952) have been subjected to a similar regimen with similar results. Iron absorption is markedly diminished in lambs by massive manganese intakes reaching 5000 mg/kg diet (Hartman *et al.*, 1955). Spontaneous lactation tetany has been seen in cows grazing on pastures high in manganese (Blakemore *et al.*, 1937). Chickens seem to tolerate 1.0 gm manganese/kg diet (Gallup and Norris, 1939a), but not 4.8 gm/kg diet (Heller and Penquite, 1942).

C. Acute Manganese Poisoning

Because of its medicinal use, $KMnO_4$ has been implicated in cases of acute poisoning. It is used internally in solutions of 0.1 gm/liter for lavage of the stomach following ingestion of alkaloids and other organic poisons. It might cause irritation or necrosis of the mucosae with which it comes into contact. It is hemolytic and injurious to capillaries, like many other strong oxidizing agents.

X. Manganese Deficiency

Observations of the manganese-deficient state have yielded considerable insight into nutritional requirements (Von Oettingen, 1942; Underwood, 1956; Cotzias, 1958). Furthermore, they have pointed to some relationships of manganese with other nutrients. Finally, they have made apparent certain physiological functions for this element, which have added weight to the argument that manganese is essential for life (Wilgus *et al.*, 1936, 1937, 1939; Van Reen and Pearson, 1955).

Though not studied in animals other than birds or mammals, manganese may also be important for the lower forms, as at least one crustacean species (*Pinna*) contains manganese instead of iron in its blood pigment.

Manganese deficiency has been studied carefully in birds and mammals. The more striking of the manifestations are indeed similar in both groups.

Birds are considered to be more susceptible to the deficiency than mammals because they require more manganese to overcome or prevent the deficiency. This is interesting in view of the higher rate of metabolism, larger oxygen consumption, and higher body temperature of birds relative to mammals. Furthermore, birds do not synthesize urea and do not contain arginase in their tissues. All this strengthens the impression that manganese may be more important in reactions involving oxidative phosphorylation (Lindberg and Ernster, 1954; Maynard and Cotzias, 1955; Zetterström and Ernster, 1956) than in urea synthesis, i.e., arginase activation (Bach and Whitehouse, 1954).

In both birds and mammals, manganese deficiency is characterized by bone abnormalities, defective growth, central nervous system manifestations, reproductive dysfunction, and anomalies in fat and lipid metabolism. The similarities between the numerous studies so markedly outnumber the differences that it is probably fair to extrapolate to man, about whom little is known in this regard.

A. Manganese Deficiency in Mammals

Kemmerer, Elvehjem, and Hart (1931) were among the first to demonstrate the essentiality of manganese in nutrition by showing that a diet composed exclusively of milk caused poor growth and poor ovarian function in mice and that both of these conditions could be overcome by manganese supplementation. Others (Waddell *et al.*, 1931) soon extended these findings to the rat and found (Orent and McCollum, 1931) that high mortality of the young, testicular degeneration, and poor lactation also accompany the deficiency.

Two groups of investigators (Skinner *et al.*, 1931; S. E. Smith *et al.*, 1944) agree that the manganese concentration in the body reaches one peak at birth and another at weaning. Supplements of manganese given to

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mothers in gestation caused the bodies of the sucklings to be rich in manganese, whereas administration of manganese to deficient mothers caused no change in the manganese body concentration of the weanlings.

Poor growth, poor reproductive capacity (Boyer *et al.*, 1942), as well as low food consumption and low food utilization, seem to accompany manganese deficiency. The offspring are debilitated and do not seem to recover when shifted from their deficient mother to a normal wet nurse (Daniels and Everson, 1935).

Barnes *et al.* (1941) and M. O. Amdur *et al.* (1945) showed that deficient rats develop bone changes characterized by short, fragile, and light bones. Hill *et al.* (1950) raised successive generations of manganese-deficient rats on a diet offering 30 μg manganese/day/adult rat. They discovered striking manifestations of apparent central nervous involvement consisting of backward gait, ataxia, loss of balance, and a severe postural reaction of these animals to external stimuli, even of their own making. While this was evident in the first generation, it became more pronounced in successive ones. The brains of these animals showed no histological changes. Shils and McCollum (1943) introduced a solid diet which provided 3 μg Mn/day/rat, i.e., one-tenth of the level used by Hill *et al.* (1950). The female animals produced defective young but were able to support the growth of young born to normal mothers. The deficient young displayed most of the symptoms and signs already discussed. Central ataxia was striking. The liver arginase activity was low but could be raised to entirely normal levels by addition of Mn^{2+} *in vitro*.

Hurley *et al.* (1958) studied the ataxia appearing in offspring of manganese-deficient rats. They discovered that the ataxia is preventable by manganese supplements given to the mothers during the first half of pregnancy, but not during the last. Hurley and Everson (1959), using indirect means, localized the ataxia in the central nervous system. Hurley *et al.* (1959) found no significant abnormalities of the plasma phosphatase levels in these animals. This would have been expected on the basis of experiences with animals rendered manganese-deficient during extrauterine life.

Wachtel *et al.* (1943) found that when high Ca/P ratios were incorporated in the diet of rats receiving 5 μg Mn^{2+}/rat/day, the disease became markedly aggravated. Severe bone changes were seen. The alkaline phosphatase activity of the sera was *elevated*. Anterior pituitary extracts ameliorated the condition. Results comparable with those obtained in the rat have been obtained with mice (Kemmerer *et al.*, 1931) and rabbit (S. E. Smith *et al.*, 1944; Ellis *et al.*, 1947).

Working with manganese-deficient *rabbits*, Rudra (1941) saw a vicarious appetite which he called *manganese hunger* because manganese supplements

prevented its appearance. Smith *et al.* (1944) found that rabbits raised on milk powder grew better if Mn^{2+} is added to their diet, but not as well as normally raised animals. The deficient animals showed a striking bowing of the legs. Careful measurements of bone weight, density, length, breaking strength, and ash content of the humeri showed them to be significantly below control levels. X-ray and histological studies differentiated this condition from rickets. The animals were anemic, but their bone marrow was abnormally overcellular. The manganese and the arginase contents of the livers were diminished. A severe *diminution* of the bone phosphatase was seen in carefully executed pair-feeding experiments.

Smith and Ellis (1947) have shown that there is a definite link between the concentrations of manganese in tissues and in the diet. In their rabbits, an almost linear relation was evident between liver (or bone) manganese concentration and that of the diet up to an intake of 4 mg/rabbit/day. Above that level, the tissue concentration fell significantly, probably due to homeostatic responses.

In the *pig*, manganese deficiency has been induced in female sucklings receiving 0.5 mg Mn^{2+}/kg diet, as compared to 50 mg/kg consumed by the controls (Plumlee *et al.*, 1956). An interesting increase in body fat was seen, involving particularly the dorsal fat. In addition, these animals had muscular weakness and short legs and displayed slow growth. Estrus cycles were irregular, and, often, the pregnancies ended with resorption of the fetus. The surviving newborn were ataxic, small and weak, and their balance and locomotion were bad. The sows produced milk poorly and secreted it ineffectively, since their udders remained underdeveloped. It is of interest that Amdur *et al.* (1946), as well as Curran (1954, 1955), have discovered biochemical links between the metabolism of fats (or lipids) and of manganese.

In *cattle*, manganese deficiency has been produced experimentally and has been reported as occurring spontaneously in animals grazing in peat (i.e., manganese-poor) pastures. The deficiency syndrome is similar to that found in the other species (Bentley and Phillips, 1951a; Gessert *et al.*, 1952; Hawkins *et al.*, 1955).

In *man*, there is now recorded indirect evidence suggesting that "apresoline disease" might well be a manganese deficiency (Comens, 1956). This is discussed under another heading (cf. Section XI, J).

B. Manganese Deficiency in Birds

As was seen in the preceding section, manganese deficiency of mammals was first induced experimentally in an effort to elucidate the role of this metal in the body. Manganese deficiency in birds, on the other hand, was

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defined as a consequence of seeking a treatment of the "spontaneous" diseases, perosis and chondrodystrophy.

The fact that manganese was linked with perosis (slipped tendon) was discovered by Wilgus, Norris, and Heuser (1936, 1937). These workers were cognizant of the fact that perotic hens become worse when given dietary supplements of calcium monophosphate. Paradoxically, a particular lot of this salt in fact *improved* their perotic hens, and a beneficial contaminant was therefore suspected. Thus, they discovered the preventive and curative effects of manganese for perosis.

It is now recognized that manganese-deficient birds are apt to develop two forms of disease: *chondrodystrophy* in embryonic life and *perosis* in postembryonic life.

1. *Chondrodystrophy*

The sporadic, genetically transmitted disease (Landauer and Dunn, 1926) is not related to known nutritional factors. The nutritional form was defined by Byerly *et al.* (1935), when they found that green vegetables offered to the hen prevented the appearance of the disease in the embryo.

Chondrodystrophy is characterized by defective growth, edema, generalized bone disease, and high mortality. The abdomen protrudes, the head is round, and the underdeveloped lower mandible makes these chickens look like parrots. After this clinical picture was reproduced by rendering laying hens manganese-deficient (Lyons and Insko, 1937), the disease became preventable either by giving manganese supplements to laying hens or by injection of 30 μg of $MnSO_4$ directly into the eggs. Analysis of eggs has shown that normal embryos contained 7.0 μg, while chondrodystrophic ones contained 2.5 μg manganese/embryo.

2. *Perosis*

Perosis is characterized by shortening and thickening of the bones, accompanied by slipping of the epiphyses and of the Achilles tendon.

The disease develops readily in chicks receiving about 10 mg manganese/kg diet, which is the level usually found in corn (Schaible and Bandemer, 1942). Feeding of high amounts of calcium and phosphorous as well as of ferrous citrate (Wilgus and Patton, 1939) tends to aggravate the disease in deficient birds and to induce it in normal chickens fed normal amounts of manganese.

In borderline cases the disease may be improved or its occurrence prevented entirely by inositol supplements (Wiese *et al.*, 1938) or by the addition of choline (Hogan *et al.*, 1941). These phenomena are attributable to "synergism" between inositol or choline and manganese, since choline has been found to be synergistic with manganese, even in its lipotropic action

on the livers of intact rats. Otherwise, the role of manganese is specific, since other elements seem incapable of preventing the disease (Wilgus *et al.*, 1939).

The degree of susceptibility to manganese deficiency is genetically controlled. The chickens displayed a considerable strained-linked susceptibility to perosis, as individual strains require different dietary levels for its prevention (Golding *et al.*, 1940). However, as a rule, 50–80 mg Mn^{2+}/kg diet is preventive, while twentyfold increases in these levels are well tolerated (Gallup and Norris 1939a,b). Perosis developing very early seems to be irreversible, but the nonaffected young of deficient mothers do not seem more susceptible to the disease than the young of normal hens.

The bodies of manganese-deficient chicks show a strikingly diminished manganese content. Normal chicken bones contain about 2.0 μg Mn/gm, while perotic bones contain one-third to one-fourth that amount. The concentrations of many other constituents of bone or of other parts of the body have been found to be similar in both normal and perotic birds (Caskey *et al.*, 1939, 1946). Some observers (Combs *et al.*, 1942; Bernard and Demers, 1953) have reported low serum alkaline phosphatase levels in deficient birds, while others (Nielsen and Madsen, 1942) have found them to be increased. Bone phosphatase activity has been reported to be low (Wiese *et al.*, 1939; Combs *et al.*, 1942).

Deficient birds produce few eggs, with a markedly reduced manganese content (Gallup and Norris, 1939b). These eggs hatch poorly and break readily. The mortality of hatched embryos is high. The survivors are often ataxic (Wilgus *et al.*, 1939).

The observations related to perosis in chickens have been essentially duplicated in and extended to turkeys (Evans *et al.*, 1942, 1943), swans (Emmel, 1944), and ducks (Bernard and Demers, 1953; Van Reen and Pearson, 1955).

XI. Interrelations Between Manganese Metabolism and Some Aspects of Disease and Therapy

This section groups together various observations that indicate possible relationships between some nonexperimental disease states (or their therapy) and manganese metabolism. By the same token, there emerge areas for further investigation.

A. Vitamins and Cofactors

1. *Choline Deficiency*

Choline deficiency markedly aggravates perosis in birds (Jukes, 1940; Hogan *et al.*, 1941; Jukes and Welch, 1942; Evans *et al.*, 1943), and some workers have found that sometimes manganese supplements alone do not

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protect from the disease (Jukes, 1940; Jukes and Welch, 1942). Hogan *et al.* (1941) discovered that choline additives were effective cofactors of manganese in preventing or reversing perosis.

Amdur *et al.* (1946) studied the deposition of fat in the livers of rats. They varied the concentration of manganese between 0.3 μg/gm diet and 649 μg/gm diet and that of choline between 0.1 and 0.8 μg/gm diet. They discovered that the amount of fat in these livers was reduced by both manganese and choline feedings. The two factors acting together produced more than the sum of the two effects.

2. *Inositol*

Inositol seems to be instrumental in the creation of manganese deficiency in a manner similar to choline. It tends to prevent perosis when dietary manganese borders the deficiency states (Wiese *et al.*, 1938).

3. *Thiamine Metabolism*

Perla (1939) and Perla and Sandberg (1939) have stated that toxic manifestations resulting from heavy loads of thiamine were neutralized by the administration of manganese additives. They surmised that the thiamine requirements of animals depend upon the inorganic manganese content of their diets. Sandberg *et al.* (1939) went so far as to indicate that manganese additives in general might have been effective as nutritional supplements by virtue of their action on thiamine utilization. This they supported by stating that the depletion period of rats on vitamin B_1-deficient diets was shortened by manganese additives and that the blood pyruvic acid level has reflected this fact. Hill *et al.* (1950) confirmed and extended the notions pertaining to metabolic reciprocity between thiamine and manganese. Holtcamp and Hill (1950) studied this effect in the rat with diets offering 0.1–20 mg Mn^{2+}/rat/day and saw no thiamine effect. However, Hill and Holtcamp (1954) analyzed the livers and intestines of rats fed varying amounts of manganese and thiamine. They found reciprocal increases in the values of both these substances when concomitantly fed. Anderson and Parker (1955) have questioned the interaction between thiamine and manganese.

These observations justify this reviewer's concern with possible differences between naturally occurring and added inorganic manganese in the context of nutrition. For instance, manganese-thiamine complexes do not appear to have been studied.

B. Hormones

Administration of estradiol increases the level of manganese in blood (Bolton, 1955). The thyroid gland tends to accumulate the metal (Ray and Deysach, 1942) which, when given in large amounts, depresses the basal metabolic rate in the guinea pig. A relationship between manganese metabolism and anterior pituitary function has also been postulated for the mouse (Kemmerer *et al.*, 1931).

Hughes and Cotzias (1960) have found that administration of glucocorticoids to mice caused depletion of the liver and enrichment of the carcass with Mn^{54} tracer. Mineral corticoids produced little effect.

C. Amino Acids

Manganese metabolism is thought to be coupled to amino acid metabolism not only because of the activation on some hydrolyzing enzymes (i.e. arginase), but also in that it forms chelates with amino acids (Christensen *et al.*, 1956; Christensen, 1957) in which *pyridoxal* may participate. These complexes of amino acid, pyridoxal, and manganese are transported in the body more rapidly than are amino acids alone. These very significant observations link protein metabolism to manganese turnover and establish the role of complexes in the metal transport (Pal and Christensen, 1959).

D. Fat and Lipid Metabolism

The relationship of fat to manganese metabolism is shown by the experiments of Amdur *et al.* (1945) already discussed. Curran (1954, 1955) has discovered that manganese increases and that vanadium depresses cholesterol synthesis. This has permitted Curran to develop an effective treatment of hypercholesterolemic states. Schroeder and associates (Schroeder and Perry, 1955; Perry *et al.*, 1954; Schroeder, 1956a,b,c) have studied hypertension and atherosclerosis as well as their treatment, viewing these diseases as metabolic errors of trace metal metabolism, including manganese. Their thesis seems ingenious and merits detailed study. Their evidence is too multifaceted for proper discussion here.

Wakil *et al.* (1957) and Tietz (1957) have demonstrated that manganese is an activator in the synthesis of fatty acids *in vitro*.

E. Antibodies

An interesting series of papers (cf. Walbum and Mörch, 1923; Walbum and Schmidt, 1925) discusses the effects of injected metal salts on serum antibody levels of immunized animals.

The diphtheria antibody titers of immunized goats and horses were markedly increased and sustained by repeated injections of manganous and cobaltous chlorides. Of the two salts, the manganous was more effective, but only when injected, not when fed. Analyses of organs from these animals showed an increase in manganese concentration which was proportional to the amount of salt injected and (in the case of the liver) directly proportional to the increment in antibody titer.

Agglutinin levels were similarly affected. Anti-redcell agglutinin titers were found to increase after injections of manganous chloride, while the effects of other metals were not clear-cut. Agglutinins against *Escherichia coli* were also found to increase following manganous chloride injections. The salts of other metals did have effects on these titers, albeit acute ones and of a kind difficult to summarize.

These early experiments appear worth pursuing further with the now available immunochemical techniques, since so much more is known both about metals and about immune processes.

F. Antibiotics

Manganese forms chelates and coordination compounds with antibiotics like tetracycline (Albert and Rees, 1956) and aureomycin (Pepper *et al.*, 1952). When aureomycin is added to deficient diets which normally produce a high incidence of disease, perosis does not occur. This probably indicates that the absorption of the element in these particular chelate forms is more effective than absorption of the element in ionic form. Concordant experiences of Christensen have been mentioned above.

G. Hematopoiesis

Small doses of manganous ions (Paterni, 1954) are thought to stimulate hematopoiesis, while manganese poisoning is often associated with a hypochromic anemia. Manganese preparations enjoyed a period of fashion as remedies for blood diseases. However, the American Medical Association (1940) has taken an unfavorable view of this practice. On the other hand, infectious anemia of cattle (Carlstrom, quoted by Underwood, 1956) tends to occur primarily in animals receiving high manganese intakes. Also, cobalt polycythemia is aggravated by a high manganese intake (Orten *et al.*, 1933). Thus an interrelationship between hematopoiesis and manganese metabolism is likely. This is supported by the discovery of a manganese containing heme in the erythrocytes of man and rabbit (Borg and Cotzias, 1958b). These latter observations were extended to the duck and were amplified by Norris and Klein (1961).

H. Carcinogenesis

This has been studied relative to manganese metabolism by Balo and Banga (1957). They found that two experimental cancers were cured or markedly inhibited by manganese maleate. Furthermore, this compound changed the respiration of these experimental cancers *in vitro* from anaerobic to aerobic. These experiments impressed the writer as being very worth pursuing further.

I. Jaundice

Jaundice has been thought to upset manganese metabolism very markedly. Lindberg and Ernster (1954) have shown that manganese may be a cofactor in oxidative phosphorylation. Zotterström and Ernster (1956) suggested that bilirubin complexes manganese, an interpretation supported by Maynard and Fink (1956). This raises the possibility that *kernicterus* is a metabolic disturbance of manganese metabolism, particularly since the clinical picture resembles that of manganism.

J. Mesodermal Diseases

Comens (1956) considers human hydralazine disease as being in essence a manganese deficiency. The disease occurs in hypertensives treated with hydralazine (Apresoline), and is very similar to naturally occurring *lupus erythematosus disseminatus*. Comens states that it can be reversed by manganese supplements. Furthermore, administration of apresoline to cockerels seems to have produced a perosis that can be prevented by supplements of manganous citrate. If Comens' results are confirmed and extended, they might not only lead to a better understanding of hypertension and lupus, but also show the importance of manganese in human metabolism.

K. Ectodermal Diseases

Injections of manganese preparations were once considered helpful in the treatment of ectodermal diseases, but such therapy is currently out of fashion (Sullivan, 1940). However, Borg and Cotzias (1958c, 1960) have discovered that phenothiazine drugs form free radicals with manganic (3+) ions *in vitro*. In searching the literature, Borg and Cotzias (unpublished) have been impressed by the extensive parallelism between the *in vivo* and *in vitro* behavior of phenothiazine drugs on the one hand and manganese additives on the other. Particularly striking was the observation that administration of phenothiazine may cause in the human the same symptoms that have been discussed under chronic manganese poisoning. These correlations and experimental results again point to a possible role of manganese in mental and nervous disease.

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Neutron activation analysis: The stability of manganese concentrations in human blood and serum

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Determinations of serum manganese by neutron activation performed at this center[1,2] have yielded consistently much lower values than those obtained by the majority of other laboratories. It was suspected that our lower results indicated losses incurred during our procedure. However, a detailed search revealed, instead of losses, a moderate, systematic contamination of the samples with exogenous metal.

The corrected procedure was applied to the question of the stability of these low concentrations of manganese in human plasma and whole blood. Repeated sampling from the same individual over long periods demonstrated a high degree of constancy of the concentration of this metal. A somewhat greater range was encountered from one person to the next.

Oral administration of manganous sulfate, in large amounts, for long time periods, showed no detectable effects on a large number of clinical parameters tested under metabolic ward conditions. However, there were observed moderate increments in the manganese concentration of whole blood and minor increments of the metal's concentration in serum. Still, additions of manganous sulfate to serum in vitro showed that the metal became extensively bound in a nondialyzable form.

By virtue of its participation in functions ranging from oxidative phosphorylation to the formation of bone, manganese seems to be essential to all living organisms.[3] Clinical interest in this nutrient has been limited by its incredibly low concentrations in the accessible liquid compartments of man. Analysis of such low concentrations required high sensitivity with exquisite control of contamination. Both specifications appeared to be met by neutron activation analysis (NAA).[4,5]

In our extensive experience with NAA of animal tissues,[1,2,6-10] we found that values for the manganese-rich solid tissues were in fair agreement with previous observations, whereas those for the manganese-poor plasma and serum were impressively lower. NAA has been employed by others,[11-16] but only one

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group obtained results as low as our own;[12, 13] another group has reported somewhat lower concentrations with a colorimetric ultramicromethod.[15] The persistence of such a sharp deviation from the experience of the majority made it mandatory that we investigate possible errors in our technique. Hence, we have dealt individually with a series of potential errors; correction of the errors actually found tended to diminish our values even further.

Application of the improved technique to analyses of human serum and blood showed a considerable constancy of this metal's concentration in the pools analyzed. This stability could be perturbed by feeding large amounts of a manganous salt. This happened, however, very slowly. The ensuing increments were much more pronounced in whole blood than they were in serum. Still, when tested in vitro, serum showed a high capacity to stabilize large amounts of manganese in a nondialyzable state. These results are discussed with regard to their bearing on the homeostatic mechanisms for the control of manganese metabolism.

Materials and methods

Method. This consisted of collection of the sample, activation in a reactor, transfer of the sample into a solution of carrier, wet digestion, oxidation to permanganate, precipitation of the latter, determination of the ^{56}Mn, and estimation of the recovered carrier.[1]

Instruments.

A. Reactors. Two reactors were compared: (1) This laboratory's Graphite Reactor (~ 20 megawatts, ~ 1.17×10^{13} total neutrons per cm.^{2} per second). The facility used (PN-3) had a cadmium ratio of about 38 and a temperature of about 90° C. (2) The Medical Research Reactor (~ 3 megawats, ~ 1.12×10^{13} total neutrons per cm.^{2} per second). The facility used had a cadmium ratio of about 71.5 and a temperature of 71° C.

B. Gamma ray spectrometer. A model 402, 400 channel pulse-height analyzer manufactured by the Technical Measurements Corp. was used in place of the spectrometer discussed earlier.[1]

C. Apparatus. The spectrophotometer, filtration apparatus, quartz pipettes, demineralizer, sample holders, and aluminum planchettes were discussed earlier.[1]

D. Sample-flushing assembly. This consisted of: (1) A 500 ml. Pyrex dispensing bottle located on a hot plate, filled with reagent grade concentrated nitric acid (~ 86° C.). (2) A Pyrex funnel (5 cm. in diameter, A. H. Thomas Co., Cat. No. 5574B) mounted on a swivel clamp to be kept horizontal while the sample holder was being attached and vertical while flushing the sample into an Erlemeyer flask. The stem of the funnel ended in a flare provided with an inner tip. These fitted, respectively, the outside diameter and the lumen of the sample holder, to prevent rinsing the outside of the holder and thus decrease the blank.

Reagents. The nitric acid, the mercuric sulfate solution, the tetraphenyl arsonium solutions, the hydrogen peroxide, the triple distilled water (DDDH$_2$O), and the manganous sulfate solutions A and B were discussed earlier.[1] Regarding the standard, it was not deemed feasible to introduce a tissue-equivalent matrix without also introducing contamination with manganese.

Sample holders. The following procedure for making sample holders appears reasonable for the present: 6 foot lengths of polyethylene tubing are rinsed with 500 ml. of DDDH$_2$O and shaken dry. Segments measuring 4 to 5 cm. are cut with newly chromium-plated scissors, placed in a rinsed 250 ml. polyethylene container, and oven-dried. Ten per cent of the segments are sampled randomly and tested for manganese. The resultant mean value constitutes a blank for the batch. Batches showing a blank of less than 10 per cent of the amount of metal expected to be present in the sample are considered as acceptable for the present.

Procedure. For collection of blood samples, there were used two procedures, both of which avoided anticoagulants: (1) the syringe and vial procedure discussed earlier,[1] and (2)

the "Peel-a-way" blood sampler. Yale sterile disposable needles were used. In case of obvious hemolysis the plasma was discarded; in case of doubt, it was tested for hemoglobin with Occultest Reagent Tablets and discarded if the test was positive. As a rule clotting did not occur during centrifugation, but if it did, serum was analyzed instead of plasma.

All samples were treated identically thereafter: the sample holders were color-coded with a Marks-a-lot spot. One sample holder contained the standard, and an empty one was used as a blank. After drying, 8 sample holders were introduced either into a nylon container (2.2 by 6 cm.) or into a 1 oz. size polyethylene vial (Nalgene Co.).

Following activation[1] each sample holder was rinsed in 40 ml. of 20 per cent reagent nitric acid followed by distilled water and wiped with Kleenex. Its color-coded end was cut off and the sample holder was introduced into the flare of the vertical funnel. The funnel was turned to the horizontal position, the remaining sealed tip was cut off and introduced into the mouth of a 250 ml. Erlenmeyer flask. This contained 1.50 ml. of solution A[1] delivered by means of a 5 ml. recalibrated burette. The funnel and flask were returned to the vertical position and flushed with 20 ml. of hot nitric acid.

Wet digestion, oxidation, and chemical separation. As opposed to other tissues, when samples of serum or plasma measuring more than 0.2 ml. were analyzed, the precipitates contained sometimes extraneous radioactivities. Hence, the millipore paper supporting the precipitate was heated in 100 ml. of oxidizing solution, resulting first in disappearance and later in reappearance of the permanganate. A second precipitation yielded permanganate containing almost no measurable extraneous radioactivities. This second purification was applied whenever the contaminating radioactivities approximated the peak of ^{56}Mn as seen on the oscilloscope of the gamma ray spectrometer.

Determination of manganese. With the exception of the introduction of a better multichannel analyzer assembly, this step was practiced as before.[1]

Remarks. The loss of carrier during the chemical separation has ranged between 0 and 10 per cent whether the second chemical separation was applied or not. Incomplete oxidation of the carrier could be induced by the addition of chloride salts but only in excess of 1 Gm. of NaCl per flask. The colorimetric estimation of the recovered carrier has provided a satisfactory means for correction of the losses of isotope under all conditions tested. The chemical separation was usually complete 2 hours following activation of the sample. The blank tubes were analyzed first. The manganese standards were corrected for decay, background, and chemical losses, but not for the day-to-day variations of the two reactors.

The following illustrate actual counting rates obtained: The mean counting rates and standard deviation (in per cent) for 21 standards activated in the Medical Research Reactor were 2.86×10^5 c.p.m. (± 9.2 per cent), whereas the corresponding numbers for 49 standards activated in the BNL Graphite Research Reactor were 2.15×10^5 c.p.m. (± 7.5 per cent). Activation of biologic samples in the Medical Research Reactor has yielded a range of 849 to 2248 c.p.m. of ^{56}Mn per 0.5 ml. of serum and 5664 to 9350 c.p.m. per 0.2 ml. of blood.

Analytical results

The contribution of ^{56}Fe to the production of ^{56}Mn. The nuclear reaction utilized in the present work is [^{55}Mn (n/γ) ^{56}Mn]. A significant contributing reaction may be [^{56}Fe (n, p) ^{56}Mn], which with other reactors has accounted for up to 40 per cent of the radiomanganese produced during activation.[18] In order to investigate here the contribution of this reaction, samples of iron wire (J. T. Baker Chemical Co., 99.8 per cent pure) weighing about 8 mg. were analyzed. After activation in either reactor, these samples were dissolved in nitric acid and counted, without chemical separation, together with manganese standards. The manganese concentrations were 187.3 and 187.5 μg per gram in one analysis, 212.3 and 212.5 μg per gram in another, and the mean and standard deviation of 12 additional analyses were 237 ± 4.9 μg per gram. The range among the various runs suggested that different segments of the iron wire contained

different amounts of manganese as a contaminant. Indeed, spectrophotometric analyses of additional wire samples showed a mean concentration of 204 μg of manganese per gram.* These analyses confirmed earlier calculations[1] which had indicated that, in the facilities used, the contribution of ^{56}Fe to the determination of ^{55}Mn was not sufficiently large to merit further definition at the present.

Sources of contamination with manganese. In agreement with others[15, 16] it seemed important to deal systematically with the definition of a "blank" for NAA by investigating all steps from collection to analysis.

The collection of blood by means of syringe and vial was compared to the "Peel-a-way." In 6 comparative tests done on separate days on the same patient no significant differences were found in the manganese concentration of whole blood or of serum.

Transfer of serum by means of Yale disposable needles showed no contamination on 4 occasions. Yet, serial transfers with these needles from one "cleaned" vial to another gave the following results: 0.930, 1.18, 4.82, and 5.20 μg per liter. Whereas on repetition the results were similar, single transfers on 5 occasions through ordinary steel needles increased the serum manganese concentration by factors ranging between 3 × and 20 ×. These tests confirmed Bowen's concern with contamination from steel.[11]

Thereafter, empty sample holders were analyzed individually soon after activation to avoid excessive decay of ^{56}Mn (T/2 = 2.586 h). The results are listed in Table I. It is noteworthy that the highest blank was due to our having cleaned these holders according to the procedure recommended earlier.[1] The resultant blank was significant if manganese-poor serum were to be placed in these "cleaned" holders, but not if the sample were manganese-rich. These results led us to study: (1) the reagents that may deliver manganese onto these holders, and (2) the optimum volume (i.e., optimum amount of manganese) for analyses of plasma or blood.

Our all-glass triple distilled water showed that the concentration increased with continued use of the double still from 0.479 to 1.17 μg per liter. Thereafter, following the distillation of 25 gallons of already distilled water, the still was washed with Alconox and rinsed with demineralized water, and the first 500 ml.

*The spectrophotometric analyses were generously performed by Mr. D. F. Leahy of this laboratory.

Table I. Manganese recovered from empty sample holders (mμg per holder)

Group*	No. of analyses	mμg Mn/holder	
		Mean	S.D.
A	10	0.068	± 0.053
B	13	0.013	± 0.009
B	23	0.024	± 0.037
B	6	0.014	± 0.012
B	10	0.009	± 0.007
C	16	0.169	± 0.114

*A, uncleaned; B, four batches rinsed with triple distilled, demineralized water (DDDH₂O); C, cleaned with distilled nitric acid and DDDH₂O.

of distillate was discarded. Subsequent distillates have ranged between 0.172 and 0.258 μg per liter. The reagent grade HNO_3 contained between 0.730 to 0.922 μg per liter. Distillation through a freshly cleaned still lowered this value to 0.450 μg per liter; use of an uncleaned still raised it to 9.83 μg per liter.

Potential failure to prevent manganese from adhering to the holders led to a search for sample holders requiring no cleaning. Teflon tubes yielded a blank of 1.7 mμg per tube. Gelatin capsules contained 0.340 μg per capsule. Quartz tubes became intensely radioactive. Cellulose acetate electrophoretic strips contained no demonstrable manganese, but they could not be molded into holders without contamination.

Accuracy of the measurement. The previous recovery experiment (experiment 1, Fig. 2) was repeated with 10 samples of serum to which manganese was added in amounts ranging from 0.25 to 400 mμg per sample. The same linearity was present.

In 10 experiments, the *volume* of the plasma sample analyzed was varied between 0.040 and 0.500 ml. and in one between 0.2 and 0.8 ml. Again a straight line fitted the data, but only in the 6 experiments which followed the control of the blank. Satisfactory linearity was obtained also when the volume of whole blood was varied between 0.1 and 0.5 ml.

The possibility of reproducible losses of serum manganese was investigated first with radiomanganese (^{54}Mn) and later with ^{55}Mn. In one experiment, a sample of serum containing ^{54}Mn was analyzed: (1) immediately; (2) following chemical separation of the isotope; and (3) following both activation in the reactor and chemical separation. The ^{56}Mn produced during activation was permitted to decay. The results are listed in Table II. Furthermore, the amount of ^{54}Mn added to serum was repeatedly quantified before and after our chemical separation without evident losses. Finally, our carrier was shown to be a true carrier of the element in tissues: ^{54}Mn was injected intravenously into rats and their blood and serum were analyzed for ^{54}Mn with and without activation and/or chemical separation.

It remained to find and to analyze a suitable biologic material for natural instead of radioactive manganese. A sample of Cohn's human plasm Fraction IV.7-2 contained only traces of Na and Cl and could be analyzed therefore for ^{55}Mn both with and without chemical separation of the ensuing ^{56}Mn. In 6 analyses without chemical separation there were found 3.44 ± 0.42 μg of ^{55}Mn per gram of dry protein *versus* 3.52 ± 0.48 μg of ^{55}Mn per gram found after chemical separation.

Table II. Recovery of radiomanganese ($^{54}Mn^{++}$) added to serum

*Group**	*No. of analyses*	*Mean*	*Range*
A	1	1.406×10^5	
B	3	1.445×10^5	$1.430\text{-}1.458 \times 10^5$
C	3	1.438×10^5	$1.432\text{-}1.446 \times 10^5$

*A, original serum; B, after digestion and chemical separation; C, after neutron activation, followed by digestion and chemical separation.

Direct comparisons of manganese analyses by NAA versus other methods. As an essential extraneous check, there were compared analyses of manganous sulfate standard by NAA versus colorimetry of permanganate produced as stated.[1] Plots of the data against each other yielded a straight line having a slope of 1, with amounts of manganese ranging from 25 to 500 μg per sample. This range is much higher than the biologic one and, therefore, samples of dried, powdered kale were analyzed thereafter. This powder was kindly supplied by Dr. H. J. N. Bowen[19] as a biologic standard. He reported it to contain 14 μg of manganese per gram of dry weight[20]; with 8 analyses with our chemical separation showed 14.1 ± 0.5 μg of Mn per gram.

The method was further compared with another by analyzing biologic samples containing even less manganese than the powdered kale. Acceptable material was obtained as follows: a unit of whole blood was separated into plasma and cells. Both were dried at 85° C. in platinum crucibles, heated in a muffle furnace at 200° C. for 36 hours, and ashed for 24 hours at 300° C. Both ashes were hygroscopic. Weighed samples were kindly analyzed spectrographically by Dr. H. Mitchell Perry, Jr.[21] He reported 0.67 (0.63 to 0.74) μg of manganese per gram of dry plasma ash and 6.3 (5.3 to 7.0) μg of manganese per gram of dry cell ash. Neutron activation analysis of 4 samples of the serum ash showed 0.70 (0.69 to 0.83) μg of manganese per gram of dry ash, but 4 analyses of cell ash showed 4.6 (4.3 to 5.3) μg of manganese per gram of dry ash. The difference between the respective values for the cell ash, as well as the ranges of these values, were ascribed to the difficulty in weighing-out samples of ash.

Two replication experiments performed recently showed a mean and standard deviation of 0.570 ± 0.070 and 0.839 ± 0.092 μg of Mn per liter, respectively.

Clinical results

The experiments which follow investigate whether the manganese concentration in some liquid pools is characterized by stability.

Normal subjects. In 14 fasting, active laboratory workers, the blood hemoglobin concentration, the hematocrit, and the total plasma proteins were determined concomitantly with the manganese concentration of whole blood and of plasma. These workers were equally divided by sex, but their ages ranged from 19 to 81 years. The data are listed in Table III. It is evident that the standard

Table III. Means and standard deviations for hemoglobin, hematocrit, and total plasma proteins of 14 active laboratory workers compared to those of the concentrations of manganese in their plasma and their whole blood

	Hemoglobin (Gm./100 ml.)	*Hematocrit (%)*	*Plasma protein (Gm./100 ml.)*	*Plasma Mn (μg/L.)*	*Whole blood (Mn μg/L.)*
Mean	14.5	44.7	7.2	0.587	8.44
Per cent	100	100	100	100	100
S.D.	±0.9	±2.5	±0.4	±0.183	±2.73
Per cent	6.0	5.6	5.5	31.2	32.3

deviation of the values for manganese was higher than that of the accepted clinical measurements listed in the table. This evoked the question of whether a higher or lower degree of constancy would be encountered if repeated analyses on the same person were done over a long period of time.

Observations on patients. All tests which follow were performed on in-patients while admitted to the metabolic wards of the Research Hospital of the Brookhaven National Laboratory. These patients were all female (Table IV) and were fasting. It is evident that the manganese concentrations in whole blood were more constant here than in the group of healthy volunteers. On the other hand, this difference was less convincing in the case of plasma.

Four patients, all female (Hospital Nos. 8062-R, 7284-R, 9212-R, and 7826-R), were given manganous sulfate orally in gelatin capsules in addition to the estimated 3 to 5 mg. of manganese supplied by the regular hospital diet.[22-24] The daily doses given ranged from 1.2 to 2.0 Gm. of $MnSO_4 \cdot H_2O$ (0.390 to 0.650 mg. of Mn^{++}). The periods of administration ranged between 34 and 570 days. A total of 8 such periods were instituted, with the patients under continuing observation in the metabolic wards. The tests* were performed periodically, with the exception of the serum iron and binding capacity determinations which were performed intermittently in one case. No changes were noted.

In 3 hospital patients an attempt was made to induce increases of the manganese concentration of whole blood and plasma. These patients, all male, were given capsules containing 300 mg. of $MnSO_4 \cdot H_2O$ (98 mg. of Mn^{++}) daily for one week. Three duplicate analyses were secured without regard to meals on 3 separate days prior to and at the end of the period during which the manganous supplement was given. The results, listed in Table V, failed to reveal a clear-cut increase in the metal's concentration in either blood or plasma. This contrasted to the obvious elevation observed in one patient (7826-R) who had been given 2000 mg. of $MnSO_4 \cdot H_2O$ (650 mg. of Mn^{++}) daily for 19 months while under rigorous observation* on the metabolic wards. During the last 9 months of this regimen there was recorded the only abnormality observed in this case, namely the high blood manganese concentration, and the minor elevation in serum manganese concentration shown on Fig. 1. Following discontinuation of this massive manganous supplement, the plasma values declined much more rapidly than those of whole blood. At the end of this latter decline we became aware of the blank in our serum analyses. Hence this same patient was fed the same manganous supplement that had been given to the three male patients discussed above, but for a long period of time. The data on Table IV were obtained prior to those shown on Fig. 2. The latter shows a slow but continuous increase in the metal's concentration in whole blood as contrasted to the much steadier concentrations of the metal in serum. Furthermore, after about 120 to 140 days of administering 300 mg. of $MnSO_4$ daily, the concentration of the

*In addition to daily visits and periodic physical examinations by a physician, the patients had periodically the following laboratory examinations: blood urea nitrogen and urea clearance, acid and alkaline phosphatase, serum iron and binding capacity, sedimentation rate, differential and direct blood counts, hemoglobin and hematocrit, total plasma proteins, A/G ratio and electrophoretic patterns, cholesterol and esters, fasting and 2 hour postprandial blood sugar, plasma CO_2 and Cl, urinalysis and culture, stool guaiac, fluid intake and output, Papanicolaou smear, EKG, chest plates, audio and visual screening, and handwriting.

Table IV. The concentration of manganese in the plasma and the whole blood of patients observed while in a metabolic ward over the time periods indicated. The data on patient 7826-R were obtained between those shown in Fig. 1 and those in Fig. 2.

Patient no.	*Diagnosis*	*Age*	*Months studied*	*No. of analyses*	*Plasma mean (μg Mn/L.)*	*S.D.*	*No. of analyses*	*Wh. blood mean (μg Mn/L.)*	*S.D.*
8-04-16R	Postencephalitic Parkinsonism	54	5	11	0.837	±0.17	11	12.36	±0.88
8-07-64R	Parkinsonism	63	2	9	0.59	±0.13	9	9.02	±0.82
8-08-08R	Rheumatoid arthritis	41	1-¼	5	0.501	±0.04	7	10.18	±0.82
8-07-37R	Lupus erythematosus disseminatus	61	1	24	0.588	±0.20	16	7.65	±0.78
78-26R	Hemiplegia (cerebral thrombosis)	79	36	11	0.83	±0.25	7	11.82	±2.00
8-02-52R	Parkinsonism	73	9	10	0.645	±0.09			

Table V. The manganese concentrations in whole blood and in serum from patients before and after administration of $MnSO_4$ (300 mg. per day) for one week

		Mn in whole blood				*Mn in serum*			
		Prior		*After*		*Prior*		*After*	
Patient no.	*Age*	*Mean (μg/L.)*	*Range (μg/L.)*	*Mean (μg/L.)*	*Range (μg/L.)*	*Mean (μg/L.)*	*Range (μg/L.)*	*Mean (μg/L.)*	*Range (μg/L.)*
7159 R *	56	5.75	5.62-5.83	5.82	5.75-5.93	0.645	0.430-0.750	0.828	0.548-1.189
8-01-86R †	60	9.18	8.48-9.78	7.99	6.91-8.99	0.697	0.556-0.847	1.160	0.678-1.139
8-07-57R ‡	57	7.67	7.49-7.85	7.33	7.11-7.47	0.793	0.593-1.190	0.886	0.686-1.020

*Hypertension and schizophrenia.
†Parkinsonism.
‡Hypertension.

metal in whole blood reached a new plateau. This plateau was lower than the one reached after the prolonged daily administration at 2000 mg. of the manganous salt.

The higher stability of the serum concentration evoked the question whether normal serum was not quite saturated with this metal. It seemed interesting therefore to determine "nondialysable" manganese (element 25) as well as "serum binding capacity" for this metal, particularly since with iron (element 26) such measurements have yielded considerable insight.

Experiments on serum in vitro. Others[14] have reported studies of the non-

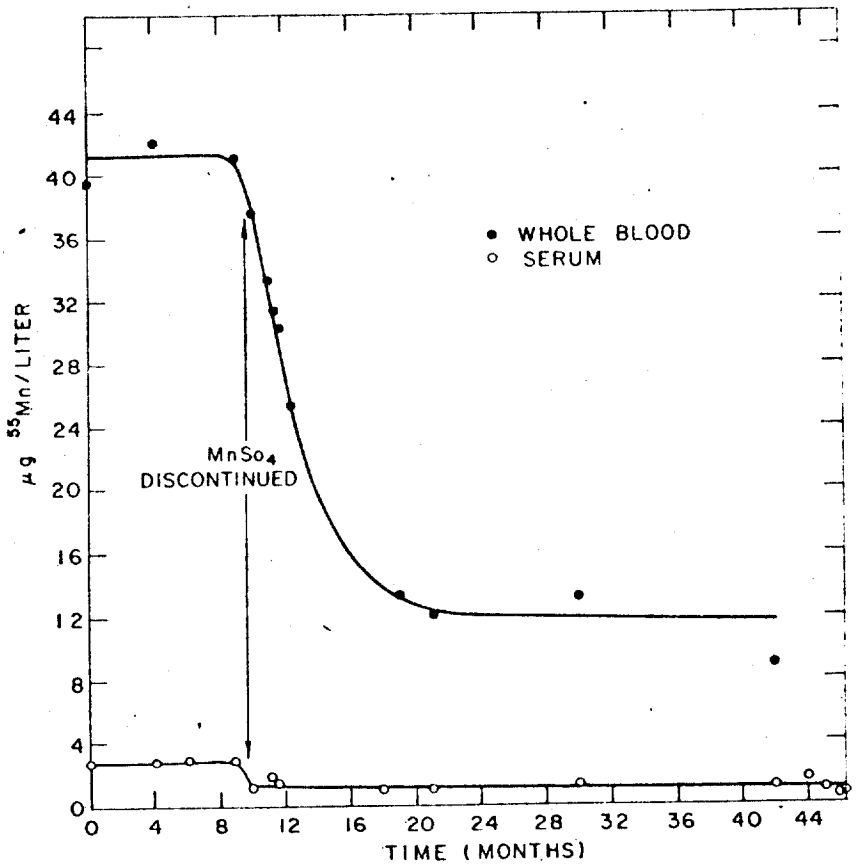

Fig. 1. The manganese concentration of whole blood and of serum following discontinuation of the manganous supplement (2.0 Gm. of $MnSO_4$ daily) in Patient 7826-R.

dialyzable manganese and copper of serum by means of neutron activation analysis. Hence their dialysis procedure appeared to constitute an appropriate method to initiate investigations of the serum's capacity to bind manganese. This procedure was used as recommended,[14] with the exception of experiments in which known amounts of manganous sulfate were added to the serum.

All data shown in Fig. 3 were obtained on sera from the same patient (8-07-64R). Since the undialyzed or partially dialyzed samples contained massive amounts of interfering radioactivities, they could be analyzed only with chemical separation of the ^{56}Mn. By contrast, the fully dialyzed samples were assayed both with [1] and without [14] chemical separation. Fig. 3 shows that shortly after the serum came in contact with the dialysis bag there occurred a sharp *rise* in the concentration of manganese. The rise continued with progressive dialysis and reached an apparent plateau, which was evident whether chemical separation of ^{56}Mn was applied or not.

The previous experiment showed that dialysis induced gross, but fairly reproducible contamination of the serum with manganese. The ammonium barbit-

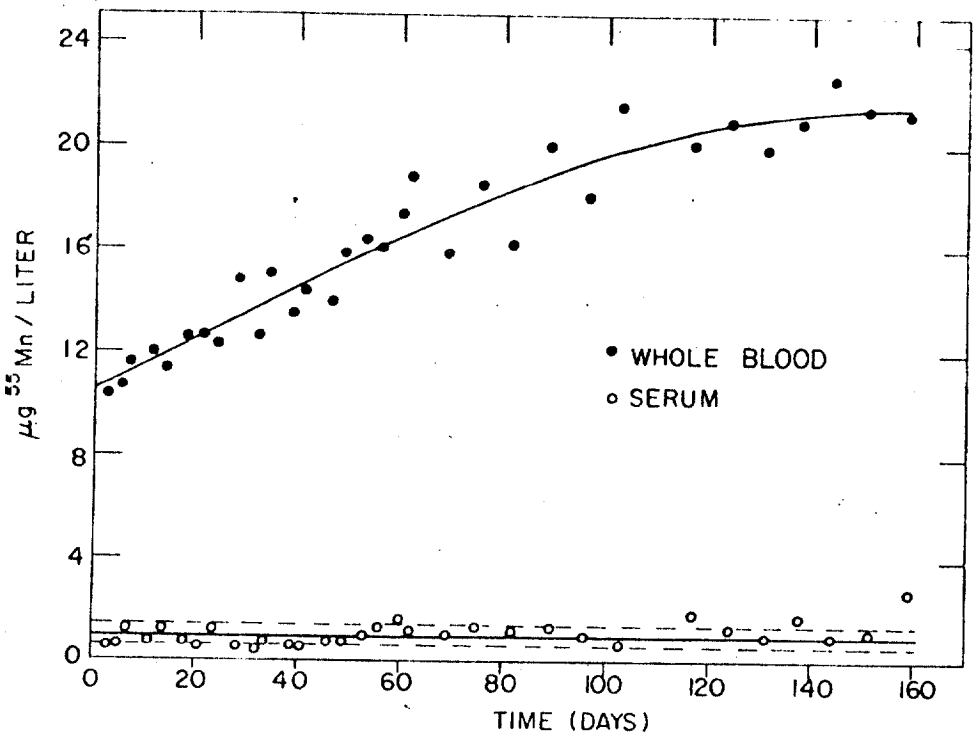

Fig. 2. The manganese concentration of whole blood and of serum following institution of continuous administration of manganous supplement (300 mg. of $MnSO_4$ daily) to Patient 7826-R, begun at time 0 of the graph. Solid lines indicate the mean values, broken lines one standard deviation. Earlier values are listed in Table IV.

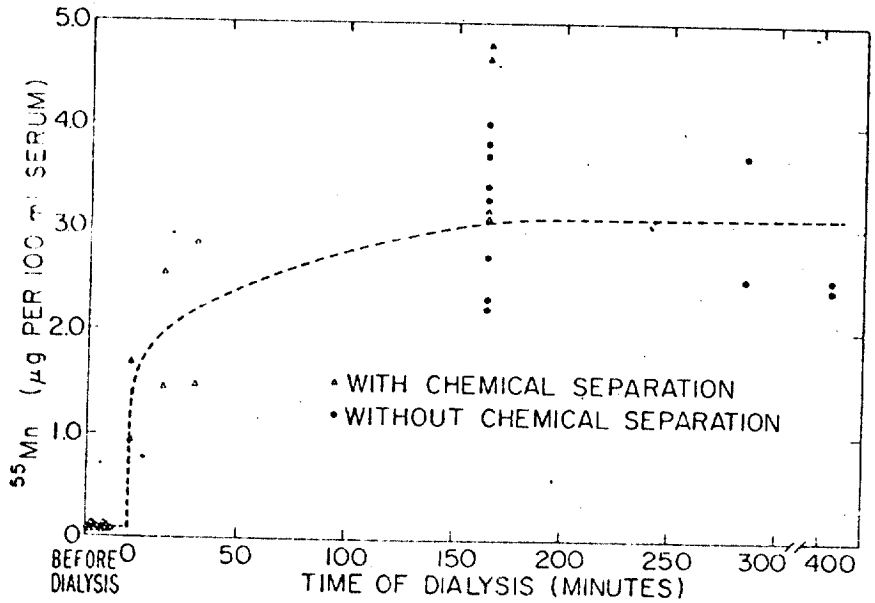

Fig. 3. Dialysis of fresh serum: note the *increase* in manganese concentration of the serum.

urate buffer contained 0.51 to 0.85 μg of Mn per liter, whether chemical separation was practiced or not, and therefore it was not a major source of contamination. The dialysis tubing showed a range of 0.086 to 0.530 μg of Mn per gram of dry tubing and was a potential source. That it was an actual source of contamination was proved in two experiments in which the manganese concentration of serum was increased tenfold after 100 mg. of dry dialysis membrane were placed in 5 ml. of serum.

Since dialyzed serum yielded a relatively constant set of manganese concentrations, the values on the plateau of Fig. 3 were thought to reflect the binding capacity of serum for this metal. Therefore, various amounts of $MnSO_4$ were added to serum prior to dialysis and the serum was analyzed without chemical separation of the radioisotope, after complete dialysis.[14] Fig. 4 shows that the limit of nondialyzable manganese was not reached in these experiments in spite of the large amounts of metal added. The suspicion that these large amounts of metal were not totally protein-bound was dispelled by direct experimentation conducted with the aid of ^{54}Mn. These data proved that manganese becomes readily stabilized by serum in one or more nondialyzable complexes. The amounts of metal thus sequestered grossly exceed the manganese concentration in fresh, unprocessed serum.

Discussion

Previous work has suggested that the metabolism of manganese might obey hitherto undefined homeostatic control mechanisms.[2, 3] The evidence was gathered with the aid of artificial radioisotopes rather than by quantifying the natural

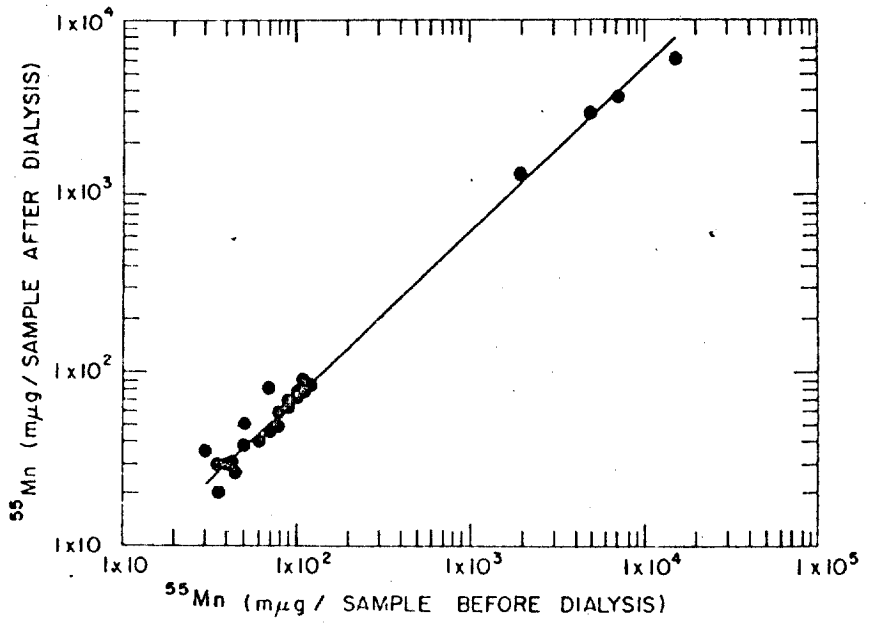

Fig. 4. Dialysis of 1 ml. samples of serum to which there were added increasing amounts of $MnSO_4$; note that the added metal remained within the serum in spite of dialysis.

species of this metal. One such observation was the marked acceleration of the tracer's excretion which followed administration of natural manganese to animals[25, 27] and man[31] either orally or parenterally. A similar behavior had characterized another essential transition metal, zinc[32-34] as opposed to a nonessential one, cadmium[35] which had shown significant differences.

The accelerated excretion of manganese following the imposition of metabolic burdens suggested that the stability of the concentration of this metal in tissues should be tested. Such studies appeared desirable, particularly in view of the changes in the tissue distribution of this metal which have followed the administration of glucocorticoid hormones,[29] the injections of ACTH[8] and the imposition of surgical stress[9] upon experimental animals. Extension of such studies to patients necessitated the quantitation of the natural manganese in the liquid pools which are accessible at the bedside. The applicability of neutron activation analysis to this problem was investigated earlier,[1] primarily because this approach offers a potential guarantee against contaminating small samples that contain scant concentrations of this trace metal. That the concentrations of this metal in liquid pools must be small indeed had been anticipated at the onset: this metal was shown earlier to concentrate rapidly in tissues, where it was preferentially localized in the mitochondria[26] and to some extent in the porphyrin of human erythrocytes.[30] Yet, the analyses of serum and plasma yielded results which were persistently and significantly lower than those obtained by the majority of laboratories. This persistent discrepancy has led to the experiments reported here.

These experiments suggested that contamination could become standardized even in ultramicroanalysis: strict adherence to procedure seems to have induced high reproducibility of the contamination present in our earlier analyses for serum manganese.[1, 2, 10] The reason analyses of blood were not affected is that the concentration of this trace metal is much higher in blood than it is in serum.[1, 10] The high but fairly constant levels reached during dialysis of serum[14] indicate that reproducible contamination must be occurring under a wide variety of circumstances in which the procedure is being rigorously followed. These circumstances must be assumed to include the collection of samples at the bedside.

Our present values for serum manganese agree with those obtained by others who used manganese-catalyzed oxidation of leukomalachite.[17] This strengthens the correlations between NAA and the other methods presented above, without constituting proof that contamination has been abolished. Hence it appears that the present work has improved the accuracy of the serum analyses, although the precision of these measurements is now somewhat lower than it was during the earlier phases of this work.[1, 2] Among the many details which resulted in improved accuracy, the definition of a blank seems to be the most important.

From the clinical point of view, the most striking finding was that the concentrations of this essential nutrient in plasma and blood are indeed very low. Still, these concentrations seemed quite steady in the serum and blood of the same individual over long periods. The values among various individuals, however, showed a higher range. Furthermore, the range of the manganese values exceeded the range of the other biologic parameters determined here. This might

well indicate that the regulation of this essential metal is less rigorous by comparison to the other parameters alluded to above. Nonetheless massive oral loads had to be given before a relatively modest increase in blood concentration was achieved. The time necessary to achieve the new plateau has approximated the life-span of the erythrocyte. Coupled with the exceedingly slow decline which has followed the interruption of excessive manganese feeding, these data might indicate incorporation of stable manganese into the heme of the erythrocyte. This is a plausible suggestion, since the in vivo incorporation of a manganese tracer into erythrocyte porphyrin has been demonstrated earlier for rabbit and man.[20]

By contrast, the concentration of this metal in serum appeared to be steadier than that of whole blood. This could not be attributed simply to saturation of the normal circulating plasma with manganese, since even the attempts to separate this metal from serum by dialysis induced marked increases in its concentration. Indeed the high avidity of serum proteins for manganese was underlined by the large excesses of metal which became nondialyzable after their addition to serum in vitro.

In vivo, on the other hand, this metal was cleared with impressive rapidity from plasma.[6, 7] Furthermore, evidence obtained in the mouse[23] suggests that manganese becomes absorbed in rough proportion to that presented for absorption. The sum of the present data is therefore compatible with the notion that regulation of this metal's concentration in tissues is accomplished primarily by active excretion. This suggestion is in keeping with current results in experimental animals, where prompt excretion of excess manganese occurred via the bile and via the intestinal mucosa.[9]

Finally, it is worth noting that no evidence of chronic manganese poisoning[3] could be elicited when massive amounts of manganous sulfate were fed to patients for long enough periods of time to induce expansion of the metal's blood pool.

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THE MINERALS OF WHEAT, FLOUR, AND BREAD[1]

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ABSTRACT

Ten commercial hard wheat blends from various growing areas, flours milled from these wheats, and bread made from the flours were analyzed for mineral elements of nutritional importance. Patent flours contained lower proportions of all minerals than the original wheat; magnesium, manganese, and cobalt less than 20%, phosphorus, potassium, zinc, iron, copper, and sodium between 20 and 32%, calcium 40%, and molybdenum 52%. In bread, sodium and calcium were present in higher concentration than in wheat. The concentration of cobalt in bread averaged 85% of that of wheat, iron and molybdenum 65%, potassium and copper 40 to 45%, zinc 28%, magnesium 19%, and manganese 13%.

Many quantitative studies have been made of the mineral composition of wheat, and there are reports of the mineral elements in flour, mill streams, and bread, as well. Bailey (1) has reviewed the literature prior to 1943. More recent research in the United States has been concerned with the relationship of mineral composition of wheat to variety and growing conditions (2,3) and to the location of the mineral elements within the wheat kernel (4). None of these studies has been concerned with the changes in content of mineral elements from wheat to bread. Consequently, data available to nutritionists have been compiled from many sources.

This paper presents quantitative data on the amounts of nutritionally important mineral elements found in commercial wheat blends from the principal hard wheat regions of the United States, commercially milled flour from each of these wheats, and bread made from each of the flours, together with a limited study of the distribution of these mineral elements in commercial flour mill streams. It represents a part of the nutrition research conducted at the American Institute of Baking (5–8).

[1]Manuscript received October 7, 1963. Contribution from the American Institute of Baking, Chicago, Illinois. This research was supported by a grant from the Max C. Fleischmann Foundation.

Materials and Methods

Sample Description. Flours, mill products, and the wheat blends from which they were milled were obtained from commercial flour mills. Bread was made from the flours at the American Institute of Baking, using the sponge and dough procedure and a representative baker's formula. The following ingredients were used, in parts per 100 parts flour by weight: compressed yeast 2.5; yeast food (composition: potassium bromate 0.11%, potassium iodate 0.10%, sodium chloride 19.4%, ammonium sulfate 7.0%, monocalcium phosphate 50%, and starch 23.4%) 0.5; sodium chloride 2, sucrose 6, nonfat dry milk 4, lard 3, water 68, calcium propionate 0.11, and an amount of commercial enrichment wafer to meet the Standards of Identity for enriched bread. Breads were sliced and dried at room temperature. Wheats and breads were ground in a hammer mill to pass a screen with 0.024-in. openings (equivalent to U.S. Standard Sieve No. 30).

Descriptive data supplied by the mills for the wheats and flours, together with our values for ash in the breads, are given in Table I.

TABLE I

DESCRIPTION OF SAMPLES

A. WHEAT, FLOUR, AND BREAD[a]

Sample No.	Wheat and Source	Flour Yield[a]	Flour Patent[a]	Ash[b] Wheat	Ash[b] Flour	Ash[b] Bread
		%	%	%	%	%
1	HRS Mont., N.D., S.D., Minn.	71.8	98.5	2.03	0.53	2.99
2	HRS Mont., N.D., S.D., Minn.	72.2	80	2.09	0.45	2.94
3	HRW Kans., Colo.	73.7	95	1.86	0.49	2.94
4	HRW Kans., Colo.	74.4	96	1.95	0.48	2.96
5	50% HRS, 50% HRW		95	1.90	0.48	2.99
6	50% Baart, Wash., 50% HRW	72.3	88	1.84	0.47	2.93
7	50% Baart, Wash., 50% HRW	72.4	90	1.82	0.48	2.97
8	50% Baart, Wash., 50% HRW	74.8	83	1.85	0.46	2.90
9	HRW Texas, Okla.	72		1.96	0.47	2.98
10	HRW Texas, Okla.	72		1.87	0.50	3.07

B. WHEAT AND COMMERCIAL FLOUR MILL PRODUCTS[c]

Product	Proportion of Cleaned Wheat[a]	Moisture As Received	Ash[b]
	%	%	%
Wheat	100.0	10.8	1.96
Farina	1.51	13.2	0.43
Patent flour, 80.1% patent	60.68	13.6	0.43
First clear flour	11.14	13.1	0.86
Low grade flour	2.42	11.4	1.98
Red dog	0.48	10.2	4.15
Shorts	9.13	12.6	5.88
Bran	14.59	8.5	7.27
Germ	0.07	8.3	3.98

[a] Data supplied by mills.
[b] Dry basis.
[c] Blend of HRS and HRW. Flour yield 75.8%.

Analytical Procedure. A search of the literature revealed that numerous methods have been suggested for the determination of major and minor mineral elements in plant products. Preliminary experiments, including recovery studies, were conducted to test the suitability of several of these procedures for the cereal products under investigation. As the result of these studies, the following procedures were selected.

Moisture and Nitrogen. Moisture was determined by the air-oven method (9) and nitrogen by a conventional Kjeldahl method. The factor used for conversion to protein was 5.7.

Ashing and Digestion. For the determination of all other elements except phosphorus, samples were ashed at 500°C. in a muffle furnace. The ash was dissolved in hydrochloric acid, evaporated to dryness, and taken up in hydrochloric acid. The solution was then diluted with water, filtered, and made up to volume quantitatively with water. For phosphorus, samples were digested with nitric and perchloric acids (10). Redistilled water was used throughout.

Phosphorus. This was determined photometrically as a phosphovanadomolybdate by the procedure of Rickey and Avens (10).

Calcium and Magnesium. A modification of the method of Patton and Reeder (11), in which calcium and magnesium are titrated with ethylenedinitrilo tetraacetate (EDTA) using Erichrome Black T indicator, was used. To prevent interference by the large amount of phosphorus present, calcium was first precipitated as the oxalate, the precipitate was ashed and dissolved in hydrochloric acid, and the analysis continued as directed after addition of a small, quantitatively determined amount of magnesium salt to improve the sensitivity of the end point. Magnesium was precipitated as magnesium ammonium phosphate from the filtrate of the calcium oxalate precipitation. This was filtered off and dissolved in 0.5N hydrochloric acid, and the analysis was continued as directed.

Sodium and Potassium. The flame photometer was used, according to the general procedure of Clifford and Winkler (12) modified as required by the instrument, a Coleman Model 21. Large amounts of both calcium and phosphate cause interference and they were quantitatively precipitated as calcium triphosphate. Readings were made on the filtrate.

Minor Mineral Constituents. Iron, manganese, zinc, copper, cobalt, and molybdenum were determined by photometric methods (13). For iron the o-phenanthroline procedure was used (14). Manganese was determined as permanganate (14). Because of the very small amounts of copper, molybdenum, and cobalt, these elements, together

with zinc, were determined by use of larger samples (25 g.). The separation procedure of Strafford, Wyatt, and Kershaw (15) was used.

Copper and Molybdenum. These elements were separated from others by complexing with diethylammonium diethyldithiocarbamate and extracting with chloroform. Copper was determined in an aliquot of the solution of carbamate complex (15). After evaporation of the chloroform and destruction of organic material by digestion with sulfuric, nitric, and perchloric acids as described above, the molybdenum salt was dissolved in hydrochloric acid and molybdenum determined as a thiocyanate (16).

Zinc and Cobalt. Diphenylthiocarbazone (dithizone) in toluene was used to extract zinc and cobalt from the solution (15). Zinc and most of the cobalt were extracted at pH 8, after which a further extraction was made at pH 9.0–9.5 for complete removal of cobalt. Zinc dithizonate was decomposed with dilute hydrochloric acid, which extracted zinc into the aqueous layer while cobalt dithizonate remained in the toluene layer.

Zinc was determined as a complex with 2-carboxy-2′-hydroxy-5′-sulfoformazyl-benzene (Zincon) by the method of Rush and Yoe (17). Toluene was evaporated from the cobalt dithizonate solution, organic matter removed by digestion with sulfuric, nitric, and perchloric acids. The cobalt salt was dissolved in hydrochloric acid and determined as an *o*-nitrosocresol complex by the method of Gregory, Morris, and Ellis (18).

Results and Discussion

Amounts of the various mineral elements found in wheat (Table II) are in the range of those reported by other investigators (1–4). The variation in amounts of individual elements in the wheat samples does not appear to be related to growing area or total ash. Previous investigators have reported that amounts of the elements are related to soil and fertilizer composition (2,3).

Flours milled from these wheats were all of approximately 72% extraction, but ranged from 98.5 to 80% patent (Table I). Neither total ash nor the content of any of the mineral elements was directly related to the reported degree of refinement of the flour. Comparison of the mean values for the minerals in flour (Table III) with those for minerals in wheat in Table II shows that the individual elements are not present in flour in proportion to their concentration in wheat. Only 11.5% of the cobalt, 14% of the manganese, and 15% of the magnesium of the wheat are found in the flour. Phosphorus, potassium, zinc, iron, copper, and sodium are present in flour to the

TABLE II

MINERAL COMPOSITION OF WHEAT, DRY BASIS

Sample No.	K	P	Mg	Ca	Na	Zn	Fe	Mn	Cu	Mo	Co
	%	%	%	%	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.
1	0.435	0.486	0.197	0.013	32	46	46	57	5.2	0.10	0.018
2	.452	.473	.195	.042	35	38	43	57	5.5	.13	.028
3	.507	.450	.162	.049	80	33	41	45	5.0	.30	.016
4	.496	.426	.153	.053	57	33	38	43	4.7	.32	.027
5	.530	.410	.175	.017	45	37	46	51	3.8	.50	.017
6	.423	.384	.158	.031	49	30	45	40	5.3	.18	.021
7	.421	.402	.209	.024	41	30	43	45	5.5	.61	.031
8	.451	.379	.153	.049	41	31	46	39	8.0	.66	.025
9	.390	.430	.229	.053	36	30	42	43	5.6	.59	.016
10	.489	.490	.181	.045	33	36	43	45	4.6	.53	.031
Mean	.454	.433	.183	.045	45	35	43	46	5.3	.48	.026

TABLE III

MINERAL COMPOSITION OF FLOUR, DRY BASIS

Sample No.	K	P	Mg	Ca	Na	Zn	Fe	Mn	Cu	Mo	Co
	%	%	%	%	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.
1	0.099	0.131	0.037	0.016	8.6	12.0	14.1	6.4	2.1	0.20	0.005
2	.086	.118	.031	.016	9.3	8.0	9.9	5.1	1.9	.26	.003
3	.123	.110	.026	.022	13.5	7.0	8.2	5.8	1.8	.21	.005
4	.123	.108	.023	.022	13.7	8.1	7.9	5.1	1.9	.16	.002
5	.085	.123	.019	.014	5.3	8.0	15.5	8.3	1.6	.20	.001
6	.097	.129	.038	.012	5.9	5.7	8.6	3.5	1.7	.34	.003
7	.084	.137	.023	.012	6.3	6.3	10.5	4.0	1.5	.39	.001
8	.118	.134	.019	.015	9.6	6.6	8.1	7.6	1.5	.35	.001
9	.097	.109	.013	.026	19.7	7.7	5.7	8.0	1.5	.18	.002
10	.139	.164	.025	.023	6.2	7.8	15.8	10.8	1.8	.14	.005
Mean	.105	.126	.028	.018	9.8	7.8	10.5	6.5	1.7	.25	.003

TABLE IV

MINERAL COMPOSITION OF BREAD, DRY BASIS

Sample No.	K	P	Mg	Ca	Na	Zn	Fe	Mn	Cu	Mo	Co
	%	%	%	%	%	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.
1	0.184	0.208	0.041	0.123	0.850	13.1	30.9	5.2	2.6	0.32	0.021
2	.168	.174	.036	.123	.840	10.1	28.1	4.7	2.3	.31	.022
3	.200	.179	.031	.123	.850	8.6	24.6	4.7	2.2	.14	.020
4	.195	.172	.025	.129	.867	10.1	28.5	4.8	2.1	.15	.023
5	.196	.180	.034	.134	.860	10.0	22.7	8.1	2.2	.31	.021
6	.201	.162	.039	.124	.839	9.3	31.8	3.1	3.0	.33	.021
7	.199	.181	.031	.115	.848	7.9	28.4	4.8	2.1	.32	.028
8	.200	.180	.035	.125	.860	8.3	28.7	7.3	2.1	.37	.020
9	.184	.210	.048	.148	.890	9.6	22.0	7.3	2.2	.39	.020
10	0.189	0.189	0.023	0.137	0.887	9.9	27.3	8.8	2.2	0.30	0.025
Mean	0.191	0.183	0.031	0.127	0.858	9.7	27.3	5.9	2.3	0.32	0.022

TABLE V

MINERAL COMPOSITION OF COMMERCIAL FLOUR MILL FRACTIONS, DRY BASIS

Fractions	Ash	K	P	Mg	Ca	Na	Zn	Fe	Mn	Cu	Mo	Co
	%	%	%	%	%	%	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.
Farina	0.43	0.115	0.065	0.021	0.019	5.0	6.6	5.4	4.6	1.6	0.17	0.004
Patent flour	0.48	0.122	0.105	.027	.021	5.2	6.1	7.4	6.0	1.5	.19	.004
First clear flour	0.86	0.190	0.196	.065	.030	7.5	15.3	20.4	9.0	2.7	.32	.012
Low grade flour	1.03	0.131	0.204	.156	.046	8.5	41.5	18.6	35.8	5.5	.39	.023
Wheat	1.96	0.155	0.380	.167	.015	12.8	31.0	37.3	49.0	4.0	.33	.021
Germ	3.98	0.889	0.923	.268	.048	27.7	100.8	66.6	137.4	7.4	.67	.017
Red dog	4.07	0.903	0.781	.342	.110	30.5	105.3	131.1	121.4	11.2	.70	.074
Shorts	5.61	1.293	1.307	.511	.133	42.2	160.1	145.7	164.7	13.3	.79	.099
Bran	7.28	1.671	1.570	.688	0.128	39.6	99.1	141.3	136.5	15.2	.83	0.109

extent of 20 to 32% of their amounts in wheat. Calcium and molybdenum are in flour to the extent of 40 and 52%, respectively, of their concentration in wheat.

The bread, which was made by a formula typical of bakery practice in the United States (19), contained a greater concentration of most of the mineral elements than the flour from which it was made (Table IV). This would be expected because of the mineral contributions of the salt, yeast food, nonfat dry milk, calcium propionate, and the iron of the enrichment wafer. The one exception was manganese, which had an average concentration of 5.9 p.p.m. in bread and 6.5 p.p.m. in flour. On the dry basis each part by weight of flour yields approximately 1.1 part of bread of this formula. Thus the manganese in the bread is entirely accounted for by that in the flour. The concentration of magnesium, zinc, copper, and molybdenum in bread averaged 120–135% of their concentration in flour, that of phosphorus was 115%, and potassium 170%. The concentration of calcium and cobalt averaged approximately 7 times and iron 2.6 times that in the flour.

Only sodium and calcium had a higher concentration in bread than in the original wheat. Cobalt in bread averaged approximately 85% of its concentration in wheat, iron and molybdenum 65%, potassium, phosphorus, and copper 40 to 45%, zinc 28%, magnesium 19%, and manganese 13%.

In Table V, which presents data on the distribution of the mineral elements in commercial flour mill fractions, the products are arranged in order of increasing ash. Values for patent flour are in good agreement with those of Table III, and the data confirm and extend the findings of Sullivan and Near (20). With increase of ash content of the fractions, the concentration of each of the mineral elements increases, although not to the same extent. For example, bran, which contains 15 times as much ash as patent flour, contains less than 10 times as much calcium, sodium, copper, and molybdenum, between 13 and 16 times as much potassium, phosphorus, and zinc, 19 to 23 times as much iron and manganese, and 25 to 27 times as much magnesium and cobalt. These results suggest that some of the commercial mill fractions such as red dog, shorts, and bran, which are produced in rather high proportions from the wheat, could serve as special dietary sources of mineral nutrients.

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MANGANESE IN CEREALS AND CEREAL MILL PRODUCTS[1]

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Introduction

Much attention has been given in recent years to the elements found in plant and animal tissues in relatively small quantities, and as a result some of the elements formerly considered non-essential in plant and animal metabolism have now been transferred to the list of essential elements. Bertrand (1926), for instance, having found cobalt and nickel in insulin, claims to have used these elements in the treatment of diabetes with a moderate degree of success. Boron (Brenchley and Warington, 1927) and manganese (McHargue, 1924) have been found to be necessary for the normal development of many plants.

The functions of these new additions to the list of essential elements have not yet been determined. Manganese, however, has been studied much more than the other elements of this group. It has been found that the manganese content of many plants is directly related to the intensity of their oxidase activity (Bertrand, 1897), (McHargue, 1914). McHargue (1922) has shown that manganese is necessary for the formation of chlorophyll in some plants, its function being analogous to that of iron. The analogy of function between these two elements is further strengthened by the fact that manganese has been found helpful in the therapy of anemia (Wood and LaWall, 1926), in the treatment of which iron has long been used. As the result of another investigation, in which he found a relative abundance of manganese in the plant and animal tissues that are rich in vitamins, McHargue (1914) came to the conclusion that the action of this element is closely associated with that of the vitamins. McCarrison (1927), having studied the comparative nutritive values of whole wheat and whole rice, also came to the conclusion that the growth-promoting properties of whole wheat are partly due to its manganese content.

The object of the work reported in this paper was to study the variation in the manganese content of cereals of several varieties, and

[1] Presented at the Seventy-fifth Meeting of the American Chemical Society, St. Louis, Mo., April 19, 1928.

of some of their extraction products, in relation to ash and protein content.

Materials and Methods

Five varieties of each cereal were used for analysis.[2] To eliminate the effect of local and yearly variations, the specimens of each cereal were selected from the harvest of the same year, and most of them from the same locality.

From 5- to 10-gram samples of the ground grains were used for ash and manganese determinations. One-gram samples were used for nitrogen determinations. Manganese was determined by the periodate method as outlined by the Association of Official Agricultural Chemists (1925).

All determinations were made in duplicate on samples ashed for the most part on different days. A Schreiner colorimeter was used for comparisons with the standard permanganate solutions. The duplicate determinations agreed closely.

Results

Correlation between manganese and ash might be expected, as manganese is a part of the total ash. The study of the correlation between manganese and protein was suggested by results obtained in this laboratory on wheat from plots to which sodium nitrate had been added at different stages of growth and which indicated that the manganese content is inversely related to the protein content (Table I).

TABLE I

MANGANESE, ASH, AND PROTEIN IN WHEAT THAT RECEIVED AN APPLICATION OF SODIUM NITRATE AT HEADING TIME

Wheat	Distance between rows	Manganese (Mn_3O_4)*	Ash*	Protein (N x 5.7)*
	in.	per cent	per cent	per cent
Control	8	0.0119	1.85	10.7
Sodium nitrate applied at heading time	8	.0094	1.68	12.5
Sodium nitrate applied at heading time	24	0.0076	1.71	13.2

*Air dry basis

The data obtained in the present work (Table II) show, however, that the manganese content of the different varieties of cereals does not vary consistently with the fluctuations in the ash content, except in the hard winter wheats. Moreover, no correlation was found between the manganese and protein in the varieties of one cereal, notwithstanding the fact that some of the differences in the

[2] The writer is indebted to J. A. Clark, C. E. Leighty, H. V. Harlan, T. R. Stanton, F. D. Richey, and C. E. Chambliss, all of Cereal Crops and Diseases, Bureau of Plant Industry, United States Department of Agriculture, for their kindness in furnishing the grains.

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protein content, especially of the wheats, were greater than those of the wheat obtained from the experimental plots of this labora-

TABLE II

MANGANESE, ASH, AND PROTEIN IN CEREALS*

Cereal	Place grown	Year	Manganese	Ash	Protein
			per cent	per cent	per cent
Hard winter wheat					
Kanred	North Platte, Nebr.	1927	0.0034	1.921	15.6
Karmont	North Platte, Nebr.	1927	.0034	2.105	17.2
Marturki	North Platte, Nebr.	1927	.0052	2.269	18.2
Buffum No. 17	North Platte, Nebr.	1927	.0068	2.344	19.8
Turkey	North Platte, Nebr.	1927	.0052	2.152	17.6
Soft winter wheat					
Chuna	Arlington Farm	1927	.0086	1.935	12.3
Currell	Arlington Farm	1927	.0086	2.002	10.8
Red Rock	Arlington Farm	1927	.0086	1.808	16.5
Leap	Arlington Farm	1927	.0086	1.816	14.2
Fulcz	Arlington Farm	1927	.0086	1.799	12.5
Hard red spring wheat					
Marquis	Dickinson, N. D.	1926	.0052	1.798	14.6
Ruby	Dickinson, N. D.	1926	.0052	1.713	14.0
Red Bobs	Moccasin, Mont.	1926	.0047	1.613	19.0
Ceres	Dickinson, N. D.	1926	.0060	1.738	17.6
Reliance	Moccasin, Mont.	1926	.0034	1.746	16.9
White spring wheat					
Quality	Pullman, Wash.	1926	.0068	2.026	14.1
Pacific Bluestem	Aberdeen, Idaho	1926	.0086	2.018	14.0
Federation	Aberdeen, Idaho	1926	.0068	1.660	13.6
Hard Federation	Aberdeen, Idaho	1926	.0086	1.723	15.8
Baart	Aberdeen, Idaho	1926	.0086	1.907	16.7
Rye					
Von Rumker	Arlington Farm	1927	.0105	2.052	11.7
Rimpau	Arlington Farm	1927	.0122	2.062	10.6
Star	Arlington Farm	1927	.0157	2.028	9.5
Rosen	Arlington Farm	1927	.0110	2.187	9.6
Abruzzes	Arlington Farm	1927	.0121	1.969	11.2
Corn					
North Dakota white flint	North Dakota	1926	.0009	1.644	11.7
Huffman	Tennessee	1926	.0009	1.440	8.5
Northwestern dent	North Dakota	1926	.0009	1.616	11.7
Iodent	Iowa	1926	.0009	1.545	10.3
Boone County white	Virginia	1926	.0018	1.519	10.0
Rice					
Fortuna	Crowley, La.	1927	.0043	1.510	8.0
Early prolific	Crowley, La.	1927	.0052	1.298	7.6
Edithe	Crowley, La.	1927	.0043	1.569	8.8
Lady Wright	Crowley, La.	1927	.0043	1.449	8.0
Blue Rose	Crowley, La.	1927	.0061	1.132	8.2
Oats					
Iowan	Dickinson, N. D.	1927	.0076	4.176	14.8
Richland	Dickinson, N. D.	1927	.0066	3.696	14.8
Red Rustproof	Arlington Farm	1927	.0204	3.947	11.0
Markton	Dickinson, N. D.	1927	.0067	3.935	11.9
Silvermine	Dickinson, N. D.	1927	.0067	3.995	14.8
Barley					
Smyrna	Aberdeen, Nebr.	1925	.0012	2.352	13.0
Meloy	Aberdeen, Nebr.	1925	.0020	3.264	12.3
Algeria	Aberdeen, Nebr.	1925	.0020	2.565	14.0
Oriel	Aberdeen, Nebr.	1925	.0020	2.902	13.9
Horn	Aberdeen, Nebr.	1925	0.0018	2.469	13.9

*Moisture free basis.

tory, mentioned above. The same lack of correlation was found between the protein content and the manganese content of different cereals. The barleys and the oats, for instance, have about the same protein content, but the manganese content of the oats is from three to six times as high as that of the barleys.

When the types of wheat are considered collectively, however, a distinct *inverse* relation between manganese and protein is found. The hard winter wheats and the red spring wheats have a higher protein content and a lower manganese content than the soft winter wheats and the white spring wheats.

Barley and corn, especially corn, are much lower in manganese than the other cereals studied. Boone County White corn, grown on the Arlington Farm, had twice as much manganese as the other varieties, but this may be due to the effect of locality. The manganese content of the Red Rustproof oats, also grown on the Arlington Farm, was about three times as high as that of the other varieties of this cereal.

Altho there is, according to Bertrand (1897) and McHargue (1914), a direct relation between the manganese content of plants and their oxidase activity, there seemed to be no correlation between the manganese content of the cereals as determined in this investigation and their diastatic properties as quoted by Levy (1899) from Moralsky and Glaser (Table III). The barleys, with a relatively low manganese content, serve as the principal source of commercial malt, which has as high diastatic properties as the malt from rye and wheat. On the other hand, corn, which has the lowest manganese content of all the cereals studied, and oats, which has a relatively high manganese content, have equally low diastatic properties.

TABLE III

MANGANESE CONTENT OF CEREALS AND ITS RELATION TO THE RELATIVE DIASTATIC STRENGTH OF THEIR MALTS

Cereal	Manganese (MnO_2)	Relative diastatic strength of malts
	per cent	
Barley	0.0012–0.0020	100.0
Rye	.0105–.0157	93.1
Wheat	.0034–.0086	107.0
Oats	.0067–.0204	30.0
Corn	0.0009–0.0018	28.9

Most of the manganese values obtained in this work are much higher than those reported by Lindow and Peterson (1927), who determined the manganese content of wheat, oats, corn, and barley, and of 79 other plant and animal foods. These higher values may be

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owing to the varieties and source of the cereals studied, as the manganese content of cereals differs with variety (Table II) and probably with locality.

Manganese, Ash, and Protein in Cereal Extraction Products

By way of comparison, manganese was also determined in the milling products of wheat and in some of the widely used breakfast foods, and its relation to the ash and protein content was considered.

The results (Table IV) show in a general way a correlation between the ash and the manganese content. The cereal products that are relatively poor in ash are also poor in manganese, the low content of both being evidently due to the removal of the germ and the outer parts of the grain in the process of preparation.

TABLE IV

MANGANESE, ASH, AND PROTEIN IN CEREAL EXTRACTION PRODUCTS*

Cereal product	Manganese (Mn_3O_4)	Ash	Protein (N x 5.7)	Moisture
	per cent	per cent	per cent	per cent
Bran†	0.0432	7.27	14.5	11.88
Shorts†	.0465	4.07	15.2	9.88
Flour†	.0027	0.57	11.0	12.45
Wheat breakfast food No. 1	.0052	1.44	11.0	9.10
Wheat breakfast food No. 2	trace	0.50	11.5	12.82
Wheat breakfast food No. 3	.0072	1.93	10.3	12.41
Rolled oats	.0052	2.13	13.8	9.67
Flaked corn	none	2.81	7.1	4.93
Uncoated rice	.0011	0.37	7.1	12.88
Pearl barley	0.0004	0.65	7.0	9.22

*Moisture-free basis.
†From a purple straw wheat rich in manganese.

Cornflakes form an apparent exception, having a high ash content and no manganese. The ash content is, however, much higher than that of whole corn, indicating that the ash is largely salt introduced in the process of manufacturing.

No correlation was found between the manganese and the protein in the cereal extraction products, except in the milling products of wheat.

It is noteworthy that both uncoated rice and corn, which are associated with the nutritional deficiency diseases beri-beri and pellagra, are extremely poor in manganese.

Summary

Manganese, ash, and protein were determined in five varieties of each of the following cereals: Hard winter wheat, soft winter wheat, red spring wheat, white spring wheat, rye, corn, rice, oats, and barley. Similar determinations were made on some of their extraction products.

No correlation was found between the manganese and the ash content either in the varieties of one cereal or in different cereals. Neither was any correlation found between manganese and protein, except when the types of wheat are considered collectively. In that case the relation was inverse, the hard winter and red spring wheats being higher in protein and lower in manganese, than the two types of soft wheat.

The manganese content of Red Rustproof oats grown in Arlington was several times higher than that of oats of the other varieties studied.

There was no distinct correlation between the manganese content and the protein content of the cereal products, with the exception of the milling products of wheat. The products having a low ash content also had a low manganese content, both being due to the low degree of extraction.

The results indicate that there is no correlation between the manganese content and the diastatic properties of the cereals studied.

Corn and uncoated rice, which are associated with nutritional deficiency diseases, were both low in manganese.

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THE DETERMINATION OF MANGANESE IN PLANT MATERIALS BY THE PERIODATE METHOD.

By Jehiel Davidson and Ruth G. Capen (Crop Chemistry Laboratory, Bureau of Chemistry and Soils, U. S. Department of Agriculture).

In connection with investigations on the manganese content of plants carried on in this laboratory, the periodate method[1] was used extensively. It was found that this method, which is outlined in the official methods[2] for plant materials, can be modified advantageously.

The fundamental principle of this method is oxidation by a periodate in an acid solution that must be free from reducing agents. In the determination of manganese in ashed plant materials, the principal reducing agent to be removed is the hydrochloric acid used in dissolving the ash. The procedure given in the official methods calls for boiling with sulfuric and nitric acids in order to expel the hydrochloric acid. Long boiling and repeated additions of nitric acid have been found necessary, however, to remove all traces of hydrochloric acid. To ascertain whether it would not be practicable to omit this troublesome reducing agent, manganese determinations were made by dissolving the ashed plant material in nitric, sulfuric, and phosphoric acids, respectively, instead of in hydrochloric acid, the periodate being added directly to these solutions. The addition of sulfuric acid to the nitric and phosphoric acid solutions was omitted because it proved to be of no advantage. This simple modification made the procedure less troublesome, and resulted in a saving of time. The quantity of chlorine in the ash of plants is generally very small and is well taken care of by the periodate, which is added in excess.

The procedure as outlined in the official methods calls for the addition of ferric nitrate to the standard permanganate solution. This precaution had been suggested by Willard and Greathouse, who worked with materials rich in iron, such as steels and ores, because the color imparted by the iron salts might interfere with the matching of the permanganate solutions. Plant materials, however, contain very little iron, and the small quantities present are generally prevented from imparting color to the solutions by the excess of phosphoric acid in plant ash. It was found quite safe, therefore, to omit the ferric nitrate. On the other hand, the addition of periodate to the standard permanganate solution was found to be very useful, since it serves as an excellent preservative.

A new precaution that was found to be necessary was the guarding of the solutions against alcohol vapors and other volatile reducing substances. The alcohol vapors in the laboratory air were found to pale or even to destroy completely the permanganate color in some cases.

[1] Willard and Greathouse, J. Am. Chem. Soc., 1917, 39: [illegible]
[2] Methods of Analysis, A. O. A. C., 1925, 42.

The color is restored on heating, but the determinations must be completed while the solutions are still hot, or the color disappears again.

Results obtained by the procedure as outlined in the official methods and by dissolving the plant ash directly in nitric, sulfuric, and phosphoric acids, are offered for comparison in Table 1.

The materials selected for comparison were two cereal grains high in manganese, two cereal grains low in manganese, and wheatstraw and cornstalks, which are much higher in manganese than the respective grains.

Table 1.

Comparison of results of manganese determinations made by the periodate method according to official and modified procedures.*

PLANT MATERIAL	OFFICIAL PROCEDURE	NITRIC ACID	SULFURIC ACID	PHOSPHORIC ACID
	per cent	*per cent*	*per cent*	*per cent*
Wheat	0.0085	0.0085	0.0073	0.0085
Rye	0.0091	0.0110	0.0110	0.0110
Rice	0.0049	0.0045	0.0055	0.0061
Corn	0.0013	0.0012	0.0012	0.0015
Wheatstraw	0.0229	0.0259	0.0224	0.0274
Cornstalks	0.0076	0.0076	0.0076	0.0076

* Mn_3O_4.

The modified procedure is as follows:

Ash the plant materials in platinum dishes in an electric muffle. Add 5 cc. of nitric, sulfuric, and phosphoric acids, respectively, and 20 cc. of distilled water to each dish. Heat the dishes on a steam bath for a few minutes and wash the contents into beakers. Heat the solutions to boiling and keep at this temperature after the addition of 0.3 gram of potassium periodate until the full development of the permanganate color occurs.

A Schreiner colorimeter was used for comparing the solutions. The differences in the results obtained are within the limits of experimental error, but, if anything, better results are obtained by the modified procedure. Of the three acids used, phosphoric acid proved to be most suitable, as it developed the permanganate color in the shortest period of time; sulfuric acid was next in order, and nitric acid was last. In the determinations recorded in the table, the maximum time required for the full development of the permanganate color in the phosphoric acid solutions was only 2–3 minutes from the time the solutions were brought to boiling.

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THE EFFECT OF MANGANESE ON THE IMMUNITY STATE

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Preliminary Remarks.

Manganese, a metal diffused in each living creature, could have a biological effect similar to the effect of iron, insofar as it can substitute iron from the viewpoint of hematogenous capacity.

Its antibacterial action likewise proved to be significant, thanks to its considerable ability to stimulate natural oxidations.

Manganese has been widely studied from the standpoint of pharmacology and toxicology. It is used as medicament in anemia therapy. It does in fact have a stimulating effect on the blood and on the hemopoietic organs, without however having the capacity, as does iron, to bind the oxygen to the hemoglobin.

As for the effect of manganese on the immunity state: a fair number of publications on the subject were received during the last fifteen years. In 1921, L.E. WALBUM, testing the action of various chlorides in goats and horses, specifically focussed his attention on manganese chloride from the viewpoint of diphtheric antitoxin and the the agglutinin of bacterium coli. He found that when using as average dose 1 - 2 cc of a n/100 $MnCl_2$ solution for each kg of animal, the rate of the specific substances in the blood underwent a rather important augmentation.

I undertook similar studies with S. SCHMID a few years after WALBUM, concerning diphtheric antitoxin as well as concerning the hemolytic antibodies, and always obtained results which were identical with those previously obtained.

in 1924 C.E. PICO, resuming previous tests on the effect of manganese chloride on immunity phenomena, reached various conclusions depending on the various examined serums (antitetanic, antidiphtheritic, antiophidianic, antimeningococcus, etc).

During the following year Hans SCHMIDT confirmed the finding of WALBURN, i.e. that 1 cc of a n/100 Mn Cl_2 solution, when endovenously applied, provokes an augmentation of the antitoxin rate: diphteric, tetatic, and typhoid (antitoxin rates). Said increase manifests itself 1 hour after the injection, and disappears quite rapidly.

E.S. HOGAN found an increase of the antityphoid agglutinins in rabbits treated endovenously with Mn Cl_2, while treatment with collodial manganese produced no effect whatsoever.

In 1906 ARCHIPOFF, a Russian, found in horses immunized against diphtheric cough an approximate increase of 19% of the antitoxin count a few hours after the injection of manganese chloride.

A. LUMIEE and R.H. GRANGE attempted to explain the mechanism through which an augmentation of the antitoxin rate is obtained, injecting Mn Cl_2, with an important hyperleukocytosis which persisted for several days.

In 1932 A. LUSZTIG, prompted by the experiments of WALBUM and BIUMERT, wanted to demonstrate the action of the manganese salts on hemolysins which, according to his previous tests, could not be favorably affected, unless X-rays were used.

He was in fact unable to determine a noteworthy increase of hemolytic antibody production after the previous endovenous triple injection administered in intervals of three days, of a 5% manganese solution.

It results from our above statements that there is no shortage of tests performed seriously and scrupulously by skilled researchers.

However, we wish to point out that the tests of these authors were in fact performed for a well-defined purpose, quite different from the goal set by us. On the whole, all of them intended primarily to utilize the stimulating effect of the manganese in animals in the course of immunization and therefore attempted to augment, with the aid of said stimulus, one or another of the antibodies; they hoped in this way to obtain a high rate, either antitoxic, or antibacterial.

Purpose.

Therefore they did not follow the effect on such organisms provoked by the augmentation of the manganese salt dose, intended to cause a vital block. Our purpose instead was: to follow the intoxication of the organism caused by the effect of stronger manganese doses, and to observe the immunitary behavior in the course of progressive $MnCl_2$ injections.

For the technique used during the immunization of the rabbits and during serological research, see: L. TORELLI, The effect of iron on the immunity state, "Journal of Bacteriology and Immunology", 1936.

Personal research work.

With reference to previous tests the bibliography of which we briefly reported above: we likewise used manganese chloride, since it is a salt with good solubility, easily injected into the veins. We also started by injecting said salt into previously vaccinated animals. We prepared four solutions for this purpose:

Solution n/100, containing 0,00063 g $Mn\ Cl_2$ in 1 cc.

Solution n/10, containing 0,0063 g of $Mn\ Cl_2$ in 1 cc.

Standard solution, containing 0,063 g of $Mn\ Cl_2$ in 1 cc.

Solution 1 : 10, containing 0,1 g of $Mn\ Cl_2$ in 1 cc.

In the course of our first test we inoculated various doses of manganese into already vaccinated animals; after five hours, we tested the agglutinative and lytic capacity. Accordingly, we applied the method used by previous researchers.

We therefore vaccinated five rabbits (three to be treated with Mn and two as controls) with choleric vibrion. Six days after the last vaccine injection, we undertook the agglutination and bacteriolysis tests (See Table I).

TABLE I

	1 AGGLUTINAZIONE						2 BATTERIOLISI					
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000			1 : 500	1 : 1000	1 : 2000	1 : 5000	1 : 10000
A_1	+ + +	— — —	— — —	— — —	— — —		A_1	+ + +	+ + —	— — —	— — —	— — —
A_2	+ + +	+ + +	+ — —	— — —	— — —		A_2	+ + +	+ + +	+ + —	— — —	— — —
3 A_3 Controlli	+ + +	+ + —	— — —	— — —	— — —	3	A_3 Controlli	+ + +	— — —	— — —	— — —	— — —
C_1	+ + +	+ + +	+ + —	— — —	— — —		C_1	+ + +	+ + +	+ + +	+ — —	— — —
C_2	+ + +	+ + +	+ + —	— — —	— — —		C_2	+ + +	+ + +	+ + +	+ — —	— — —

1) Agglutination. 2) Bacteriolysis. 3) Controls.

Next day we administered to the rabbits A_1, A_2, A_3 an endovenous injection consisting of 1 cc n/100 $Mn\ Cl_2$ solution, and five hours later we performed the blood count. The following data was obtained concerning the agglutination and the lysis (see Table 2):

TABLE 2.

1 AGGLUTINAZIONE						2 BATTERIOLISI					
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 500	1 : 1000	1 : 2000	1 : 5000	1 : 10000
A_1	+ + +	+ + +	+ + +	+ — —	— — —	A_1	+ + +	+ + +	+ + +	+ + —	— —
A_2	+ + +	+ + +	+ + +	+ + —	— — —	A_2	+ + +	+ + +	+ + +	— — —	— —
A_3	+ + +	+ + +	+ + +	+ — —	— — —	A_3	+ + +	+ + +	+ — —	— — —	— —
C_1	+ + +	+ + +	+ + +	— — —	— — —	C_1	+ + +	+ + +	+ + +	+ + +	— —
C_2	+ + +	+ + +	+ + +	— — —	— — —	C_2	+ + +	+ + +	+ + +	+ — —	— —

1) Agglutination. 2) Bacteriolysis.

As indicated in the preceding examples, the agglutinative and bacteriolytic capacity remained almost stable in the control rabbits; on the other hand, in rabbits treated with a small dose of manganese chloride (mg 0,63), the quantity of the agglutinin as well as the quantity of the lysine proved to be considerably increased five hours after the injection of the manganese.

After five days we administered to the rabbits A an endovenous injection of 1 cc n/10 Mn Cl_2 solution and we determined the agglutinative and lytic capacity five hours later, as shown on the following Tables (see Table 3.)

It follows that mg 6,3 of Mn Cl_2 likewise provokes an increased production of antibodies.

After 5 additional days, when testing with the standard Mn Cl_2 solution, we still obtained an augmentation of the immunizing capacity which, however, was slight (see Table 4).

After another period of five days, we injected 1 cc of the 1 : 10 solution, and obtained the results shown below during agglutination and lysis tests, performed five hours after the endovenous injection (see Table 5).

TABLE 3

	1 AGGLUTINAZIONE						2 BATTERIOLISI				
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 500	1 : 1000	1 : 2000	1 : 5000	1 : 10000
A_1	+ + +	+ + +	+ + +	+ + +	— — —	A_1	+ + +	+ + +	+ + +	+ + +	— — —
A_2	+ + +	+ + +	+ + +	+ + +	— — —	A_2	+ + +	+ + +	+ + +	+ + +	+ — —
A_3	+ + +	+ + +	+ + +	+ + —	— — —	A_3	+ + +	+ + +	+ + —	— — —	— — —
C_1	+ + +	+ + +	+ + +	+ — —	— — —	C_1	+ + +	+ + +	+ + +	+ + +	— — —
C_2	+ + +	+ + +	+ + +	+ — —	— — —	C_2	+ + +	+ + +	+ + +	+ + —	— — —

TABLE 4

	1 AGGLUTINAZIONE						2 BATTERIOLISI				
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 500	1 : 1000	1 : 2000	1 : 5000	1 : 10000
A1	+ + +	+ + +	+ + +	+ + +	+ + —	A1	+ + +	+ + +	+ + +	+ + +	+ — —
A2	+ + +	+ + +	+ + +	+ + +	+ — —	A2	+ + +	+ + +	+ + +	+ + +	+ + —
A3	+ + +	+ + +	+ + —	+ + +	+ — —	A3	+ + +	+ + +	+ + +	+ + —	— — —
C1	+ + +	+ + +	+ + +	+ — —	— — —	C1	+ + +	+ + +	+ + +	+ + +	— — —
C2	+ + +	+ + +	+ + +	— — —	— — —	C2	+ + +	+ + +	+ + +	+ + —	— — —

TABLE 5

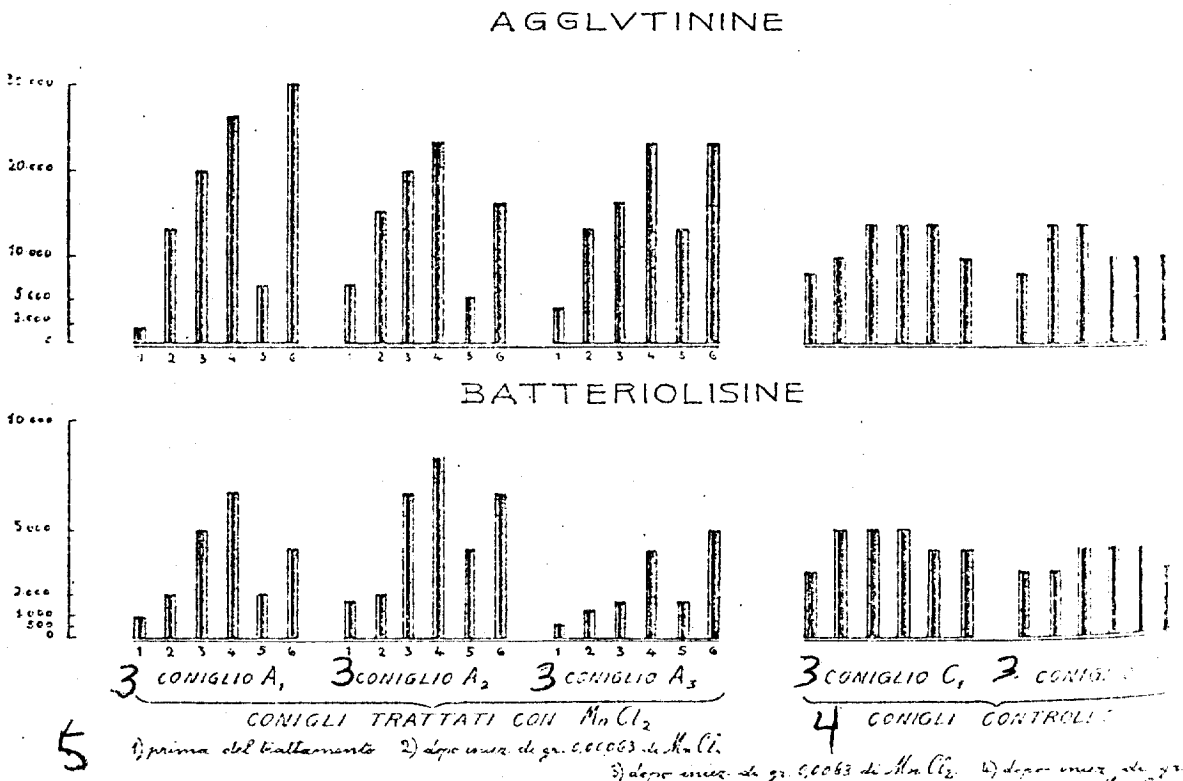
	1 AGGLUTINAZIONE						2 BATTERIOLISI				
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 500	1 : 1000	1 : 2000	1 : 5000	1 : 10000
A_1	+ + +	+ + +	— — —	— — —	— — —	A_1	+ + +	+ + +	+ + +	— — —	— — —
A_2	+ + +	+ + +	— — —	— — —	— — —	A_2	+ + +	+ + +	+ + +	+ + —	— — —
A_3	+ + +	+ + +	+ + +	+ — —	— — —	A_3	+ + +	+ + +	+ + —	— — —	— — —
C_1	+ + +	+ + +	+ + +	+ — —	— — —	C_1	+ + +	+ + +	+ + +	+ + —	— — —
C_2	+ + +	+ + +	+ + +	— — —	— — —	C_2	+ + +	+ + +	+ + +	+ + —	— — —

1) Agglutination. 2) Bacteriolysis.

Since the agglutinative and lytic values remained near-constant for the control rabbits, we can reach the conclusion that in the last -mentioned test the endovenous injection of 0,1 g $Mn\ Cl_2$ administered to each rabbit, causes a considerable decrease of the agglutinin and lysin rate within five hours.

Four days after the last injection we performed the agglutination and bacteriolysis of the five animals which had not been additionally treated, and obtained the following results (see Table 6)

1 AGGLUTINAZIONE						2 BATTERIOLISI					
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 500	1 : 1000	1 : 2000	1 : 5000	1 : 10000
A_1	+ + +	+ + +	+ + +	+ + +	+ + +	A_2	+ + +	+ + +	+ + +	+ + —	— — —
A_2	+ + +	+ + +	+ + +	+ + —	— — —	A_2	+ + +	+ + +	+ + +	+ + +	+ — —
A_3	+ + +	+ + +	+ + +	+ + +	+ — —	A_3	+ + +	+ + +	+ + +	+ + +	— — —
C_1	+ + +	+ + +	+ + +	— — —	— — —	C_1	+ + +	+ + +	+ + +	+ + —	— — —
C_2	+ + +	+ + +	+ + +	— — —	— — —	C_2	+ + +	+ + +	+ + +	+ — —	— — —



1) Agglutination. 2) Bacteriolysis.
3) Rabbit . . 4) Control rabbits
5) (1) before treatment; (2) after injection of 0,00063 g of $Mn\ Cl_2$; (3) after injection of 0,0063 g of $Mn\ Cl_2$; (4) after injection of 0,063 g of $Mn\ Cl_2$; (5) after injection of 0,1 g of $Mn\ Cl_2$; (6) four days after the last injection of $Mn\ Cl_2$.

Synthesis.

We now recapitulate with diagrams the data reported so far, so as to deduce subsequent logical conclusions from them.

Conclusions.

It is obvious from the graphs that small and average doses of $Mn\ Cl_2$ (0,03 mg to 6,3 ctg) powerfully stimulate the production of both antibodies under consideration. Strong doses, on the other hand, cause a rather high degree of agglutinin and lysin rate decrease. Said decrease is definitely evident five hours after the injection of $Mn\ Cl_2$; but after four days the agglutinative and lytic value of the serum dropped to the initial level, and in some cases even surpassed the latter.

No reference can in this case be made to a negative phase because a negative phase is understood to be the moment of diminished immunitary resistance which immediately follows the vaccinating injection, i.e. the negative phase manifests itself at a time when specific antibodies do not yet exist.

In our case instead the antibodies are already formed, and only the decrease is at issue.

With what mechanism can a strong manganese chloride dose provoke a rapid and temporary decrease of specific antibodies? The endothelial reticulum or the histiocytic reticulum, in a certain sense, or the other tissues engaged in the formation of antibodies were not lastingly affected, at least not insofar as their antibody-producing activity is concerned, when the agglutinative and lytic rate increased again only four days later.

We consider the occurrence of said temporary arrest to be a natural phenomenon under the intoxicating effect of the manganese the level of which is comparatively high at that moment. We are therefore of opinion that said temporary decrease is the result of an inhibitory phase of the agglutinin and lysin production in the circulatory system,

which could in a certain sense also be compared with the negative phase in the course of the specific vaccination. This is a negativism linked, however, to the injected manganese dose; whenever said negative phase is eliminated or somehow overcome by adaptation of the organism to the toxin, even if (the adpatation) is only temporary, the negative phase becomes positive, or at least returns to the rate which existed prior to the injection.

But at the moment when the organism of the animal is overwhelmed by the violence of the manganese injection--a moment which we compared with the negative phase--classic colloidal events undoubtedly occur, especially in the blood, which are reflected in the decrease of the agglutinin and lysin rate.

Therefore, so as to attempt an explanation of the problem at issue, we placed a few drops of manganese chloride--diluted in three forms: n/100, standard, and 1 : 10--into test tubes containing blood serum; after five minutes we found evidence of turbidity in the serum which had come into contact with the 1 : 10 solution of the manganese salt, while a very slight turbidity was present in the serum treated with the standard $Mn\ Cl_2$ solution; no turbidity whatsoever occurred in the serum treated with the n/100 solution of $Mn\ Cl_2$. The conclusion is reached from the above that a strong dose of $Mn\ Cl_2$ causes a temporary precipitation of the protein fraction in the blood serum. It is therefore conceivable that the regular production of antibodies is interrupted, which explains the rapid decrease of their rate.

We wish to point out that when these tests terminated, the general conditions of the rabbits remained good, and even the rabbits treated with $Mn\ Cl_2$ had achieved a weight increase of approximately 200 g each.

Manganese intoxication and immunity state.

After having carried out research work as indicated concerning the effect of more or less large doses of $Mn\ Cl_2$ on the formation of antibodies, we proposed to investigate what effect a continuous treatment with the salt under examination had, when repeatedly applied, on the immunity state, and accordingly to examine whether manganism could be an unfavorable or a favorable factor for eventual infectious processes in human pathology.

Tests.

We used 11 gray rabbits; their average weight was 1800 g. We divided these animals into three series: A) including five rabbits; B) four rabbits; C) two rabbits. We treated the rabbits of series A) with $Mn\ Cl_2$ prior to the vaccination; those of series B) after the vaccination, and the rabbits of series C) served for control purposes.

The treatment of the rabbits of series A) was executed as follows: A_1 and A_2 received six 1 cc endovenous injections of the n/100 $Mn\ Cl_2$ solution on alternate days . After the fifth and sixth injection both rabbits seemed depressed, with moderate dyspnea; they scratched their noses repeatedly with their hind feet. The rabbits A_3 and A_4 received on the first day an endovenous 1 cc injection of the 1 : 10 $Mn\ Cl_2$ solution (0,1 g of the salt). The animal A_4 became sluggish a few minutes after the injection, with dyspneic respiration, trembling of the head and violent diarrhea. This condition lasted for approximately six hours. The animal A_3, on the other hand, showed a much more serious symptomatology: as soon as the injection was given, it was overcome by clonotonic convulsions, with complete respiration arrest; the respiration resumed after approx. thirty seconds, slowly at first, with dyspnea .. but the rabbit appeared to suffer seriously for an additional period of fifteen minutes. It was subsequently affected by diarrhea and died 18 minutes later.

During the necroscopic examination I observed a diffuse reddening of the intestinal ansae, injections of the vessels of the gastric and vesical serous membrane. The bladder was filled with urine (approx. 70 cc); the urine was limpid, of normal color, containing 1/2 °/oo of albumin and its sediment contained a moderate number of granulous cylinders. The microscopic examination of the liver showed nothing worth mentioning, while the microscopic examination of the kidneys showed the presence of glomerulonephritis, with numerous hemorrhagic centres.

Arguments.

It was found at this point that while during previously performed tests the dose of 0,1 g $MnCl_2$ had not caused any morbid episode, it proved to be lethal here. This can be explained by a certain habituation to the toxin which the organism can acquire.

In the course of the preceding tests the dose of 0,1 g $MnCl_2$ was in fact applied after the administration of increasingly large doses, while in this case it was administered to animals which had not been subjected to said toxin before.

It is on the other hand necessary to note that during these tests even small doses provoked, after the fourth injection, morbid phenomena consisting in a slight depression and moderate dyspnea. How can this fact be explained? We believe that an explanation is possible, in view of the circumstance that the injections were applied on alternate days; therefore the manganese chloride was not completely eliminated during the brief interval. During the previous tests, on the other hand, we administered the injections in intervals of five days. Despite the increasingly high doses which we injected, no accumulation of the toxin

occurred in the organism; instead, since the organism was already completely free from the toxic substance, a gradual state of tolerance developed against the subsequent larger doses.

Subsequent tests.

The rabbit A_4 which survived the injection of 0,1 g $Mn\ Cl_2$ and which recovered well, to the extent that its weight showed no decrease whatsoever, received--jointly with the rabbit A_5, an animal that had not been treated before--an endovenous injection of 1 cc standard $Mn\ Cl_2$ solution (0,06 g).

With the above injection, containing less $Mn\ Cl_2$ than the dose which had killed the rabbit A_3, disorders similar to those described earlier became evident as well, although their intensity was minor: both animals appeared depressed, subject to slight tremors and dyspnea. A_4 was diarrheic as well.

They recovered very soon, to such an extent that we could give them a second injection five days later, using the standard $Mn\ Cl_2$ solution. This endovenous injection caused the same morbid symptoms: A_4 showed some clonic shocks as well; both animals were considerably depressed for several hours after the injection, so that their death could be expected. We therefore suspended the administration of $Mn\ Cl_2$ since it seemed to us that it was not possible to proceed any further with the intoxication.

However, when the effect of the injection ceased, the general condition of all four rabbits was very good; no weight loss occurred.

At this point, we undertook the vaccination, involving two endovenous injections of the vaccine, applied to rabbits of the series A) and to those of the series B) and C).

Nine days after the second vaccinating injection we sampled blood from ten rabbits and we determined the agglutinative and lysine count (see Table 7).

As generally evident , the formation of antibodies occurs in the same way in rabbits already treated with $Mn\ Cl_2$ as in those (series B and C) not yet intoxicated with the salt at issue.

After the vaccination we started the intoxication of the rabbits from series B) and specifically, of (rabbits) B_1 and B_2, with injections of n/100 $Mn\ Cl_2$ solution; B_3 amd B_4 received the standard solution of $Mn\ Cl_2$. The rabbits B_3 and B_4, however, died within 12 to 24 hours after the injection, showing before their death symptoms idetical with those which the rabbits of series A), treated with a very strong dose of $Mn\ Cl_2$, had presented.

It seems to us that the above fact likewise demonstrates that 0,06 g (i.e. approx. 0,04 g per kg of body weight) of manganese chloride injected into animals not accustomed to this toxin represents a lethal dose, while animals previously treated with minor doses become accustomed to the toxin and are not damaged by larger doses.

We administered three endovenous injections on alternate days to the rabbits B_1 and B_2, using the n/100 solution of $Mn\ Cl_2$, after which we again determined the quantity of agglutinin and lysin contained in the circulating blood (see Table 8).

Conclusions.

These Tables indicate that the augmentation of the antibody rate in the rabbits of the series A) (already intoxicated) and in the control rabbits, was moderate, and generally occurred to the same extent.

TABLE 7

	AGGLUTINAZIONE 1						BATTERIOLISI 2				
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 200	1 : 500	1 : 1000	1 : 2000	1 : 5000
A_1	+ + +	+ — —	— + —	— — —	— — —	A_1	+ + +	+ + +	+ + —	+ — —	— — —
A_2	+ + +	+ + +	+ + —	— — —	— — —	A_2	+ + +	+ + +	+ — —	— — —	— — —
A_4	+ + +	+ + +	+ + —	— — —	— — —	A_4	+ + +	+ + —	+ — —	— — —	— — —
A_5	+ + +	+ + +	+ + —	— — —	— — —	A_5	+ + +	+ — —	± — —	— — —	— — —
B_1	+ + +	+ + +	+ — —	— — —	— — —	B_1	+ + +	+ + —	+ — —	— — —	— — —
B_2	+ + +	+ + +	+ + —	— — —	— — —	B_2	+ + +	+ + —	— — —	— — —	— — —
B_3	+ + +	+ + —	— — —	— — —	— — —	B_3	+ + +	+ + —	— — —	— — —	— — —
B_4	+ + +	+ + +	+ + —	— — —	— — —	B_4	+ + +	+ — —	— — —	— — —	— — —
C_1	+ + +	+ + +	+ + —	— — —	— — —	C_1	+ + +	+ + —	— — —	— — —	— — —
C_2	+ + +	+ — —	— — —	— — —	— — —	C_2	+ + +	+ + +	— — —	— — —	— — —

TABLE 8

	AGGLUTINAZIONE 1						BATTERIOLISI 2				
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 200	1 : 500	1 : 1000	1 : 2000	1 : 5000
A_1	+ + +	+ + —	— — —	— — —	— — —	A_1	+ + +	+ + +	+ + +	± — —	— — —
A_2	+ + +	+ + +	+ + —	+ — —	— — —	A_2	+ + +	+ + +	+ + —	— — —	— — —
A_4	+ + +	+ + +	+ + +	— — —	— — —	A_4	+ + +	+ + +	+ + —	— — —	— — —
A_5	+ + +	+ + +	+ + +	— — —	— — —	A_5	+ + +	+ + —	+ — —	— — —	— — —
B_1	+ + +	+ + +	+ + +	+ + —	— — —	B_1	+ + +	+ + +	+ + +	— — —	— — —
B_2	+ + +	+ + +	+ + +	+ + +	— — —	B_2	+ + +	+ + +	+ + +	+ — —	— — —
C_1	+ + +	+ + +	+ + +	— — —	— — —	C_1	+ + +	+ + —	+ — —	— — —	— — —
C_2	+ + +	+ + —	— — —	— — —	— — —	C_2	+ + +	+ + +	+ — —	— — —	— — —

1) Agglutination. 2) Bacteriolysis.

On the other hand, a more significant increase was verified in the rabbits of the series B), comparable to the increase already verified in connection with small doses of manganese chloride in the course of previous tests.

We then applied two additional injections endovenously to the rabbits B_1 and B_2, using the n/100 $MnCl_2$ solution; the injections were given in an interval of two days. We performed the agglutination and bacteriolysis for eight rabbits approximately ten days after the last injection.

The last-mentioned tests to which the following Tables refer, showed in all rabbits a slow degradation of the antibody count, as standard after a certain period of vaccination therapy (in our case, we reached the 33rd day after the last vaccine injection). (See Table 9).

We present below a few summarizing diagrams of the data obtained in the course of the second test group concerning the effect of manganese chloride on the formation of antibodies.

The diagrams in question make it obvious that on the whole, the fluctuations of the agglutination and lytic rate in previously intoxicated and subsequently vaccinated rabbits are identical with the fluctuations observed in the control rabbits.

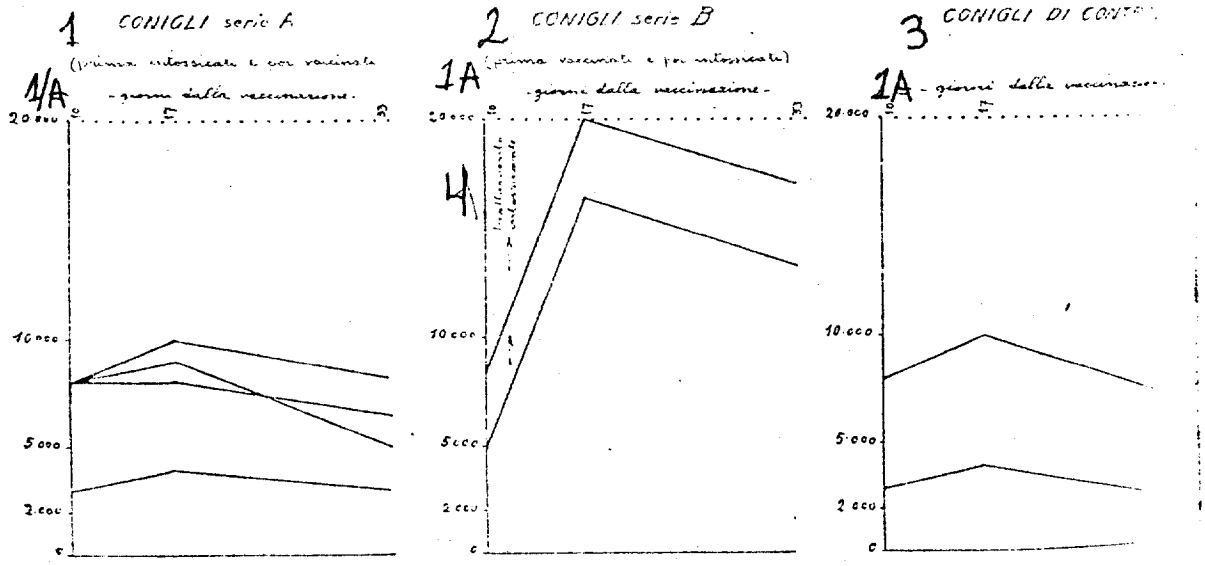
The rabbits of the series B), on the other hand, which were first vaccinated and subsequently intoxicated, show the formation of a large quantity of antibodies (specifically of agglutinin) as compared to the controls: the descending antibody line for these rabbits B) follows a course more or less identical with the course of the line for the controls.

TABLE 9.

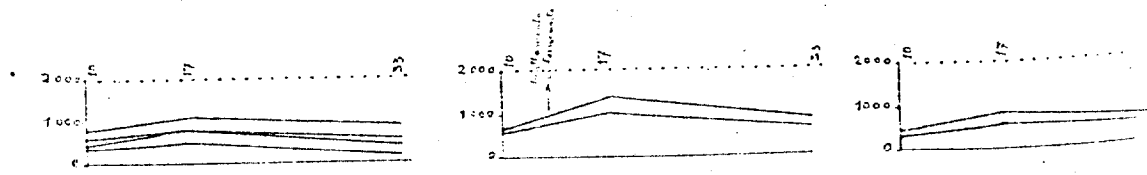
AGGLUTINAZIONE 1						BATTERIOLISI 2					
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 200	1 : 500	1 : 1000	1 : 2000	1 : 5000
A	+ + +	+ + +	— — —	— — —	— — —	A_1	+ + +	+ + +	+ + —	— — —	— — —
A_2	+ + +	+ + +	— — —	— — —	— — —	A_2	+ + +	+ + +	+ — —	— — —	— — —
A_4	+ + +	+ + +	+ + —	— — —	— — —	A_4	+ + +	+ + +	— — —	— — —	— — —
A_5	+ + +	+ + +	+ — —	— — —	— — —	A_5	+ + +	+ — —	— — —	— — —	— — —
B_1	+ + +	+ + +	+ + +	— — —	— — —	B_1	+ + +	+ + +	+ — —	— — —	— — —
B_2	+ + +	+ + +	+ + +	+ — —	— — —	B_2	+ + +	+ + +	+ + —	— — —	— — —
C_1	+ + +	+ + +	+ — —	— — —	— — —	C_1	+ + +	+ — —	— — —	— — —	— — —
C_2	+ + +	— — —	— — —	— — —	— — —	C_2	+ + +	+ + —	— — —	— — —	— — —

1) Agglutination. 2) Bacteriolysis.

AGGLUTININ



BACTERIOLYSIN



1) Rabbits series A (intoxicated first, then vaccinated)
1A) days after vaccination

2) Rabbits series B (vaccinated first, then intoxicated)
1A) days after vaccination

3) Control rabbits 4) Intoxicating injection.

CONCLUSIONS

In vaccinated animals, manganese chloride doses of 0,63 mg to 0,063 g cause, five hours after endovenous injection, augmentation of the agglutinins as well as of the lysins. On the other hand, a manganese chloride dose of 0,1 g provokes a rapid decrease of the antibodies under examination; however, the antibody rate rapidly climbs again to very high figures.

In animals which were previously intoxicated, either with small or with high doses of manganese chloride, vaccination is followed by the establishment of an immunity state which is not perceptibly different from the immunity state of the control rabbits.

In vaccinated animals, intoxication due to manganese chloride causes a considerable increase of the antibody rate (specifically of the agglutinins), followed by a normal decrease, i.e. comparable to the decrease in the control rabbits.

It follows from the above that the preventive intoxication of the animal organism with manganese does not leave indelible traces in all organic systems which are involved in the production of agglutinin, provided that the latter occurs normally, while in animals which were vaccinated first and subsequently intoxicated, the intoxication favors the already established production of antibodies (at least to the limit of a specific maximal manganese dose).

Our results correlated to the last-mentioned point coincide with the conclusions reached by the authors cited by us in the bibliogrpahy who studied the stimulus of manganese in the course of immunization.

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L'influenza del manganese sullo stato immune

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PREMESSE.

Il manganese, metallo diffuso in ogni essere vivente, ha, forse, un'azione biologica simile a quella del ferro, tanto che può sostituire il ferro dal punto di vista del potere ematogeno. Si è dimostrata anche importante la sua azione antibatterica, grazie al suo elevato potere stimolante delle ossidasi naturali.

Il manganese è stato largamente studiato dal lato farmacologico e tossicologico. Come medicamento esso viene usato nella cura delle anemie. Infatti esso ha azione stimolante sul sangue e sugli organi emopoietici, senza avere però la proprietà, come il ferro, di legare l'ossigeno all'emoglobina.

Per quello che riguarda l'azione del manganese sullo stato immune, si è avuto in questi ultimi quindici anni un discreto numero di pubblicazioni. Nel 1921 L. E. Walbum, sperimentando su capre e cavalli l'azione di vari cloruri, si è soffermato in special modo sul cloruro di manganese in riguardo alla antitossina difterica e all'agglutinina del bacterium coli, ed ha osservato che, usando come dose media 1-2 cc. di soluzione n/100 di $Mn\,Cl_2$ per ogni chilo dell'animale, il tasso delle sostanze specifiche del sangue subiva un aumento piuttosto rilevante.

Tali studi proseguì qualche anno dopo il Walbum, insieme a S. Schmid, sia ancora in riguardo alla antitossina difterica, sia contro gli anticorpi emolitici, e sempre ebbe risultati analoghi ai precedenti.

Nel 1924 C. E. Pico, riprendendo le precedenti esperienze sull'influenza del Cloruro di manganese sui fenomeni immunitari, giunse a conclusioni diverse, a seconda dei vari sieri presi in esame (antitetanico, antidifterico, antiofidico, antimontone, antimeningococcico, ecc.).

L'anno successivo Hans Schmidt confermò la osservazione di Walbum, e cioè che un cc. di soluzione n/100 di $Mn\,Cl_2$ per via endovenosa provoca un aumento del tasso delle antitossine difterica, tetanica e tifica. Tale aumento si manifesta un'ora dopo l'iniezione e ben presto scompare.

E. S. Horgan trovò un aumento delle agglutinine antitifiche in conigli trattati per via endovenosa con $Mn\,Cl_2$, mentre il trattamento con manganese colloidale non aveva alcun effetto.

Nel 1906 il russo Archipoff constatò in cavalli immunizzati contro la tossina difterica un aumento di circa il 19 % del titolo antitossico dopo due ore dalla iniezione di cloruro di manganese.

A. Lumière e R. H. Grange hanno cercato di spiegare il meccanismo per cui si ottiene un aumento del tasso antitossico, iniettando $Mn\,Cl_2$, con una iperleucocitosi importante e persistente per più giorni.

Nel 1932 A. Lusztig, spinto dalle esperienze di Walbum e Bickert, volle provare l'azione dei sali di manganese sulle emolisine, le quali, secondo sue esperienze precedenti, non potevano venire influenzate favorevolmente se non dai raggi X. Effettivamente non ebbe a constatare aumento degno di nota nella produzione degli anticorpi emolitici previa triplice iniezione endovenosa, a tre giorni di distanza l'una dall'altra, d'una soluzione di manganese al 5 %.

Da quanto abbiamo esposto risulta che non mancano esperimenti portati a termine da valenti ricercatori con serietà e scrupolo.

Però ci permettiamo di far notare che le esperienze di questi Autori sono state fatte in realtà con uno scopo ben determinato e ben diverso da quello che ci siamo proposti noi. Essi volevano, in complesso, tutti usufruire più che altro dell'azione stimolatrice del manganese su animali in corso di immunizzazione, onde cercare di aumentare con detto stimolo aspecifico questo o quello anticorpo, nella speranza di realizzare un alto titolo, sia antitossico, sia antibatterico.

Scopo.

Non hanno quindi seguito l'effetto che su tali organismi provoca l'aumentare della dose di sale di manganese, fino ad ottenerne il blocco vitale, come era invece il compito nostro, che intendeva seguire l'intossicarsi dell'organismo per l'effetto di dosi più forti di manganese, e osservare il comportamento immunitario durante il corso di iniezioni progressive di $Mn\,Cl_2$

Circa la tecnica seguita nella immunizzazione dei conigli e nelle ricerche sierologiche, vedi: L. Torelli: *L'influenza del ferro sullo stato immune.* « Giornale di Batteriologia e Immunologia », 1936.

Ricerche personali.

Riferendoci alle precedenti ricerche di cui sopra abbiamo dato brevi cenni bibliografici, abbiamo anche noi usato il cloruro di manganese, essendo questo un sale ben solubile e iniettabile nelle vene. E anche noi abbiamo cominciato con l'iniettare questo sale in animali già precedentemente vaccinati, saggiando via via dosi maggiori. A questo scopo abbiamo preparato quattro soluzioni di cloruro di manganese, e cioè:

Soluz. n/100 contenente in 1 cc. gr. 0,0063 di $Mn\,Cl_2$.

Soluz. n/10 contenente in 1 cc. gr. 0,0063 di $Mn\,Cl_2$.

Soluz. normale contenente in 1 cc. gr. 0,63 di $Mn\,Cl_2$.

Soluz. 1:10 contenente in 1 cc. gr. 0.1 di $Mn\,Cl_2$.

Abbiamo prima esperimentato, inoculando le varie dosi del manganese in animali già vaccinati e saggiando dopo cinque ore il potere agglutinante e quello litico: ci siamo cioè attenuti al metodo seguito dai precedenti ricercatori.

Abbiamo quindi vaccinato cinque conigli (tre da trattare poi col Mn e due per controllo) col vibrione colerigeno e dopo sei giorni dall'ultima iniezione vaccinante abbiamo fatto le prove dell'agglutinazione e della batteriolisi (vedi tab. 1).

TABELLA 1.

	AGGLUTINAZIONE						BATTERIOLISI				
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 500	1 : 1000	1 : 2000	1 : 5000	1 : 10000
A_1	+ + +	— — —	— — —	— — —	— — —	A_1	+ + +	+ + —	— — —	— — —	— — —
A_2	+ + +	+ + +	+ — —	— — —	— — —	A_2	+ + +	+ + +	+ + —	— — —	— — —
A_3	+ + +	+ + —	— — —	— — —	— — —	A_3	+ + +	— — —	— — —	— — —	— — —
Controlli C_1	+ + +	+ + +	+ + —	— — —	— — —	Controlli C_1	+ + +	+ + +	+ + +	+ — —	— — —
C_2	+ + +	+ + +	+ + —	— — —	— — —	C_2	+ + +	+ + +	+ + +	+ — —	— — —

Il giorno successivo abbiamo praticato ai conigli A_1, A_2, A_3 un'iniezione endovenosa di 1 cc. di soluzione n/100 di $Mn\,Cl_2$ e, dopo cinque ore abbiamo praticato il salasso: dalla agglutinazione e dalla lisi si sono ottenuti i seguenti dati (vedi tab. 2):

TABELLA 2.

	AGGLUTINAZIONE						BATTERIOLISI				
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 500	1 : 1000	1 : 2000	1 : 5000	1 : 10000
A_1	+ + +	+ + +	+ + +	+ — —	— — —	A_1	+ + +	+ + +	+ + +	+ + —	— — —
A_2	+ + +	+ + +	+ + +	+ + —	— — —	A_2	+ + +	+ + +	+ + +	— — —	— — —
A_3	+ + +	+ + +	+ + +	+ — —	— — —	A_3	+ + +	+ + +	+ — —	— — —	— — —
C_1	+ + +	+ + +	+ + +	— — —	— — —	C_1	+ + +	+ + +	+ + +	+ + +	— —
C_2	+ + +	+ + +	+ + +	— — —	— — —	C_2	+ + +	+ + +	+ + +	+ — —	— — —

Come dimostrano i precedenti specchi, mentre nei conigli controllo il potere agglutinante e quello batteriolitico sono rimasti quasi stazionari, nei conigli trattati con una piccola dose di cloruro di manganese (mmgr. 0,63) sia la quantità di agglutinine quanto quella di lisine si è dimostrata notevolmente aumentata dopo cinque ore dalla iniezione del manganese.

Dopo cinque giorni abbiamo praticato ai tre conigli A un'iniezione endovenosa di 1 cc. di soluzione n/10 di $Mn\,Cl_2$, e dopo cinque ore abbiamo determinato il potere agglutinante e quello litico, secondo quanto è esposto nelle seguenti tabelle (vedi tab. 3):

Dunque anche con mmgr. 6,3 di $Mn\,Cl_2$ si provoca una maggiore produzione di anticorpi.

Dopo altri cinque giorni, sperimentando con la soluzione normale di $Mn\,Cl_2$, ottenemmo ancora un aumento, sia pur lieve, del potere immunitario (vedi tab. 4).

Ancora, dopo un altro periodo di cinque giorni, con l'iniezione di 1 cc. della soluzione 1:10, abbiamo avuto i seguenti risultati nelle prove della agglutinazione e della lisi, praticate cinque ore dopo l'iniezione endovenosa (vedi tab. 5).

TABELLA 3.

	AGGLUTINAZIONE						BATTERIOLISI				
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 500	1 : 1000	1 : 2000	1 : 5000	1 : 10000
A_1	+ + +	+ + +	+ + +	+ + +	— — —	A_1	+ + +	+ + +	+ + +	+ + +	— — —
A_2	+ + +	+ + +	+ + +	+ + +	— — —	A_2	+ + +	+ + +	+ + +	+ + +	+ — —
A_3	+ + +	+ + +	+ + +	+ + —	— — —	A_3	+ + +	+ + +	+ + —	— — —	— — —
C_1	+ + +	+ + +	+ + +	+ — —	— — —	C_1	+ + +	+ + +	+ + +	+ + +	— — —
C_2	+ + +	+ + +	+ + +	+ — —	— — —	C_2	+ + +	+ + +	+ + +	+ + —	— — —

TABELLA 4.

	AGGLUTINAZIONE						BATTERIOLISI				
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 500	1 : 1000	1 : 2000	1 : 5000	1 : 10000
A1	+ + +	+ + +	+ + +	+ + +	+ + —	A1	+ + +	+ + +	+ + +	+ + +	+ — —
A2	+ + +	+ + +	+ + +	+ + +	+ — —	A2	+ + +	+ + +	+ + +	+ + +	+ + —
A3	+ + +	+ + +	+ + +	+ + +	+ — —	A3	+ + +	+ + +	+ + +	+ + —	— — —
C1	+ + +	+ + +	+ + +	+ — —	— — —	C1	+ + +	+ + +	+ + +	+ + +	— — —
C2	+ + +	+ + +	+ + +	— — —	— — —	C2	+ + +	+ + +	+ + +	+ + —	— — —

TABELLA 5.

	AGGLUTINAZIONE						BATTERIOLISI				
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 500	1 : 1000	1 : 2000	1 : 5000	1 : 10000
A_1	+ + +	+ + +	+ — —	— — —	— — —	A_1	+ + +	+ + +	+ + +	— — —	— — —
A_2	+ + +	+ + +	— — —	— — —	— — —	A_2	+ + +	+ + +	+ + +	+ + —	— — —
A_3	+ + +	+ + +	+ + +	+ — —	— — —	A_3	+ + +	+ + +	+ + —	— — —	— — —
C_1	+ + +	+ + +	+ + +	+ — —	— — —	C_1	+ + +	+ + +	+ + +	+ + —	— — —
C_2	+ + +	+ + +	+ + +	— — —	— — —	C_2	+ + +	+ + +	+ + +	+ + —	— — —

Essendo rimasti quasi costanti i valori agglutinarti e litici dei conigli controllo, possiamo concludere in quest'ultima prova che l'iniezione endovenosa di 0,1 gr. di $Mn\,Cl_2$ per ogni coniglio provoca, entro cinque ore, un abbassamento assai marcato del tasso delle agglutinine e delle lisine.

Dopo quattro giorni dall'ultima iniezione, senza più trattare i conigli, abbiamo eseguito l'agglutinazione e la batteriolisi dei cinque animali, avendone i seguenti risultati (vedi tab. 6):

SINTESI.

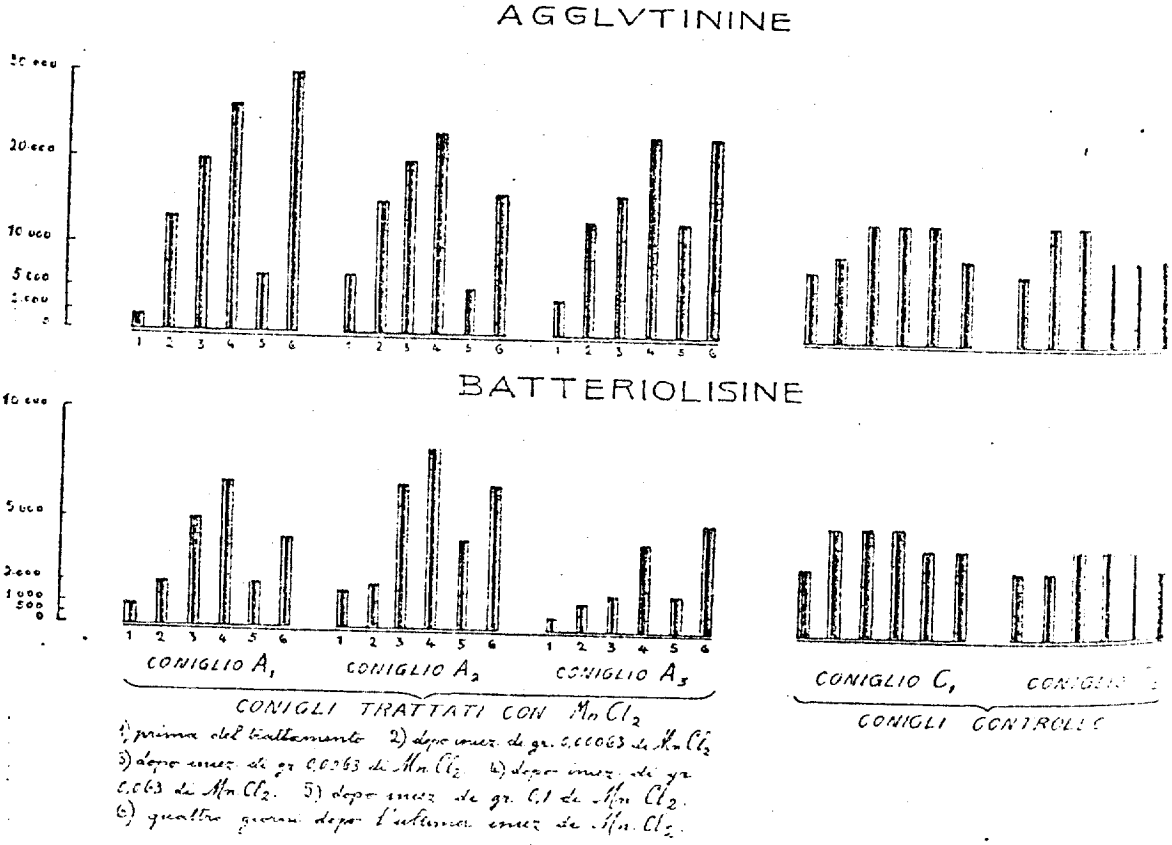
Riassumiamo adesso *con grafici* i dati fin qui riportati per trarne poi le logiche deduzioni.

DEDUZIONI.

Chiaramente si vede dai grafici che le dosi piccole e medie di $Mn\,Cl_2$ (mmgr. 0,63; ctgr. 6,3) stimolano assai fortemente la

TABELLA 6.

	AGGLUTINAZIONE						BATTERIOLISI				
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 500	1 : 1000	1 : 2000	1 : 5000	1 : 10000
A_1	+ + +	+ + +	+ + +	+ + +	+ + +	A_2	+ + +	+ + +	+ + +	+ + —	— — —
A_2	+ + +	+ + +	+ + +	+ + —	— — —	A_2	+ + +	+ + +	+ + +	+ + +	+ — —
A_3	+ + +	+ + +	+ + +	+ + +	+ — —	A_3	+ + +	+ + +	+ + +	+ + +	— — —
C_1	+ + +	+ + +	+ + +	— — —	— — —	C_1	+ + +	+ + +	+ + +	+ + —	— — —
C_2	+ + +	+ + +	+ + +	— — —	— — —	C_2	+ + +	+ + +	+ + +	+ — —	— — —



1) prima del trattamento 2) dopo iniez. di gr. 0,00063 di $Mn\,Cl_2$ 3) dopo iniez. di gr. 0,00063 di $Mn\,Cl_2$ 4) dopo iniez. di gr. 0,063 di $Mn\,Cl_2$ 5) dopo iniez. di gr. 0,1 di $Mn\,Cl_2$. 6) quattro giorni dopo l'ultima iniez. di $Mn\,Cl_2$.

produzione di ambedue gli anticorpi presi in considerazione. Le dosi forti abbassano invece in misura piuttosto elevata il tasso delle agglutinine e delle lisine. Tale diminuzione è nettamente evidente dopo cinque ore dalla iniezione di $Mn\,Cl_2$, ma dopo quattro giorni il valore agglutinante e litico del siero si è riportato al livello iniziale e anzi in qualche caso lo ha superato.

Non si può in questo caso parlare di fase negativa, perchè per questa s'intende quel momento di minorata resistenza immunitaria aspecifica che segue immediatamente un'iniezione vaccinante, cioè la fase negativa si manifesta quando ancora anticorpi specifici non esistono.

Nel caso nostro invece gli anticorpi sono già formati e si tratta solo di una loro diminuzione.

Con quale meccanismo una dose forte di cloruro di manganese può provocare una rapida e momentanea diminuzione di anticorpi specifici? Il reticolo endoteliale o istiocitario che dir si voglia e gli altri tessuti deputati alla formazione degli anticorpi certo non ne hanno sofferto in modo durevole, almeno per quanto riguarda la loro attività sulla produzione degli anticorpi, se dopo soli quattro giorni il titolo agglutinante e litico è di nuovo risalito.

Noi riteniamo naturale che questo arresto temporaneo sia avvenuto sotto l'influsso intossicante del manganese, la cui soglia era in quel momento relativamente alta. Noi pensiamo quindi che tale momentanea diminuzione sia l'effetto di una fase inibitoria della produzione delle agglutinine e delle lisine in circolo, che in un certo qual modo si potrebbe anche paragonare alla fase negativa nel corso della vaccinazione specifica. È questa una negatività legata però alla dose da manganese iniettato, che quando venga eliminato, o, comunque, vinto da un adattamento, sia pur momentaneo, dell'organismo al veleno, passa alla positività o, per lo meno, al ritorno del titolo preesistente all'iniezione stessa.

Ma nel momento in cui l'organismo animale rimane sopraffatto dalla violenza dell'iniezione di manganese, in quel momento, che noi abbiamo paragonato alla fase negativa, avvengono indubbiamente fatti colloido-clasici, nel siero di sangue specialmente, che si riverberano sull'abbassarsi del titolo delle agglutinine e delle lisine.

Anzi, per tentare di spiegare questo problema, abbiamo lasciato cadere in tre provette contenenti siero di sangue, alcune gocce di cloruro di manganese nelle tre diluizioni n/100, normale e 1 : 10, e dopo cinque ore abbiamo osservato che esisteva un evidente intorbidamento del siero che era stato messo in contatto con la diluizione 1 : 10 del sale di manganese, mentre un intorbidamento molto lieve esisteva nel siero trattato con la soluzione normale del $Mn\,Cl_2$ e nessun intorbidamento nel siero trattato con la diluizione n/100 di $Mn\,Cl_2$. Se ne deduce che il $Mn\,Cl_2$ a dose forte provoca una precipitazione temporanea della frazione proteica del siero di sangue, per cui si potrebbe pensare che la regolare produzione degli anticorpi venisse interrotta, spiegandoci così il rapido diminuire del loro tasso.

Desideriamo far notare che alla fine di queste esperienze le condizioni generali dei conigli si erano mantenute buone, e che anzi i conigli trattati con $Mn\,Cl_2$ erano aumentati di peso circa 200 grammi ciascuno.

Intossicazione da manganese e stato immune.

Esaurite così le ricerche riguardanti l'azione di dosi più o meno grandi di $Mn\,Cl_2$ sulla formazione degli anticorpi, ci siamo proposti di vedere quale azione abbia sullo stato immune un trattamento continuato per diverse volte col sale in esame; e ciò per vedere se in patologia umana il manganismo possa rappresentare un fattore sfavorevole o favorevole su eventuali processi infettivi.

Esperienze.

Abbiamo preso undici conigli grigi, il cui peso si aggirava in media su 1800 grammi; abbiamo diviso questi animali in tre serie; *A*) di cinque conigli; *B*) di quattro; *C*) di due. I conigli della serie *A*) li abbiamo trattati col $Mn\,Cl_2$ prima della vaccinazione; quelli della serie *B*) dopo la vaccinazione; e i conigli della serie *C*) facevano da controllo.

Il trattamento dei conigli della serie *A*) si è svolta nel seguente modo: A_1 e A_2 hanno avuto sei iniezioni endovenose di un cc. di soluzione n/100 di $Mn\,Cl_2$, a giorni alterni. Dopo la quinta e la sesta iniezione ambedue i conigli si sono mostrati abbattuti, con modica dispnea e si grattavano ripetutamente il muso con le zampe anteriori. I conigli A_3 e A_4 hanno ricevuto il primo giorno un'iniezione endovenosa di un cc. della soluzione 1 : 10 di $Mn\,Cl_2$ (gr. 0,1 del sale). L'animale A_1 dopo pochi minuti dall'iniezione si presentò in stato di torpore, con respiro dispnoico, tremore della testa, forte diarrea. Tale stato durò circa sei ore. L'animale A_2

presentò invece una sintomatologia ben più grave: appena eseguita l'iniezione, venne colto da convulsioni clonotoniche, con arresto completo del respiro; questo, dopo circa trenta secondi, riprese, dapprima lentamente, poi dispnoico; ma il coniglio per ancora quindici minuti rimase di aspetto molto sofferente. In seguito venne colto da diarrea, e dopo diciotto ore morì.

All'esame necroscopico potei osservare un arrossamento diffuso delle anse intestinali, iniezioni dei vasi della sierosa gastrica e vescicale. La vescica si mostrò piena di urina (circa 70 cc.); la urina era limpida, di colorito normale, contenendo il 1/2 ‰ di albumina, e il suo sedimento conteneva un discreto numero di cilindri granulosi. L'esame microscopico del fegato non rilevò niente di notevole, mentre quello del rene dimostrò la presenza di glomerulonefrite, con numerosi focolai emorragici.

CONSIDERAZIONI.

A questo punto vien fatto di rilevare come nelle esperienze precedentemente eseguite la dose di gr. 0,1 di $Mn\,Cl_2$ non avesse provocato alcun fatto morboso, mentre qui si è mostrata mortale. Questo si può spiegare con una certa assuefazione che l'organismo può acquistare per il tossico.

Infatti negli esperimenti precedenti la dose di gr. 0,1 di $Mn\,Cl_2$ era stata eseguita dopo le somministrazioni di dosi crescenti, mentre in questo caso è stata eseguita in animali nuovi a questo tossico.

D'altra parte bisogna osservare che in queste esperienze anche le dosi piccole hanno dato, dopo la quarta iniezione, dei fenomeni morbosi consistenti in un po' di abbattimento e in modica dispnea. Come si può spiegare questo fatto? Noi pensiamo che si possa dargli una spiegazione, osservando che le iniezioni venivano praticate a giorni alterni, e in così breve spazio di tempo non veniva completamente eliminato il cloruro di manganese; nelle prove precedenti invece avevamo praticato le iniezioni a distanza di cinque giorni l'una dall'altra e, benchè iniettassimo dosi sempre più elevate, non si arrivava ad un accumulo del tossico nell'organismo, ma anzi, essendo questo già completamente libero della sostanza tossica, si instaurava mano a mano uno stato di tolleranza alle maggiori dosi successive.

ULTERIORI ESPERIENZE.

Il coniglio A_4, sopravissuto alla iniezione di gr. 0,1 di $Mn\,Cl_2$ e rimessosi bene, tanto che il suo peso non dimostrava alcuna diminuzione, dopo nove giorni ricevette, insieme al coniglio A_5, animale non ancora trattato, una iniezione endovenosa di 1 cc. di soluzione normale di $Mn\,Cl_2$ (gr. 0,06).

Anche con questa iniezione, inferiore in contenuto di $Mn\,Cl_2$ a quella che ci aveva ucciso il coniglio A_3, si verificarono inconvenienti simili ai già descritti, benchè di intensità assai minore: ambedue gli animali si presentarono accasciati, in preda a lieve tremore e dispnea. A_4 fu anche diarroico.

Ben presto però si rimisero, tanto che dopo cinque giorni potemmo praticar loro una seconda iniezione di soluzione normale di $Mn\,Cl_2$. Anche questa iniezione endovenosa provocò gli stessi fenomeni morbosi; A_4 presentò in più qualche scossa clonica; ambedue gli animali furono assai abbattuti per varie ore dopo la iniezione, tanto da temere della loro morte. Sospendemmo quindi la somministrazione di $Mn\,Cl_2$ perchè ci parve di non potere andare più avanti nella intossicazione.

Però tutti e quattro i conigli trattati, cessata l'azione della iniezione, stavano assai bene di condizioni generali; e il loro peso non subì alcuna diminuzione.

A questo punto praticammo la vaccinazione mediante due iniezioni endovenose di vaccino dei conigli della serie *A*) e di quelli della serie *B*) e *C*).

Dopo nove giorni dalla seconda iniezione vaccinante praticammo il salasso ai dieci conigli e determinammo il titolo agglutinante e quello delle lisine (vedi tab. 7).

Come si vede in linea generale, la formazione degli anticorpi avviene in modo uguale sia nei conigli già trattati con $Mn\,Cl_2$, sia in quelli (serie *B* e *C*) non ancora intossicati con questo sale.

Dopo la vaccinazione abbiamo cominciato a intossicare i conigli della serie *B*) e precisamente B_1 e B_2 con iniezioni di soluzione n/100 di $Mn\,Cl_2$; e B_3 e B_4 con la soluzione n di $Mn\,Cl_2$. Però i conigli B_3 e B_4 sono morti fra le dodici e le ventiquattro ore dall'iniezione, presentando, prima della morte, gli stessi sintomi che avevano presentato i conigli della serie *A*), trattati con la dose assai forte di $Mn\,Cl_2$.

Questo fatto ci pare che stia ancora a dimostrare che gr. 0,06 (cioè circa gr. 0,0 pro chilo corporeo) di cloruro di manganese iniettati in animali non abituati a questo tossico, rappresentano una dose mortale; mentre animali trattati precedentemente con

TABELLA 7.

	AGGLUTINAZIONE						BATTERIOLISI				
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 200	1 : 500	1 : 1000	1 : 2000	1 : 5000
A_1	+ + +	+ — —	— + —	— — —	— — —	A_1	+ + +	+ + +	+ + —	+ — —	— — —
A_2	+ + +	+ + +	+ + —	— — —	— — —	A_2	+ + +	+ + +	+ — —	— — —	— — —
A_4	+ + +	+ + +	+ + —	— — —	— — —	A_4	+ + +	+ + —	+ — —	— — —	— — —
A_5	+ + +	+ + +	+ + —	— — —	— — —	A_5	+ + +	+ — —	± — —	— — —	— — —
B_1	+ + +	+ + +	+ — —	— — —	— — —	B_1	+ + +	+ + —	+ — —	— — —	— — —
B_2	+ + +	+ + +	+ + —	— — —	— — —	B_2	+ + +	+ + —	— — —	— — —	— — —
B_3	+ + +	+ + —	— — —	— — —	— — —	B_3	+ + +	+ + —	— — —	— — —	— — —
B_4	+ + +	+ + +	+ + —	— — —	— — —	B_4	+ + +	+ — —	— — —	— — —	— — —
C_1	+ + +	+ + +	+ + —	— — —	— — —	C_1	+ + +	+ + —	— — —	— — —	— — —
C_2	+ + +	+ — —	— — —	— — —	— — —	C_2	+ + +	+ + +	— — —	— — —	— — —

dosi più piccole si abituano al tossico e non sentono danno dalle dosi maggiori.

Ai conigli B_1 e B_2 abbiamo fatto tre iniezioni endovenose a giorni alterni di soluzione n/100 di $Mn\,Cl_2$, dopo le quali abbiamo di nuovo determinato la quantità di agglutinine e di lisine contenute nel sangue circolante (vedi tab. 8).

grado e, come linee generali, in egual misura.

Nei conigli della serie *B*), invece, si è verificato un aumento più notevole, paragonabile a quello che avevamo già riscontrato in riguardo alle piccole dosi di cloruro di manganese nelle precedenti esperienze.

Abbiamo eseguito poi altre due iniezioni

TABELLA 8.

	AGGLUTINAZIONE						BATTERIOLISI				
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 200	1 : 500	1 : 1000	1 : 2000	1 : 5000
A_1	+ + +	+ + —	— — —	— — —	— — —	A_1	+ + +	+ + +	+ + +	± — —	— — —
A_2	+ + +	+ + +	+ + —	+ — —	— — —	A_2	+ + +	+ + +	+ + —	— — —	— — —
A_4	+ + +	+ + +	+ + +	— — —	— — —	A_4	+ + +	+ + +	+ + —	— — —	— — —
A_5	+ + +	+ + +	+ + +	— — —	— — —	A_5	+ + +	+ + —	+ — —	— — —	— — —
B_1	+ + +	+ + +	+ + +	+ + —	— — —	B_1	+ + +	+ + +	+ + +	— — —	— — —
B_2	+ + +	+ + +	+ + +	+ + +	— — —	B_2	+ + +	+ + +	+ + +	+ — —	— — —
C_1	+ + +	+ + +	+ + +	— — —	— — —	C_1	+ + +	+ + —	+ — —	— — —	— — —
C_2	+ + +	+ + —	— — —	— — —	— — —	C_2	+ + +	+ + +	+ — —	— — —	— — —

DEDUZIONI.

Da questi specchietti si può rilevare come l'aumento del tasso degli anticorpi nei conigli della serie *A*) (già intossicati) e di quelli di controllo, sia avvenuto in modico

endovenose della soluzione n/100 di $Mn\,Cl_2$ ai conigli B_1 e B_2, a distanza di due giorni l'una dall'altra; dopo circa dieci giorni dall'ultima iniezione abbiamo eseguito l'agglutinazione e la batteriolisi agli otto conigli.

Quest'ultime prove, di cui riferiscono gli

specchi seguenti, hanno mostrato, in tutti i conigli, un lento degradare del titolo degli anticorpi, come avviene di norma dopo un certo tempo del trattamento vaccinante (nel nostro caso siamo a 33 giorni dall'ultima iniezione di vaccino) (vedi tab. 9).

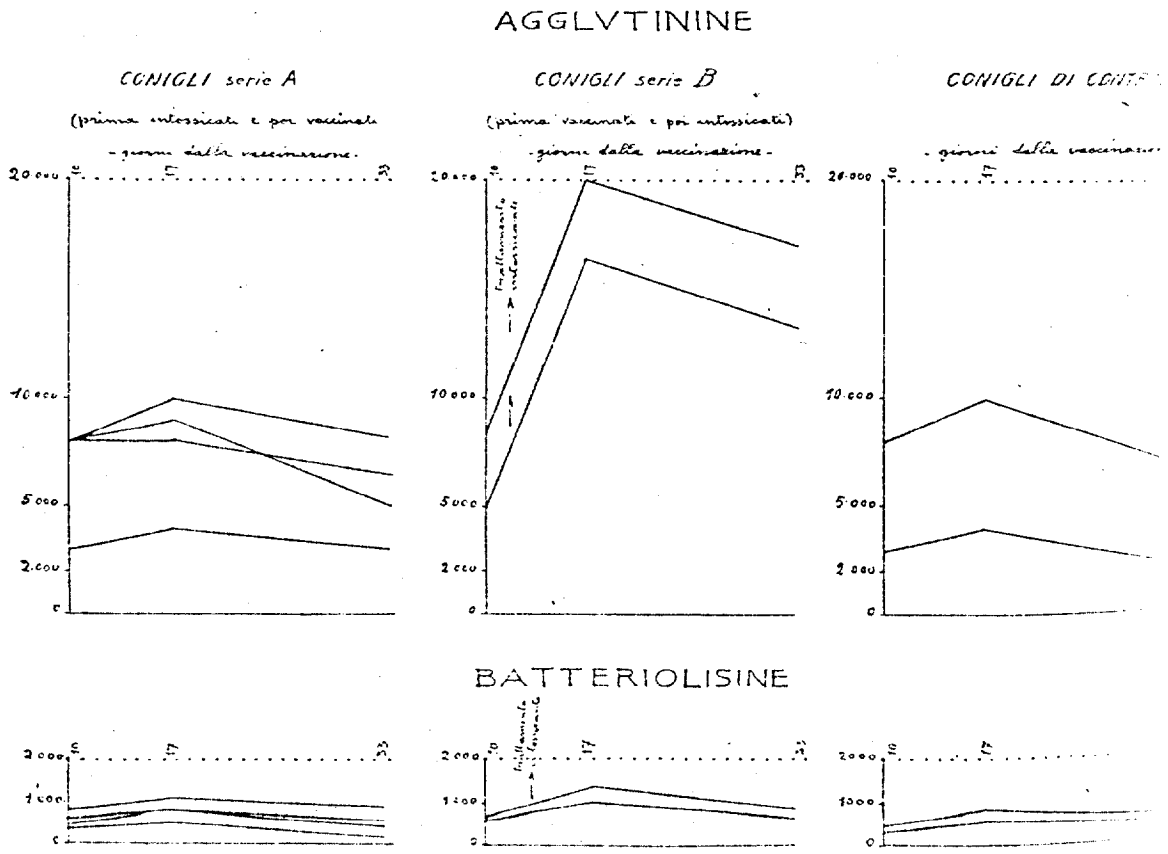
condo gruppo di esperienze, in riguardo all'azione del cloruro di manganese sulla formazione degli anticorpi.

Da questi grafici si vede bene come nei conigli prima intossicati e poi vaccinati, in complesso, le oscillazioni del tasso agglutinante e litico sono uguali a quelle che si osservano nei cogli controllo.

TABELLA 9.

	AGGLUTINAZIONE						BATTERIOLISI				
	1 : 2000	1 : 5000	1 : 10000	1 : 20000	1 : 30000		1 : 200	1 : 500	1 : 1000	1 : 2000	1 : 500[illegible]
A	+ + +	+ + +	— — —	— — —	— — —	A_1	+ + +	+ + +	+ + —	— — —	— — —
A_2	+ + +	+ + +	— — —	— — —	— — —	A_2	+ + +	+ + +	+ — —	— — —	— — —
A_4	+ + +	+ + +	+ + —	— — —	— — —	A_4	+ + +	+ + +	— — —	— — —	— — —
A_5	+ + +	+ + +	+ — —	— — —	— — —	A_5	+ + +	+ — —	— — —	— — —	— — —
B_1	+ + +	+ + +	+ + +	— — —	— — —	B_1	+ + +	+ + +	+ — —	— — —	— — —
B_2	+ + +	+ + +	+ + +	+ — —	— — —	B_2	+ + +	+ + +	+ + —	— — —	— —
C_1	+ + +	+ + +	+ — —	— — —	— — —	C_1	+ + +	+ — —	— — —	— — —	— — —
C_2	+ + +	— — —	— — —	— — —	— — —	C_2	+ + +	+ + —	— — —	— — —	— —

Riportiamo qui di seguito alcuni grafici riassuntivi dei dati ottenuti da questo se-



Nei conigli della serie *B*), invece, che sono stati prima vaccinati e poi intossicati, si ha la formazione di una maggiore quantità di anticorpi (specialmente agglutinine) rispetto ai controlli: la discesa del tasso degli anticorpi anche in questi conigli *B*) avviene presso a poco con una linea simile a quella dei controlli.

CONCLUSIONI

In animali vaccinati, dosi da mmgr. 0,63 a gr. 0,063 di cloruro di manganese provocano, cinque ore dopo l'iniezione endovenosa, un aumento sia delle agglutinine che delle lisine. La dose di gr. 0,1 di cloruro di manganese provoca invece una rapida diminuzione degli anticorpi presi in esame, ma ben presto il tasso degli anticorpi risale a cifre assai elevate.

In animali precedentemente intossicati, sia con dosi piccole che con dosi alte di cloruro di manganese, la vaccinazione è seguita dall'instaurarsi di uno stato immunitario non diverso in modo apprezzabile a quello dei conigli controllo.

In animali vaccinati l'intossicazione da cloruro di manganese provoca un forte innalzamento del tasso degli anticorpi (specialmente delle agglutinine), seguito da una discesa normale, cioè paragonabile a quella dei conigli controllo.

Ne consegue perciò che: la preventiva intossicazione dell'organismo animale con manganese non lascia tracce indelebili in tutti quei sistemi organici deputati alla produzione della agglutinine, tanto che questa avviene come di norma, mentre negli animali prima vaccinati e poi intossicati l'intossicazione favorisce la già instaurata produzione degli anticorpi (almeno fino a una certa dose massima di manganese).

In quest'ultimo punto i nostri risultati concordano con le deduzioni tratte da quegli autori da noi citati nella Bibliografia, che si sono occupati dello stimolo del manganese nel corso di un'immunizzazione.

RIASSUNTO

Gli AA., studiando il comportamento della produzione degli anticorpi nei conigli immunizzati contro il vibrione colerigeno e trattati per via endovenosa con cloruro di manganese, hanno potuto dimostrare:

1°) La somministrazione di piccole dosi del preparato (mmgr. 0,63-gr.0,063) provoca, dopo solo cinque ore, un aumento degli anticorpi, mentre la dose di gr. 0,1 ne provoca una rapida diminuzione, seguita però da un considerevole aumento.

2°) L'intossicazione con $Mn\,Cl_2$, in animali già vaccinati esalta lo stato immune.

3°) L'intossicazione con $Mn\,Cl_2$, seguita da vaccinazione non agisce in alcun modo sullo stato immune.

RESUME

Les AA., en étudiant le comportement de la production des anticorps dans les lapins immunisés par le vibrion cholérigène et traités par la rue des veines avec $Mn\,Cl_2$, ont pu démontrer:

1) L'administration du préparat en de petits doses (mmgr. 0,63, gr. 0,063) provoque, seulement après cinq heures, une augmentation des anticorps, tandis que la dose de gr. 0,1 en provoque une rapide diminution, suivie cependant de une augmentation considerable.

2) L'intoxication avec $Mn\,Cl_2$ dans les animaux déja vaccinés augmente les defenses immunitaires.

3) L'intoxication avec $Mn\,Cl_2$, suivie de la vaccination n'a aucune action sur les défences immunitaires.

SUMMARY

The Authors studing the behaviour of the production of antibodies in rabbits, reduced immune to choleric vibrion and injected into the veins with $Mn\,Cl_2$, have been able to show.

1) that the administration of small portions of the composition (milligrams 0,63 - grams 0,063) provokes, after five hours only, a augmentation of the antibodies, while the portion of grams 0,1 provokes a rapid reduction, that therefore is pursued with a considerable augmentation.

2) that the poisoning with $Mn\,Cl_2$ in vaccinated rabbits, raises the immunitary defence.

3) that the poisoning with $Mn\,Cl_2$ pursued with the vaccination, has not any effect on the immunitary defence.

ZUSAMMENFASSUNG

Die AA. haben das Verhalten der Antikörperbildung bei Kaninchen studiert, die mit Choleravibrio immunisiert und mit Manganchlorür intravenös behandelt wurden. Sie konnten beweisen.

1) dass kleine Mengen (mmgr. 0,63 - gr. 0,063) des Präparates, eine Steigerung der Antikörper bereits nach 5 h hervorruft, während eine Dosis von gr. 0,1 eine rasche Verminderung, die alsbald von einer starken Zunahme gefolgt ist, verursacht;

2) dass die Intoxication mit Manganchlorür in bereits vakzinierten Tieren die Antikörperbildung reizt;

3) dass die Intoxication mit $MnCl_2$, die von einer Vakzination gefolgt ist, in gar keiner Weise die Immunisierungskurwe beeinflusst.

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INDUSTRIAL AND CLINICAL ASPECTS OF OCCUPATIONAL POISONING WITH MANGANESE

Industrijsko-higihenski I Klinichi Aspekti Profesionalnog Otrovanja Manganom.

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By

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INDUSTRIAL AND CLINICAL ASPECTS OF OCCUPATIONAL POISONING WITH MANGANESE.

(With presentation of ten examples observed in actual conditions).

From 1949 to 1953 ten cases of manganese occupational poisoning were observed in one manganese alloy plant, where the operators were exposed to manganese ore dust and smoke. The men's exposure period varied from 18 months to 12 years. Clinical findings corresponded to the symptoms of light parkinsonism. The intoxication with two operators was manifested by hemopark--insonism; one of these men exhibited a so called "cock walk", exhibited lesions of the pyramidac tract, and the other symptoms of hemihypaesthesia. Very often, their symptoms were muscular cramps, and uncontrollable laughter, while only one patient exhibited symptoms of uncontrollable weeping. Blood analysis showed slight leukopenia (4,200-4,700 leukocytes) in five patients. Functional liver tests gave normal results. Liver biopsy tests were performed with five patients, only one exhibited strong accumulation of pigments which did not show the positive reaction for iron. Four other patients showed normal reactions. The Cerebrospinal fluid was probed using eight patients. Three of them demonstrated normal results. With the other five, the following was discovered. One case of light Pleocytolysis; Pandy reaction was positive in one case and plus/minus with three patients; in one case total albumin was slightly increased; and five patients showed small changes in collorgol reactions. Pneumoencephalograph was performed using two patients. One showed normal results and the other showed first symptoms of a diffused cerebral atrophia. The electroencephalogram was performed on six patients. Only one demonstrated modest and diffused irregularities. These seven patients were treated with "Bal" by the method suggested by Thompson; one was treated with pyridoxime, but no effect was observed. Detailed literature presentation demonstrated the note of manganese under natural conditions, industrial use of manganese, causes and effects of poisoning with manganese in industrial usage and pharmacology of manganese. The symptoms of manganese poisoning are presented, and manganese effect on the

liver, blood, lungs and other organs are shown as well. Several recommendations to prevent manganese poisoning are briefly outlined at the end of this article. The cases of manganese poisoning which we have observed in our practice are appended to this paper.

The element manganese belongs to a group of those metals which are considered as a typical cause of an occupational poisoning. Almost all known manganese poisonings are of industrial sources and all have a longlasting chronic effect. Accordingly, manganese poisoning is a typical occupational disease. Comparing it with lead poisoning, manganese occurs very rarely in spite of large production and a large usage of manganese. From 1837, when the English pharmacologist John Couper first observed manganese poisoning, eg. during a period of 116 years, only approximately 400 cases were discovered. Not many physicians have an opportunity to study this specific and interesting disease. Because of that, this disease still remains relatively unknown and not adequately investigated while its diagnosis is difficult to establish.

Manganese intoxication has one more particular interest in our work. Just before World War II, manganese poisoning attracted much public attention in our country. In 1939, in Sibenik (Yugoslavia) at the plant "La Dalmatienne", a large number of workman became sick with a strange neurological and psychical symptoms. Two opposite opinions were formed about their affliction. One thought, was that it was manganese intoxication and the other argued that intoxication was caused by carbon monoxide. Concerning this, Slezinger published an article in 1940, Ivancevic in 1941, and Karminski published two detailed papers in 1949. We are not going to discuss these cases at this time. The articles of Ivancevic and Karminski are very detailed and all data pertinent to this case can be found in the above articles. However, during the last four years we had an opportunity to observe several cases of manganese poisoning at the Department of Occupational disease at the Neuro-Psychiatrical Clinic. Our patients were also from the same plant in Sibenik and with the exception of one, all are employed at the same location and were working under the same conditions of mangansese

contact. Besides the clirical testing, we have observed their working place and conditions to establish possibility of manganese poisoning and to compare our data with information already published by other authors.

Up to now, all efforts to discover devices for early diagnosis of manganese poisoning have failed. This diagonsis is, unfortunately, possible only when chronic changes have already occured in a human body. Therefore we feel that there is a great need not just to investigate the toxicological but also biological and physiological behavior of manganese.

I. Manganese in Nature.

Occurence

There is no elemental manganese in nature, but it is wide spread in a great number of its compounds: 0.08% of the earths core. Manganese combounds can be found everywhere in rocks, in all iron ores, and in many silicates. There is manganese in sea water and in many spring waters.

A. Minerals of Manganese.

There are a great number of minerals in which manganese is either a main component or one of the constituents. Out of many terro-manganese ores, manganese dioxide-pyrolusite-, dark brown or gray rock should be mentioned first, which is most important and is the most used manganese mineral in industry. This mineral was known to Plyny who called it black "female" rock because it did not exhibit magnetic behavior of iron minerals. Because of that, some called this mineral "magnesia nigra" or "pseudomagne". In the glass industry this ore was named "magnes" or "lapis manganesis", in Germany "maganesium" (French: manganese, English: manganese) (173).

Other manganese minerals are presented in Table I which also shows the manganese content in each of the presented minerals. The following should also be noted: Albandite and hauserite, then silicates tephroite, knebetite and rhodonite and phosphate triphylite etc.

One third of manganese ores are located in Russia, and the rest in India and Brazil. Smaller manganese ore deposits are found in many other countries. However, ore characteristics particularly ones regarding economical processing

are important factors. We will show later that the geographical origin is very important from our point of view in order to organize safety measures.

TABLE I

The most important manganese ore and respective manganese content (23).

1 Imenuje	Formula	2 Sadržaj Mn u %
3 *Oksidi:*		
Pirolozit	MnO_2	63,2
Braunit	$Mn_2O_3 + xSiO_2$	64,3
Manganit	$Mn_2O_3 + H_2O$	62,5
Hausmanit	Mn_3O_4	72,0
4 *Manganati:*		
Psilomelan	$(Mn, Ba, K_2)Mn5_3 + xH_2O$	45-60
Vad	MnO_2xMinO sa 10-15% H_2O	5-15
5 *Karbonat:*		
Rodokrozit	$MnCO_3$	47-48

1. Ore name, 2. Mm content, %, 3. Oxides, Pyrobisit, Braunit, Manganite, Hausmknnite, 4. Manganate, Psilomclane, Pyrochroite, 5. Karbonat, Rhodochrosite.

Ferro-manganese ores should contain at least 25% manganese composition to be considered economically attractive for processing. Ore of the very costly items in production is the transportation cost. For example, India does not mine very much manganese ore because that country is too distant from processing sites and transportation costs are prohibitive. This also applies to Brazil ores which are bought mainly and almost exclusively by the United States. On the contrary, Russian manganese, from the Georgian Republic (Caucasia) is predominant on the Europian market. Caucasian manganese is of excellent qualtity with 60-65% manganese. In addition, this ore does not contain calcium carbonate, has a relatively small iron content, and "the peroxide" content is fairly high. Also, the Caucasian ore (as well as Indian ores) does not desintegrate easily into dust, (like the Chilean ores), and because of that it is considered more suitable for transportation.

A considerable amount of manganese ore is located in South Africa, Gold Coast, Cuba, Morocco, and smaller quantities in Turkey, Germany, Spain, Chile, Sweden, Norway, Hungary, England, British Borneo, Holland and the State of Colorado (United States).

World production of manganese was 4,000,000 tons per year (150) before World War II. Of the total production Russia contributed 50%, India 17%, Gold Coast 13%, South Africa 10%, Cuba and Brazil 10%. Production decreased after the war in India and South Aferica and was increased in Marocco and appears that the total production is about the same as it was 4,000,000 to 5,000,000 tons per year. In our country (Yugoslavia), manganese ores are mined at the places shown in Table II.

TABLE II.

Manganese mines in Yugoslavia (by Prof. L. Maril).

1 Vrsta rudišta	2 Nalazište	3 Manganske rude
4 Hipotermalno-metasomatno rudište	*Cer, Nebojša* kod Kičeva	5 Piroluzit
6 Epitermalno rudište (mala)	*Dobrevo* kod Zlotova	7 Piroluzit i Psilomelan
	Stari Trg (Trepča)	8 Rodohrozit (uz Pb i Zn)
9 Subvulkanska vulkanska rudišta	*Čevljanovići*	10 Piroluzit
	Ivanjska u Kozari	Psilomelan i
	Begunje	Vad
	Uroč i Ljubija	

1. Deposit type, 2. Location, 3. Manganese ore, 4. Mortic, 5. Pyrolusite, 6. Small, 7. Pyrolusite and Psilomelane, 8. Rhodochrosite, 9. Volcamic, 10. Pyrolusite Psilomelane Pyrochroite.

Besides those mentioned in Table two, manganese was discovered at Vitoliste in Macedonia, Nova Varos, Bar, Tanjevo etc.

B. Manganese in Living Plants.

Sheele, who discovered manganese in pyrolaisite in 1774, had argued that plants can assimilate manganese from soil. Later, manganese was found in many plants as barley, wheat, rice, rye, grapes, cocoa, coffee and tea, and can have manganese content of up to 10% on a dry basis. Some plants which show certain healing effects as cinnamon and leaves of digitalis contain manganese.

Manganese is accumulated particularly in those plant parts which are in an active vegetation. The reproductive parts have the most manganese, for example

new leaves contain more manganese than the old ones and as a seed becomes mature the manganese content increases. Some plants such as Leguminosae are very sensitive to a lack of manganese. It seems, that plants need manganese for growth. However, the mechanism of manganese effect on plant growth is not clearly explained yet. Is it the effect on the soil (85), is it a catalytic effect on oxygen absorption (19), or is it the effect of manganese salts on the soil ammonification and nitrification (27)?

Very interesting data concerning manganese effect on plants and fungi (especially Aspergillus niger) and on manganese content of many plants, is published by Bertrand (20,21,22). There were attempts to use manganese salts as fertilizer constituent to promote the plants growth. Dosages should have been taken into consideration, because it showed that when an overdosage occured manganese toxicity was observed (178).

Manganese is needed by plants and it is usefull to them but it becomes toxic over the physiological concentrations.

C. Manganese in Animals.

It also appears that manganese plays an important role in animal organisms. Manganese was discovered in human blood in 1830 (53) and then in the blood and tissues of many animal species: horses, cows, pigs, rabbits, hens, sheep, tunas, carnaries (53). The oyster pinna squamosa has in its blood manganese protein compound pinnaglobulin. Later, it was proved (178) that manganese is present in almost all normal tissues. Today the opinion (34) is that manganese plays a role in some redox system. It was shown that manganese can have an important role in the synthesis of ascorbic acid. It was discovered that there is a correlation in methabolism of the vitamine B_1 and manganese (153). Also, it is known that manganese is needed as a co-ferment to the ferment orginase and many other ferments (phosphatase in bones are more active in the presence of manganese (34)). Besides tissues, manganese was found in gall-liquid, gallstone, womans milk(in the colostrum five times more), and urine of humans and horses. In eggs, only the yolk has manganese (148). It is obvious that

manganese is the very important mineral constituent of living organisms. Manganese content in living organisms depends on the species itself and location of the habitat.

Distribution of manganese within the same species varied greatly in different organs. According to the date presented in Table Three, liver contains most manganese both in mammals and birds. The data differences for the same species were therefore significant, information should be considered with some caution and as a relative one. For example, manganese rabbit liver content which is used by Fabre (according to Bertrand and Lemosa) is 0.81 mgm per one kilo weight (71) which is quite different than the value of 0.17 mgm per one kilo of weight shown in out Table Three (118). It is believed that a difference occured due to different analytical methods used in this case. Also, in Table Three, we see that in a guinea pig lung, no manganese was found by Linda (a) and a considerable amount was found by Heinea(b). Manganese promotes growth with some animal species and it is believed to be the same with plants. It was demonstrated with rats (111, 126 and many others) and mice (100). A conclusion can be made (on the basis of the above results) that manganese plays a critical role for optimal growth. Small deficeincy of mamganese can be tolerated, but significant deficeincy undoubtedly showed an adverse effect on growth (184). When rats were fed with food without manganese they showed degeneration of the testes, decreased lactation and their new born grew slower than normally expected. In Holland, it was observed that some cows became sterile because they were fed with food deficient in manganese (163). With chickens, there was observed a peculiar disease "perosis", a congential malformation of the tibial joint, which is believed to be caused by deficeincy of manganese in food. In fact, if animals are kept on a diet without manganese they develop short leg bones (34). Some contend that manganese has some role in the life of a human fetus. It is mentioned as a possibility that during the last three months of pregnancy, within the uterus the fetus has, increased manganese content in the liver (117). Therefore, it was suggested that a baby

diet should have controlled amounts of manganese.

TABLE III.

Distribution of Manganese in various organs by Cettingenn (178)
Expressed in milligrams per 100 grams of natural tissue.

1 Vrsta životinje	2 Lit.	3 Pluća	4 Jetra	5 Bubrezi	6 Ostali organi
7 Kunić	20		0,237	0,087	8
			0,351	0,101	
Kunić	118	0,36	0,178	0,147	
Mačka	118	0,031	0,341	0,136	Medulla spinalis 0,059
Pas	20	0,010	0,306	0,106	
Pas	118	0,023	0,238	0,087	
Zamorac (a)	173	0,00		0,000	
Zamorac (b)	88	0,136	0,221		
Štakor	118	0,00		0,110	
Macaca rhesus	129	0,055	0,120	0,155	
Govedo	20	0,010	0,298	0,084	
Tele	20	0,011	0,290	0,053	
Svinja	20	0,023	0,203	0,109	
			0,317	0,122	
Konj	20	0,006	0,280	0,027	
Kokoš	20	0,010	0,416	0,217	jaje 0,063
Kanarinac	20	0,010	0,380	0,238	
Grlica	118	0,00			
Čovjek	146	0,20	0,170	0,061	
9 Granične vrijednosti*	178	0,00 —	0,120 —	0,061 —	
		0,055	0,351	0,155	
Srednja vrijednost za sisavce*	178	0,016	0,273	0,104	za mozak 0,035
Srednja vrijednost za ptice	178	0,010	0,398	0,217	

1. Species 2. Literature number 3. Lung 4. Kidney 6. Other 7. Rabbit, Rabbit, Cat, Dog, Dog, Guinea pig, Guinea pig, Rat, Cow, Young cow, Pig, Horse, Hen, Canary, Dove, Human 8. Egg 9. Limits, Average for mammals, Average for birds 10. Brain.

* Values "b" for the guinea pig not included.

It was found in rats (4) that manganese also has a lipotropic effect e.g. to protect the liver against a build up of fat.

Thus, manganese has undoubtedly both a biological and a physiological effect on animals. Later, we will see that this metal also has a toxicological effect on all tested animals and humans at higher concentrations.

II. Industrial use of Manganese.

Elemental manganese can be strained from many manganese minerals. It has an atomic weight of 54.93 and a specific gravity of 7.2 at 20 deg. C. The pure

metal is produced most frequently by the thermal method of Goldschmit. In this procedure, the aluminum metal reduces manganese oxide according to the equation.

$$3MnO_2 + 2Al_2 = 2Al_2O_3 + 3Mn.$$

Thus produced, manganese is a reddish gray color, it is relatively hard but softer than iron. Melting point is 1,260 Deg. C., boiling point 1,960 Deg. C. It oxidizes easily in air. Elemental manganese is seldom used, but its compounds and alloys have a widespread use in modern industry. This was followed by a constant increase in manganese production since the beginning of this century.

Manganese is 2, 3, 4, 6 and 7 valent element, thefore it is very reactive. Of particular interest are a series of manganese compounds with oxygen: Simple oxide manganosite (Mn_2O_3), hausmannite (Mn_3O_4) manganese oxide (Mn_2O_3), dioxide (MnO_2), trioxide (MnO_3), heptoxide (Mn_2O_7) and tetraoxide (MnO_4).

Of all manganese compounds, manganese dioxide is most often used, both as a natural mineral pyrolustic or as a synthetic product. By order of importance, then follows: oxide MnO, Chloride $MnCl_2$, sulfate $MnSO_4$ etc.

The most important uses of manganese are:

A. In Metallurgy

About nine tenths of the manganese production is used for metallurgical purposes, to produce manganese alloys which play very inportant roles in the steel industry. By addition of manganese alloys into steel, desoridation and recarborrization occurs which improves the quality or changes steel characterstics (hardness, stress resistance, elasticity) in a controlled way. The most known among these alloys are: Ferro-manganese with 25-80% of manganese and Spiegeleisen (Spiegel) with 9-25% of manganese. Also known are alloys of silicone and manganese. Siliconmanganese which contains besides iron and silicon about 50-55% of manganese, and "Silicopiegl" which contains up to 20% of manganese. There are alloys of manganese with zinc, nickel, copper, and aluminum. Among them, the most known is Manganin made of 83% Cu, 13% Mn and 4% Ni and is used for producing electrical filaments.

B. In the Glass Industry.

The oldest use of manganese occurred in the glass industry. The mineral pyrolusite was named after its use in the making of glass (of two Greek words; "fire" and "follow").

Pliny had described the use of manganese in glass industry in the middle ages, glass-soap was mentioned for decoloring glass. Natural manganese dioxide is most often used in the glass industry, if it has at least 50% Mn and not more than 1% of iron/oxide. When manganese is melted in glass at high temperatures, it releases oxygen which purifies (washes) glass so that glass loses its greenish, bluish-green or dark brown colors caused by impurities of iron, sulfides and ferrisilicates. On the other side, glass can be colored violet, black or brown by the addition of a pyrolusite.

C. In the Dye Industry.

The most common manganese colors, some of them natural are; chromic-brown (basic manganese chromate), manganese-brown (manganese oxide hydrate), manganese-green (the product of desintegration of barium manganate), manganese-violet (armonium manganophrophosphate), and manganese-white (manganese carboriate). It is also known as a dark-brown natural color "umber" (named after the Italian province Umbria) which is an iron ore with some contents of manganese, aluminum and calcium. This color has some significance because of similar characteristics with ocher-clay. Manganese colors are used for mosaics, in enamel production, for linoleums etc. (23,173).

Manganese is also used in the chemical industry. For example, manganese dioxide is used for oxidation of the leuko-compounds to introduce a hydroxyl group and for transformation of the methyl group to aldehyde or of the carboxyl groups.

Manganese dioxide is also used in the production of various oils, varnishes together with other manganese salts(Corated, Sulfates, Linoleates, Oxalates), which promote transformation of unsaturated acids into resins.

D. In the Ceramic Industry.

Manganese is used in the production of faience, porcelain and earthware. Manganese minerals are in additive in bricks and cement etc. The ceramic coats also contain manganese.

E. Manganese in the Electro Industry.

1. A mixture of manganese and graphite is used for the Leclanche Cell (dry cell), for depolarization of hydrogen, to prolong a battery's life. In the latter case manganese is wrapped around graphite. For this purpose a purefied manganese oxide is used which does not contain either iron oxide or elemental iron (such as the ores from Caucasia, Java, Philipsburg, Cuba and Puerto Rico) generaly in mixture with a synthetic pyrolusite. With recent development of radio-communication and a demand for anodic batteries, the use of manganese is constantly increasing.

2. Welding electrodes and rods sometimes contain manganese in a significant amount (101) particularly when "hard welding" is necessary. The purpose of manganese addition is to deoxidize during the welding process.

3. In electrolysis of zinc sulfate and copper nitrate, the electrodes of manganese dioxide are sometimes used.

F. Manganese in the Chemical Industry.

Before methods to produce chlorine electrolitically was developed, manganese was used for producing chlorine from hydrochloric acids, as it was done by Scheele who boiled hydrochloric acid with manganese dioxide, according to the reaction.

$$MnO_2 + 4HCl = MnCl_4 + 2\ H_2O$$

Then at higher temperatures manganese tetra chloride releases chlorine.

$$MnCl_4 = MnCl_2 + Cl_2$$

Manganese dioxide was also used in the production of aniline and alizarine leangan. Manganese dioxide is used in the production of matches as an oxygen carrier. Manganese persulfate is used as an oxidizing agent in the production of many organic compounds e.g. Saccharine, Benzoic acid, etc. Manganese has many other used: Powdered pyrolusite is used for purification

of drinking water; in Germany manganese sulfate is used as additives to fertilizers: manganates and permanganates for wood conservation, textile bleaching, various manganese salts are used for impregnation of raw leather, for disinfection, in deodorants, in flotation, for many medical and photographic reagents, etc.

III. The Source of Manganese Poisoning in Industry.

A. Grinding of raw Pyrolusite.

Most frequent cases of manganese poisoning occurred in locations where "the brown rock" is ground, because of that manganese poisoning is called in German literature "Braunstein-Mullkrankheit. In the grinding process there are many working places where exposure to manganese is rather considerable. The ore is crushed, ground, screened and then various fractions are mixed. Particularly during mixing, much dusting occurs. This dust has a high percentage of manganese dioxide. Indirectly, contamination with manganese can occur even in areas where the ore is washed. During washing, water is spilling or spraying around the working places and after drying time dust is left (53). In oneof our plants, the workmen call the grinding area "black room", because it is completely covered with black dust of manganese dioxide. Bickert, described in detail the conditions in milling areas and he classified the process phases in the following order of contamination: grinding, packing, screening, mixing, washing and drying in furnaces. Therefore, grinding is the most dangerous, while drying is the least dangerous. In pyrolusite mills, some cases of manganese pneumonia are also observed.

B. Mining of Manganese Ores.

It is interesting to point out that manganese poisoning in manganese mines are not so frequent as in industrial plants. Probable explanation is that the manganese ores are usually mined wet,they contain up to 40% moisture (72), so that dust formation is very limited. Freise, spent many years in one of the Brasilian mines and he did not observe any of the neurological symptoms in spite of the fact that the health and safety conditions on the work were very poor.

Just recently, since 1935, the manganese poisoning in mines became more often (in Spain, Egypt before the war and in Chili and Morocco after the war). Baaderu found that in Egyptian mines there were no manganese poisonings until they started to use the pneumatic drills and then about 30 workmen were manganese poisoned in a short period of time, due to formation of dust. Alvarez says, that his patients came from the Chilean mine producing the ore with 87% MnO_2 which can be easily pulverized.

Since manganese poisoning is always a massive one, a total number of poisoned persons are considerably great. In mines, the manganese pneumonia occurs more often than other forms of the manganese poisoning which is explained by specific climate conditions in mines.

According to J. and M. Rodier, the manganese intoxication is very closely related to, (a) manganese content in the ore, (b) oxidation state and extent of the ore and (c) fineness of the manganese dust. They attribute to these factors, more often manganese intoxication in the mines Imini and Tiouine then in the Bou Afra mine.

C. Production of the Leclanche Battery Cell.

About eight publications are related to the manganese poisoning in connection with production of the Leclanche Cell. Although, these cases are discussed often they do not involve many patients, only about 14 cases. In production of the Leclanche Cells, the dust formation is very extensive during mixing and processing of the MnO_2 mass. Nosheim (131) describes that his patients were working "in the clouds of the manganese dust". He observed some cases of the manganese pneumonia. The most exposed working places are weighting, charging, and discharging of the mixer, screening and transportation of the mixture (53).

D. Classification of Ores which contain Manganese.

Other ores, not just manganese ores, can be the source of the manganese poisoning. It is first observed by the American authors (36,58) which published some manganese poisoning of workmen on the electromagnetic separation of ores containing manganese only as impurities. One of these articles (58) presents 38

cases of poisoning while the ore with only 9% manganese was processed. The same possibility is later observed by Ehrismann in Germany (62).

We do not know whether the manganese pneumonia was observed in the above cases.

E. Loading and Unloading of the Manganese Ores.

Freise in Brazil and Bubareu in the Soviet Union did not observe the manganese poisoning with men working on loading and unloading, but Baader (11) and Trendtel observed a few cases under such circumstances. It sounds a little bit strange because in question is an ore of the same origin. However, loading and unloading jobs are more often accompanied by the manganese pneumonia which is also caused in some extent by specific climatic conditions.

F. Metallurgy.

Although manganese is mostly used in metallurgy, the manganese poisoning is very rare in this field. The cases which were described by Schlockow in 1879 should be now considered as the manganese poisonings in metallurgy. After that, only ten cases in production of the ferro-manganese alloys in an electric furnace (51) and one case at work with "the manganese furnace" (130). These three cases were caused by the gases in the furnace smoke, which probably contained manganese as manganese oxides. Seven other cases occured by breathing the dust during grinding ferro-manganese. In these cases manganese was in the metallic form (88,186).

We observed several of this rather rare manganese poisoning in metallurgy. Our patients came from "the factory of ferroalloys and electrodes" in Sibenik. This factory produces ferromangan, siliconmangan and "Silicospiegl". It started production in 1930 but closed in 1931 to start again in 1938. During the war, the plant was closed and was reopened in 1945 with production of the ferro alloys.

Fig. I.

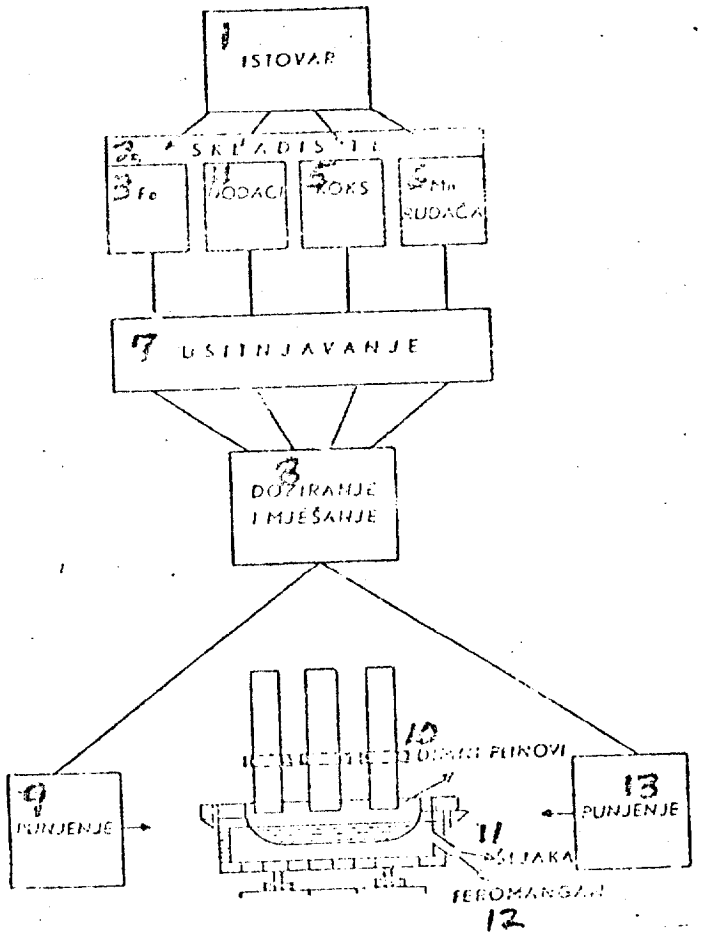


Fig. I. The Flowsheet of the Production of Ferromanganese.

1. Unloading 2. Storage 3. Iron 4. Additive 5. Coal 6. Mn Ore 7. Grinding 8. Mixing 9. Charge 10. Waste Gases 11. Sludge 12. Ferromanganese 13. Charge

The process flowsheet of the production of manganese alloys is presented in Fig. I. The ore is brought in front of the factory by the railroad or by the ships. After unloading, this ore and other process constituents are crushed, ground and mixed. The mixture of a specific composition is then melted and the alloys is casted in the molds. Dust id forming during unloading, grinding and furnace charging, also we have waste gases from the furnace. Both the dust and the waste gases contain manganese. An older literature data (173) shows how much the waste gases can contain manganese. Of the total manganese input into the

furnace, 75% is in the produced alloy, 15% in the sludge and 10% (1) is lost in the waste gases. This smoke, which in enormous amounts covers the near by city, is described picturesquely by Karminski.

We were observing in person the working conditions in the factory. All patients, except one which was a plumber, were either the transportation workers or the furnace stockers. They load the mixture into the small carriages, they drive them to the furnace, they unload the carriages in the vicinity of the furnace and sometimes they charge the furnace. They push the carriages through the tunnel which is so full of smoke that one man cannot see another man by-passing him. The atmosphere in the tunnel consists of both dust and the waste gases, which have high content of manganese oxides, mostly dioxides. In such an atmosphere, the operators rest sometimes, they even sleep during the night shift waiting to be called to charge the furnace.

According to not yet finished investigation (done by Kraljic) the many working places are exposed to rather high manganese concentrations. The highest concentration was found in the vicinity of the three phase furnace (about 63 mg/cubic meters), at the mons-phase furnaces it was found about 7.5mg/ cubic meter, while the least concentration was found in the yard atmosphere, about 0.5 mg/cubic meter. The maximum allowed concentration according to the American standard is 6.0mg/ cubic meter (183).

G. Electrowelding.

Electrowelding, in our opinion, is a very rare source of the manganese poisoning. Only one article (18) and two cases are described. The welding electrodes can contain, as we mentioned, either manganese dioxide or ferro-manganese. During welding, in the electric arc at a temperature of 2,300 to 2,700 deg. C.,the electrode metals do vaporize and condense in the near area polluting the air with very fine smoke which contains manganese probably in the form of the oxides. Thus, manganese can be brought into human bodies by the respiratory system. However, the manganese pneumonia was not observed in connection with welding jobs.

H. Production of Potassium Permanganate.

Pneumonias are often observed in production of potassium permantanate (112,113) and they are considered the manganese pneumonias. The chronic manganese poisoning was not detected.

Also, in many other areas where manganese is employed, no poisoning was observed. Consequently, not all forms of exposition to manganese can be considered poisonous. Small amounts of manganese, used in the ceramic and glass industries, and in the dye industry or during electro welding, are insufficient to cause the chronical manganese poisoning (72) and not even the manganese pneumonias as well.

According to all that we presented about this subject, we see that the manganese poisoning in industry comes by the exposition to the dust and smoke containing manganese. Manganese is believed to be in the form of the oxides in either of the above medias. In some cases, manganese can be in an elemental form as it is in the ferromanganese dust, to cause poisoning.

IV. Toxicology and Pharmacology of Manganese.

The toxicological investigation and experimental poisoning with manganese, has started very early, at the time when not much was known about the clinical poisoning in general. Couper points out, to the Gmelin experiments with animals which were performed in 1824. Then in the 19th century, a series of investigations were performed. Kobert points to several authors, which performed experiments in order to cause an acute poisoning. At the end of the century, several publications deal with poisoning caused by heavy metals including manganese (32, 87). The experiments with manganese were done mainly because of its pharmacological similarity with iron.

A. Toxicology of Manganese.

Whether or not all manganese compounds are toxic was the first question to be answered. We have presented in this paper that the chronic manganese poisoning were caused mainly with the same manganese compounds, with emphasis particularly with pyrolusite. On the other hand, in many other areas where

manganese is used, the manganese poisoning was not observed. It is not determined accurately yet which factor contributes to the poisonous characteristics of manganese. It is the nature of the chemical compound or the amount or concentration to which one is exposed, or the manganese toxic characteristics itself. It is prevailing opinion (72) that the exposition to small concentrations of manganese as it is in the ceramic and glass industries, is not dangerous to cause the chronic poisoning.

Already Van Jaksch claimed that the two-valent ion is more toxic than the three-valent ion. This was proved later by other authors (154,178). It is generally considered that the manganese compounds in the higher oxidation state are less poisonous (they can cause only local acute changes) than the compounds in the lower oxidation state which cause the resorptive chronic poisoning (53). The latter should be considered the exclusive cause of the chronic intoxication while the first ones are of importance if they can harm the respiratory system causing the "bronchial catarrhs and pneumonias". It was thought, that the poisonous degree of a manganese ore depends on the manganese monoxide content. The caucasian ore with a small manganese monoxide content should be less dangerous than the ones from Thuringia (East Germany) and Japan (53,128). Some authors think that only the manganese dioxide content in the ore is the crucial factor (58,161). They point out to the "Merk" pyrolucite with 82.7% MnO_2 which is more toxic than the natural pyrolucite which contains about 70% manganese dioxide (53). Let us add to it, that there is also the possibility of considering, that the toxicity of manganese ores depends on the manganese content and not of the chemical nature of manganese. According to Heineu, even the ferro-manganese dust is poisonous which contain manganese in a metallic form. This is in discrepancy with the Baader theory, that metallic manganese does not "have an importance for the health hazard" and "that it becomes poisonous only in specific oxidation states".

There is not much of an experimental work done to investigate the manganese oxides toxicity. Schwarz and Pagels showed on cats, that the manganese toxicity

depends on the manganese dioxide content and on the manganese compound solubility. They obtained various toxical effects using the pure manganese dioxide. Heine, on the contrary, in his experimental work with guinea pigs, concluded that the manganese toxicity does not depend on the manganese oxidation state but on the manganese concentration involved. He was experimenting with ferromanganese and he compared the toxicity of the various manganese compounds and forms, (metallic manganese with 95% Mn, manganese from the furnace with 75% Mn, pyrolucite with 66% Mn, manganese silicate with about 60% Mn, the manganese ore with 50% Mn, and half of that was in the form of MnO_2, vanadium sludge with 29.5% Mn, manganese as MnO and with Thomas sludge with 6.7% Mn in the form of MnO). With birds (70), the peroral treatment with metallic manganese did not cause intoxication.

There are only a few data of the lethal dosage of manganese. The earlier investigation was performed with the manganese compounds which are of no importance in a industrial use, such as manganese citrate, manganese chloride and potassium permanganate. In Table IV are shown the lethal dosages for some manganese compounds.

TABLE IV.

The Lethal Dosages for some Manganese Compounds.

1 Spoj	2 Lit.	3 Species	4 Način aplikacije	Letalna doza na kg tjelesne težine 5	6 Opaska
7 Manganov Florid	178	riba 8	u vodi	0.3 g	na litru 10
	86	miševi zamorci kunići	supkut. 9	50 mg	
	178 108	psi kunići	intrav.	56 mg 18 mg	
Manganov citrat 11	103	psi mačke mačke kunići zamorci 12	supkut. 13	6–8 mg 13–14 mg 12–13 mg 28–30 mg	u roku od 2 dana u roku od 24 h konvulzije i paraliza, u manjim dozama progrs depresija 14
15 Kalijev permanganat	111	16 konj kunić čovjek	oralni 17	10 g 0.34 g 5 i više g	Apsolutna doza 18

(1) Compound (2) Literature Number (3) Species (4) Application in Water

(5) Lethal Dosages per 1 Kilo of Weight (6) Mark (7) Manganese Chloride (8) Fish, Mice, Guinea Pig, Rabbit, Dog, Rabbit (9) Subcut. (10) Per Litre (11) Manganese Citrate (12) Dog, Cat, Cat, Rabbit, Guinea Pig (13) Subcut. (14) In two Days, In 24 Hours, Convulsion and Paralysis in a Smaller Dosage Depression (15) Potassium Permanganate (16) Horse, Rabbit, Human (17) Oral (18) Absolute Dosage

The data presented in Table IV, show that the manganese toxicity does not depend on chemical nature but also on the species involved, and on the way manganese was applicated, and in the first place on the amount of manganese applied.

B. Absorption and Resorption of Manganese.

It is well known, that the toxicity of any poison depends on its solubility in the biological liquors. Thus the effort was made to determine the solubility of manganese compounds in the stomach secretion particularly because it was believed that the digestive tract is the main entrance to introduce manganese compounds into the body and cause poisoning. For example, McCord says, while working with the manganese dust, some dust can be inhaled but more dangerous is a part of dust, which enters into the mouth and is swallowed into the stomach. The same opinion was shared with Casamajor and later by Leclerg. About the manganese solubility in the gastrointestinal canal there are opposite views.

The earliest toxicological investigation already proved that introducing the simple manganese compounds into the stomach, showed toxic symptoms only if they were given in very large dosages. The effect is mainly due to the corrosive effect of manganese compounds on the stomach and the intestinal walls. At that time it could have been possible to see that the resorptive effect did not occur even with a high manganese dosage, because of a very low solubility of the particular manganese compound used in testing. Later, investigation showed that there is a great difference in solubility of various manganese compounds, particularly of the oxides. Korber gave the manganese citrate to the rabbits and he observed a very weak resorption. Resorption occurred only with large dosages,

which caused the gastritis. The similar results were obtained by Cahn, Reiman and Minot in 1921. They were determining the solubility of the franklite, rhodonite and manganese doixide in the stomach fluid. They found, that the solubility of the manganese compounds depend on the stomach fluid acidity and the contact duration and that resorption in the blood stream is possible. One of the authors took eight grams of franklite suspended in water and then the manganese concentration in his blood was assayed for some period of time. They found that the manganese concentrations increased considerable during the first few hours and then it suddenly dropped back to the normal.

It is generally accepted (69,88), that the manganese dioxide is only slightly soluble in the stomach fluid (gastric juice) and consequently that the intoxication through the digestive tract is possible only with the lower oxidation compounds of manganese (MnO, Mn_3O_4). This was also the reason to believe that these oxides are more poisonous. However, with some animals it was possible to cause the resorptive symptoms by peroral treatment with pyrolusite (114).

Von Oettingen, in his detailed publication about toxicology and pharmacology of manganese, concluded that the manganese resorption by means of the gastrointestinal tract is under normal conditions, a very slow process and causes only the temporary increase of manganese concentration in blood. In spite of this statement, he also pointed out that the gastrointestinal tract is the main route to resorb manganese "although one part can be inhaled by the lungs". There is no certain proof that the human can be poisoned through the digestive tract. However, several newer articles (35,77,104,120,186) are mentioning the ingestion besides the inhalation, as the possible way of intoxication. The ingestion is mentioned, probably because in some experiments with animals, it was possible to cause poisoning in this way. Fairhall, 1949, says that a possibility of poisoning by the ingestion is negligible. If we look at the sources of the manganese poisoning in industry, we see that the poisonings are more often at places where there is a lot of the manganese dust.

So, the absorption of manganese through the respiratory tract is probably at the greatest practical importance. Many recent authors (14, 58, 69, 72, 88), believe that the professional poisoning can occur by the absorption through the respiratory tract only. Already Von Taksch thought, that manganese is tied in the lungs by one albumin by which it is transported through the body.

C. Manganese in Bodies of Living Species.

We do not know what is happening with manganese in the body. We know that manganese appears in the blood, reaching a limited level, and according to Reiman and Minot, it again disappears from the blood. There is an experiment possibility to stop poisoning before manganese is excreted out and then to analyze the specific organs to determine the distribution and accumulation of the poison in these organs. However, that accumulation in the various organs depends on many factors such as: animal species tested, method of the manganese application, manganese chemical nature, manganese concentration applied. Due to this, the results are discrepant, uncertain and contrast with each other.

Handowski and others, showed that the manganese distrubution is different in case of subcutaneous application then obtained by the oral one, specially concerning the manganese concentration in the liver. Kobert had infected subcutaneously the manganese citrate into rabbits, and he found that most manganese was concentrated in the liver, kidneys and intestines. Cahn had injected intravenously the same manganese compound, and he found that most manganese was in the intestine walls, liver and kidneys. Drinker injected intravenously, the suspended manganese dioxide into cats, and he found about 90% of the total manganese infected in the liver, lungs and pancreas. The manganese concentration in the intestinal tract, was not as high as when the oral application was used.

Handowski, after the subcutaneous injection of the manganese chloride and citrate, found most manganese accumulation in the bones, pancreas and the brain, different amounts in the liver and relatively very small concentrations in the lungs. Harnack and Schreiber, after a peroral application of manganese peptonate to rabbits (two grams per day for three to four weeks), found high concentra-

tions of manganese in the pancreas, liver, stomach and less amounts in the kidneys. Lemos observed the manganese accumulation in the brain and liver after the peroral application, and in the lungs after application is performed through the respiratory tract. However, Van Bouguert and Dallemagne claim that they did not find any traces of manganese in the heart, liver, lungs, brain, skeleton etc. of a monkey poisoned through the respiratory tract. The very changing manganese content in the liver was explained by Handowski and others, by different "local conditions of a specific organ". They also claim that the amount of manganese accumulated, does not cause an animal's death, or anatomic change in the picture of poisoning. This conclusion, the authors made, on the basis of the clinical observation where they found that some of the heavily poisoned patients had twice the normal amount of manganese in the blood, while the others had a normal amount of manganese concentrations in the blood (146). Concerning the manganese content in the lungs, the experimental data does not agree with the clinical findings. An autopsy of one patient, who died of the manganese poisoning in 1940, showed that the lungs contained most of the manganese, more than any other organ, even though the patient was not in contact with manganese for seven years. The lungs of this man were considered as "the manganese storage" (77). We will talk later in this article, about the mangnaese affinity toward the Crain substance.

D. Manganese Excretion.

The experimental results and clinical observations are in good agreement, concerning the mechanism of the manganese excretion, only disagreement appears concerning the excretion rate. Already in 1860, Wichert discovered manganese in the bile of the poisoned cats. Cahn found manganese in a large amount, in the stomach content only a short period of time since it was intravenously injected. Kobert found manganese in the feces and only traces in the urine. Handowski and others, proved that about 50% of the manganese injected, is excreted quickly with the feces and only in long lasting experiments, some traces of manganese were found in the urine ("die Niere ist also Mangandicat"). Even when the subcutaneous injection is employed, the small part of manganese being resorbed is

excreted into the feces through the intestines and not through the kidneys. Manganese is partly excreted by the bile fluid (20,21,22,119). Thus, the experiments proved that manganese similarly to other heavy metals, is excreted through the intestinal walls and by the feces. Similar observations to the above ores, are made with human beings (150). Baader (11) found 3.93% manganese in the feces 16 months after the exposition time. Leschke and also Grewel, Sassen and Voss (181), obtained similar results. Bodier concludes, that manganese excretion occurs mainly by the bile fluid and by the feces. Very rarely is mentioned the excretion by the urine with the clinicaly observed cases, and it mentioned only traces of manganese are involved. Many authors did not find even traces of manganese in the urine. Wagner explains, that urine analysis are performed too late. However, in one case (77), the patient kept excreting manganese in very small concentrations in the urine seven years after he discontinued working with manganese. But in a practical sense, the excretion by urine is of no importance.

V. The Clinical Observations of the Professional Poisoning with Manganese.

A. The History.

Manganese poisoning was first described by T. Couper, in 1837, who observed poisoning of men working on crushing "the black manganese oxide", in the production of a bleaching powder. His first two patients showed the symptoms of a paraplegia, then signs of propulsions and stumbling. Three other workmen became slightly ill, but recovered after they discontinued to work. The sickness of all five patients', was attributed to the toxic effect of manganese. This Couper article is the first published warning, that manganese is an active poison to human beings. He also showed the difference between the manganese poisoning on one side, and poisoning with mercury and lead on the other side. He said, manganese similar to mercury and lead, attacks the central nervous system, but does not cause the colics as lead does, and does not cause "the metallic tremor".

For a long time after the Couper publication there was no literature data

about manganese poisonings until the begining of this century. In fact, Schlockow in 1879, described some diseases of the medulla spinals of several workmen in the zinc smelter, at the mines in Schleswig (Germany). Casamajor, Gross (53) and Heine, thought that these cases were manganese, rather than zinc poisoning as the author believed. But one should be aware, that the processed ore contained besides manganese and zinc, also arsenic and lead.

The manganese poisoning was forgotten for a while. Kobert, in his first book about toxicology in 1893, claims that the manganese compounds are not hazardous to human beings, and he had to correct this statement in the second edition of his book (53). R. VonJaksch in 1901, described (75) first two cases, and later in the same year one more case of the manganese poisoning with workers employed on drying of manganese dioxide. The same year, in Hamburg, Embden (67,68) described four more cases of the manganese poisonings in the pyrolusite mill. So in this year, has started a new era of the clinical observation of the manganese poisoning.

While Embden connected manganese etiologically to all patients, V.Jalsch thought in the beginning, that the disease was caused by coldness. He corrected himself in 1907, admitting relation between the diseases and the work with manganese, still assuming the possibility of some other factors. V.Jaksch, was the first one who warned about the possibilities of the neurosis in the manganese mills, which might appear similar to the manganese poisoning. He called this neurosis manganophobia. Both V.Jaksch and Embden, described the symptoms of the manganese poisoning as we know them today. Until 1907, VonJaksch collected from literature, 15 cases of the manganese poisoning.

It is interesting, that the authors in the beginning had compared the manganese poisoning with multiple sclerosis. Although the pathological-anatomic results were not available, it was supposed (194) that the anatomic symptoms should be similar to ones observed with the multiple sclerosis. However, today all authors make a comparison between the manganese poisoning and the Parkinson's syndrome (disease). It is somewhat hard to understand that the earlier

authors did not notice this connection. The explanation might be, that the pathology and phusiology of the extra-pyramidal system were not known at that time. The famous neurologist Edinger in 1911, claimed that nothing is known about the function of the "corpus striatuma". The only disease which we today consider as the extrapyramidal disease, and which which was known in that earlier time, is the Parkinson's disease, Paralysis Agitans. This disease was first described by Parkinson in 1817, but it was to become more known after Charcota (1875) who classified it as some sort of Neurosis. Even in 1913, Oppenheim wrote that "essentially this disease is completely unknown". But, after Wilson published in 1912, the pathological-anatomic results of the symmetrical softening in Nucleus Lenticularis, and the liver cirrhosis of one patient with the clinical picture of the "parkinosonism" (hepatolenticular degeneration), the more intensive investigation of the basal ganglion began. The work of Hunta in 1917, and C. and O. Vogta in 1920, and then the appearance of a great number of the extrapyramidal symptoms following the big epidemic of the encephalitis after World War I, helped to get deeper in the pathology of the extrapyramidal systems.

Soon after the above mentioned Wilson publication, two new publications about manganese poisoning appeared (36,162), both in 1913. Seelert described one case which was already described by Friedel in 1903, with no improvement during the ten year period. Since the V.Jaksch publication in 1907, to the Seelert's one, there was not published any case of manganese poisoning. Seelert still sees similarity between the multiple sclerosis, explaining some symptons by the probable lesion of the cerebellar system. He also pointed out, that some symptoms are similar to the symptoms of the Wilsons disease, and he thought that the manganese poisoning was caused by the lesion of the basal ganglions. He also mentioned that there is no liver lesion with the manganese poisoning. The year, 1913, Casamajor published the first American case of the manganese poisoning. He mentioned nine cases of poisonings but described only one. This case is the first one with the results of an autopsy. The patient was manganese poisoned but died of pneumonia. An autopsy found an increase in the perivascular space of the

thalamus and of the lenticular nuclei.

In Germany, after the first cases were published (78), there were no other cases until 1922, supposedly because of the strict safety rules, and in that year only one case of the manganese poisoning was announced.

After World War I, many poisonings were reported from other countries. In 1919 it was reported, 38 new cases of the manganese poisoning in the United States from the same plant where the Casamajor's patients were employed (processing of the zinc ore which contained manganese). The next year, Sato presented the first case in Japan. In 1921, Davis and Huey described two cases of manganese poisoning in production of ferromanganese, and in 1922 Embden appeared again with a case of poisoning in Hamburg, with a worker on grinding of the Brazilian ore, which has just replaced the caucasian ore. The same year, Charles (41) described after Couper, the first case of manganese poisoning in Great Britain. From World War I until 1940 in each year, at least one case was reported, or about two thirds of all cases. Planques and Salmon in 1934, announced the first case in France, and Dragonneti in 1937, the first case in Italy. During World War II Heine described in Germany, five cases of poisoning occurring in grinding of ferromanganese, and in 1944 in Chili, 64 cases were reported in one mine. In 1948, two cases were reported in Sweden (186). Among articles after World War II, it should be emphasized, the cases reported in the mines in Morocco (Marakesh) which was published in 1949 (150). From the same place, four new cases were reported in the last year 1952 (133).

Very detailed presentations of the manganese poisonings were given by Dhersa in 1934 in France, by McNally in 1937 and by Fairhall and Neal in 1934 in the United States, and by Vossa (180) in 1939 and by Heine in 1943 in Germany.

B. Exposition Time.

How long does the contact last to cause the disease? The literature data showed, that the first symptoms can appear only after a short exposition of about three months, but no strict defenition of the exposition time can be made.

McNally, looking at the 133 cases in literature, found that exposition varied from six weeks to 29 years; Baader, states time span from four months to 20 years. The average time is from one to three years.

The exposition time for our patients is given in Table V, and we see that it lasted from 1.5 to 12 years. The same circumstances related to our patients, were in some aspects specific, particularly regarding discontinuance of work. The plant started production in 1939, but it was shut down from 1942 to 1945. The plant also discontinues production each year during the summer, due to water deficiency. Also, our patients changed their working places from time to time.

TABLE V

The Exposition Time in Years for our Patients.

2 Rad prije rata	5	—	4	2	—	—	3	—	—	—
3 Prekid u ratu i poslijeratnom periodu	2	—	6	5	—	—	7	—	—	—
4 Rad poslije rata	7	4	4	4	2	4	2	3	1,5	6
5 Sveukupna ekspozicija	12	4	8	6	2	4	5	3	1,5	6

1. Name Initials 2. Exposition before World War II 3. Discontinuance in an employement during the war and after the war 4. Exposition after World War II 5. Total Exposition.

Four of our patients had discontinued work for several years during the War. What the effect of these interuptions on the disease development and clinical observations, is hard to answer. Baader (11) mentioned an interesting case of a very heavy manganese poisoning, where the total exposition time was only a few weeks, during a time period of four years.

C. Dispositon.

Very early it was noticed, that the number of people intoxicated was relatively small, considering the number of people exposed. Heine wrote that in Germany, in a period from 1929 to 1937, only one case was registered per 1000

of the exposed workers.

On the basis of literature data (14,36,53,68,78,80,93,103,146,150,185), it appears that the manganese intoxication is a more individual disease than any other intoxication. Already Embden (67), in his first publication pointed out "that no small number of people are tolerant to the gray rock". This very different disposition, misled VonJaksch to not etiologically connect manganese with his first case of the manganese disease. He thought about the manganese poisoning as a possible diagnosis, but he rejected it because many other workers stayed healthy, although they were exposed to the same manganese dust. The different disposition is particularly observed with sporadic workers, for example with workers in sea ports, which perform unloading occasionally. This is described by Baader (11) and mentioned by Trendtel. In both instances only one worker got sick out of a group of 12 to 16 workers. Flintzer mentioned the interesting case, in which two brothers were involved. They were working under the same conditions, in the same pyrolusite mill, the elder stayed healthy all the time, but the younger brother became ill after one year with symptoms of a heavy manganese poisoning. We might add, a sporadic appearance of the manganese poisoning in production of the Leclanche cells were observed, usually in one (12, 55,119,170), and seldom in two (131), three (166) or four (138) cases of the poisoning. A great variation of an exposition time, can be partly explained by different disposition of individuals.

To find what is in the substance of variety of disposition, would be of great practical significance, in order to prevent the professional manganese poisoning. Unfortunately, nothing is known about it. Some authors are only guessing and are making indirect conclusions. Because of a great practical significance, we will present some opinions about the roles some organs have in building a resistance e.g. creating disposition. Similarly to other poisons, which enter a human body by inhalation, it was thought that the respiratory tract of some individuals is more suitable for an easier manganese resorption. So, a malfunctioning of a nose was first considered as the cause for an increased

disposition. It was assumed that the nose, which does not filter manganese dust efficiently, opens the posibility for better resorption of poison through the inflamed mucous of the respiratory system (81,150). Baader explained the manganese poisoning with the mentioned sporadic workers (sporadical unloading of the manganese ore in sea ports), and that their respiratory tracts were inflamed by other non-manganese dust, and the dampened mucous make easier manganese resorption. Baader also warns, that the ore origin can have some effect, and claimed that the Cypriote ore effect the respiratory organs more than other ores. Trendtel also thinks, that the Cypriote ore (from Cyprus) is more dangerous because of the high sulfur content, which irritates the respiratory tract and makes it suitable for manganese resorption. Hilpert(93) thinks about a constitutional weakness of the respiratory tract, because he found a relatively high percentage of cases of tuberculosis and the lung careinoma in the patients' families. It is interesting the relationship between disposition, found in manganese poisoning (which effects chronically the central nervous system) and the manganese pneumonia. While the manganese poisoning occurs in a relatively small number of the exposed workers, the manganese pneumonia can appear in a high percentage of workers exposed to the manganese dust. The probable explanation might be, the stronger disposition toward pneumonia. Manganese role in causing pneumonia, is not explained yet, so neither is the disposition toward pneumonia. In addition, there is not a device to prove that the pneumonia cases started because of the manganese role. There is no doubt, that climate factors play some role in the cases of manganese pneumonias. They are occurring mostly in mines, on outside transportation jobs, and in other words, in such places where increased numbers of the respiratory tract diseases are registered. May be, the same factor (the extreme temperature change) is responsible for an increased occurrence of the manganese pneumonia in metallurgy.

If we accept the opinion, that manganese can be resorbed through the digestive tract, then we can also speak of some kind of disposition of the digestive tract itself. Reiman and Minot, found that manganese concentration in

in blood, increases two-fold with some individuals, and does not change at all with others when manganese was ingested. They concluded, that the individuals of the first group are more sensitive to manganese. Since manganese is excreted by the bile fluid, the healthy liver should be considered as one link in a mechanism against the intoxication (120). So we can expect, that the liver damaged either by endogenous means (unknown), or by exogenous ones (alcohol, phosphorus, arsenic), cannot perform normally, the function of elimination of a poison from the body. This can lead indirectly, to an increased possibility for the manganese poisoning.

Manganese resorbed and retained in the body, is in the first place, a poison of the nervous system. So it was thought, that traumata or other diseases of the central nervous system, can aid developing of the manganese poisoning, but this has not been proven. Also, it was thought that the mentally retarded persons, were less resistant toward poisoning because their nervous system is of some kind less value. One of our patients was mentally deficient, and we think that the appearance of the mentally retarded amoung the manganese poisoned persons, can be explained by a simple fact that the job usually does not require any qualifications, so that the mentally retarded can obtain such jobs easier than others. It is also thought, that the stronger disposition of the mentally retarded person to the poisoning, is because they cannot realize the importance of the safety and operational rules (78).

Also mentioned, are general infections (syphilis (78), malaria (150), intoxication with alcohol, nicotine (78) and tea(150)) as the elements, which can increase disposition. It was not proven that the chronical effect of carbon monoxide in mines, increased disposition toward the manganese poisoning (150). Of all the above factors, alcoholism is mentioned more often, but Gayle showed, that the alcoholism does not play an important role in developing the poisoning. This excludes the fact, that an indirect damage of the liver was done by using alcohol.

It seems, that the females are less sensitive to manganese than the males.

Several more questions can be raised. Is there a process of an accomo-

dation to manganese? Is there an insensibility toward manganese? Is there any endogenous factor of disposition, or is it just an individual dosage of the received manganese? May be, the earlier authors omitted the fact, that all workers were not exposed equally. Wilbur thinks, that the manganese dust concentration is the critical factor, and that the number of poisonings is proportional to the manganese concentration in the dust. Flinn and others share the sare opinion. They were observing two groups of workers, one group was exposed to high manganese concentrations, and the number of the poisoned workers kept increasing as manganese concentrations increased. In the other group, which was exposed to low manganese concentrations, not one case of poisoning was detected.

We think, that we cannot give any additional contribution in determining disposition. It would be possible, if we could have determined or made equal, all exogenous factors for all patients, and then to analyze the endogenous factors individually. In closing, we can only conclude that nothing is known with certainty, about natural or developed disposition toward the manganese poisoning.

D. Symptomatology.

1. Effect on the central nervous system.

When we talk about the manganese poisoning, we think first about manganese effect on the nervous system. Manganese poisoning is a relatively rare phenomenom so there are not many authors who gave us a complete picture of this disease, on the basis of their own experience. Some cases were not investigated critically from the neurological standpoint. There is sometimes a disagreement, particularly concerning the interpretation of the anatomic-physiological symptoms. Never the less, the symptomatology of the manganese poisoning can be considered well known, and this disease is clinically well defined.

The clinical symptomatology of the manganese poisoning varied very much, but some symptoms appear with more or less regularity, and they are the nuclei of the clinical picture. That the manganese poisoning is accompanied with

characteristical symptoms is illustrated by the fact, that the crucial symptoms are described by the earliest authors (46,67,68,78,175,176,177,185). However, V.Jaksch, Embden and others in their time, evaluated the clinical results wrongly. They compared the manganese poisoning with the multiple-sclerosis, although they did not identify the manganese poisoning and the multiple-sclerosis. Later, it was realized that the main symptoms are similar to the ones of the Parkinson's syndrome, and some called this disease "manganese parkonsonism". As in the case of the parkonsonism etiology, we also have in this case, a series of the negative symptoms which are attributed to the lesion of the cerebral system,mainly of the hypothalamic nuclei of the autonomous nervous system. The clinical symptomatology, is not always strictly elective to the clinical results, do not give always "a clear picture", compared with the above mentioned syndrome. Besides the symptoms of the Parkinsons syndrome, there are other symptoms outside of the nervous system. The significance of these symptoms varied. Some of them give to the clinical picture a specific "coloration", which is very helpful in establishing a diagnosis. Other symptoms, so called "accessory are not so characteristic, as it is for example, in a pain in the lower back, which was mentioned already by Embden, and also, some of out patients complained about the same symptom. These uncharacteristical symptoms, which are not understood well, might be pure coincidental, or based on the very specific individual disposition. As an example, one of our patients (M.B.) had an epileptic symptom, besides the characteristical symptoms of the manganese poisoning. We can consider the epileptic symptoms as the specific disposition, but also there is some basis to connect this epilepsy with the manganese poisoning.

It is hard to relate the symptoms characteristics with the sensitivity of poisoning, or with the exposition time, or with the disease duration because of great individual differences in sensitivity toward manganese. The only fact, is that the symptoms intensities and their order of appearance can vary greatly. The beginning of the disease is hard to observe, and symptoms are very subjective. Among them most often mentioned: a general weakness, tiresomeness, sadness,

sleepiness, nervousness and apathy. These symptoms are called by one name, "general signs of the intoxication". They can be considered, as the initial encephalitis, and as the begining of the lesion of the central autonomous centers. The "general signs", are not always characteristical for the disease begining, and the disease can start with more characteristical abnormality of movements.

Some of our patients did not complain about "the general signs". It might be explained by the lack of self observation, due to the fact, that our patients were of low intellectual levels (most without any school education). The moment we had the opportunity to observe them, might have been too late to detect those general signs. For example, one patient denied any of the general signs, but from other sources we obtained information, that he was for some time an extremely slow moving person, and he fell asleep many times on the job. However, all of our patients complained about difficulties with legs and arms. The same symptoms were observed by many other authors. The difficulties in the legs, are stronger and come earlier than the ones in the arms. These are "the signs of the disturbed movements", which are the main and most important signs, in the symptomatology of the manganese poisoning. Difficulties in the legs are manifested first by the walk. Sometimes it is the first sign, but it is always one of the signs shown during the sickness. In the begining, it might be only a feeling of tiresomeness, and of "heavy legs", and a diminished ability to walk long distances. Obvious clumsiness going up or down the stairs. Sometimes from the very begining, the patients have a problem to keep themselves in balance. They stumble, and they can fall down in a forward or a backward direction. Difficulties with hands, usually come later, and they are not so intensive. Sometime they can appear simultaneously with the difficulties in the legs.

These difficulties can be in the begining of the subjective nature, but as the disease develops, they become the objectively observed symptoms. The patient walks slowly, he pulls his legs and does not lift them, in the knee or the hip joints. The steps become very short. Vondaksch described, a special degeneration

in walking which he called "the cock walk". This degeneration was observed only with some of the patients in the later stage of the disease. The feet and toes are contracted in a position of "equinovarus". The patient while walking, supports himself first by the metatarsusphalanx joint and with the outside edge of the feet. We observed such a case with one of our patients, on one foot. All these difficulties are the consequences of the muscular tonus ("the rigor"), and the hypokinesis. The rigor can be observed (felt), by performing the passive motions in the big joints of the limbs, on moving the patient's head very gently. We can feel continiously a resistance against the motion. If the tremor exists, then we can feel a broken rhythm of the motion ("the gear phenomenon"). The rigor can be intensive in both legs and arms. The rigor is usually stronger in the legs. Crouzon, Desoille and de Lisi found an increased tonus only with legs and with arms in the hypotonia. These symptoms are rarely manifested on the limbs of only one side, but it might occur. We observed with several of our patients, more or less asymmetry, but we always observed the symptoms on both sides. The rigor and the hypokinesis might not occur simultaneously. The same was experienced with the Parkinson's syndrome. Lyon and Caen, did not find an increased tonus with their patient. We did not find the extensive tonus by performing the passive motion although the symptoms were obvious. According to Loebe, this does not exclude the tonus in the vertical body position.

Other symptoms related to the rigor and to the hypokinesis would be the following. A patient keeps his body more or less stiffened and inclines the upper part forward. The natural elasticity of movements disappear when a patient turns around, he does it as if he were made of one block. The arm motions during walking is very poor and if the arms are affected by the rigor they stay motionless, tightly along the body. If the patient, while standing, inclines forward or backward too much, he looses his balance and he has to take several steps before he regains his balance or falls down (propulsion retropulsion). Flintzer thinks that the propulsion and retropulsion can be the early signs of the disease. The patients are not able to perform quickly antagonistical

movements, this so called "adiadochokinesis", was observed by many authors. Flintzer, underlines that the difficulties are greatest at the begining of a motion. It is particularly hard to make large movements. The patient's face is expressionless because of the limited mimic. (hypomimia, face appears like a mask). The movement of the eye balls are free, but the eye lids move less frequently. The speech becomes changed early during the disease. Our patient complained, that they cannot speak as loudly as they used to, and that they cannot always speak smoothly. The speech becomes quite slow, montonous and in an extreme case hard to understand, mumbled. The patients answer questions very shortly. While they speak, the lips and jaws move little. It seems, that there are a few variations of the speech difficulties. Hilpert (93) says, that the patients start a phrase normally and become non-understandable at the end. David and Huey gave somewhat different descriptions. The patient has difficulties to start speaking, then he stops speaking and then he speaks out in a series of words very fast. Very often, a patient repeats a syllable or a short word of the phrase begining (palilalia, manganese stuttering, psellismus mangalis, Embden (67)). The typical way of writing is that the letters are getting smaller and less readable as writting progresses, particularly when a tremor exists.

FIG. 2

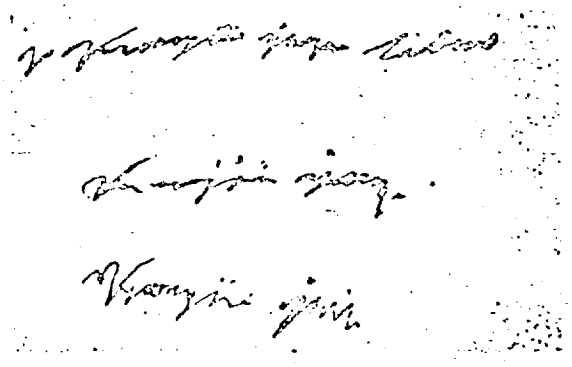


Fig. 2 A sample of the hand writting of one of the patients observed.

Difficulties in swallowing are observed by many authors (62,78,93,132). If we exclude the Vossov cases (181) of the bulbar paralysis and of the ameotrophic lateral sclerosis, all other cases can be considered as of the

extrapyramidal source. Because of difficulties in swallowing, the saliva is accumulating in the mouth and leaks from the mouth resembling the hypersalvation. Other changes in the area of the cranial nerves are not observed, except that Hilpert observed decreased convergency in two cases. Two of our patients complained of having double vision. Casamajor, described some difficulties in hearing in one case and Flitzer, the narrowing of the sight field. The vertigo is very rare, and was mentioned by Nosleim (132). The tremor is not as typical or regular, as with the postencephalitical parkonsonism or with the paralysis agitans. It can appear in a form typical for the paralysis agitans, as the rhythmic tremor in a motionless state. This form is mentioned by Flitzer, Muller and Christaens (135). More often the tremor is "a typical one", then it appears as a fine tremor in motion, or when the limbs are stretched out (64,65, 80). The tremor can be local or general, and can appear only on one side of the body or on both sides. Sometimes, the tremor occurs on the tongue, on the eyebrows and on the lips. The intentional tremor does not occur. The tremor is intensified when a person is tired or psychically excited. Flintzer, observed the tremor in the early symptoms, but it can appear anytime during the disease duration. Also, the tremor can disappear during sickness, as it was observed by Hilpert in his two cases (93). We observed the tremor with several of our patients, but it was not very intensive. Tara (1948), did not observe the tremor in his case.

As a rule, the manganese poisoning does not cause an atrophy. Couper talks about a paraplegia and Embden mentioned a paresis, but their analysis of the symptoms are not satisfactory. Flitzer says, that in the case of heavy poisoning, a body gets weaker, loosing muscular strength. It is not a characteristic symptom to point to the paresis, because the similar symptoms can appear with the parkinson syndrome.

The signs of lesion of the pyramidal tract, are first mentioned by Embden and VonJaksch, and after them by a majority of those who dealt with the mangnaese poisoning. These signs do not appear with all patients, and they are manifested

by increased reflexes, by the occurrence of a clonus, and by the phenomenon, Babinsky. A change of the stomach reflexes are not mentioned often, and the other pyramidal symptoms are not mentioned at all. The pyramidal signs are usually found on the lower extremities. If the reflexes are somewhat stronger, it should not be immediately taken as a lesion of the pyramidal tract, since this symptom is common for the Parkinson's syndrome. The strong paresis does not occur and a light paresis, or a slight increased tonus (spasm), can be covered, the extrapyramidal symptomatology. We found discrete symptoms of the pyramidal tract lesion on one patient. When these symptoms are combined with the extrapyramidal symptomatology, they give specifics to the manganese poisoning picture. Hilpert (93), thinks that the pyramidal tract lesion can occur only at the very late stage of the disease.

Reflexes can be normal, but also in a lesser number of cases they were weakened or even completely omitted (44,135). Sometimes asymmetrical reflexes can be observed.

A lesion of the peripherial neuromotorial system does not happen with the manganese posioning. Only Baader found (55) in one case a degenerative electric reaction of the fibular muscles. Salmon and Planque published about four cases, with symptoms of the polyeuritides. Probably in question, was a combined intoxication with both manganese and arsenic. Wilkinson from Hong Kong, also found some symptoms of the polyeuritides, but he believed that it was caused by the beri-beri diseases, which was numerous amoung workers.

The loss of sensitivity is rare and less impotant. Among the painful phenomena, headache is mentioned only in a few cases (12,93). Our patients did not complain about the headache by themselves, but when asked, they were describing slight pain sensation in the head. The hip-ache (lower back), was described first by Erbden, but little attention was paid to it by the other authors. Our patients complained many times of the unpleasant, almost painfull sensation of a heavy load in the lower back. Gayle, talks about a neuritial pain in the upper extremities, and Baader about the lower extremities. Baader also described, the

symptoms of the paresthesia. Schwarz (160), described the patient with the hemihypaesthesia, and with a pain in one arm. DeLisi commenting, this patient thinks it might have been a case of the psychoneurotical obstruction of a sensitivity. Cohen, tried to prove an existence of the lesion of the spinal sensitivity.

The painful cramps, are the regular symptom of the manganese poisoning. We observed them with a majority of our patients, partly with the first signs of the poisoning. The cramps occur very often, and they can effect healthy persons under sub-physiological circumstances as are cold, heat and tiresomeness. They can be associated with many other diseases, like diabetes, muscular abnormalities and the affected peripherial or central nervous system. The cramps are also described in connection with the Parkinson's syndrome. Thus, the cramps cannot be considered as the specific symptom of the manganese poisoning. However, the cramps combined with other symptoms can be helpfull to make a diagnosis, because some form of the Parkinson's syndrome (the second etiology) is associated with much less cramps. The cramps, occur most often in the legs, and sometimes in the arms or other muscles. They strike during day time or during the night, and sometimes occur several times daily. Some patients complained, that they could not sleep because of the cramps frequency during the night.

The symptoms of the damaged cereberal system, have not been proven with full certaintly. Some authors mentioned them (45,55,116) and Bogaert and Dallamagne (25), had caused the cereberal ataxia with one monkey by an experimental manganese poisoning. We did not observe the symptoms of the cereberal lesion on our patients, but a possibility of cereberal lesions cannot be rejected completely. It is possible, that discrete cereberal symptoms are covered by other more intensive symptoms. The occurences, related to the lesion of the autonomous and particularly of the diencephalic centers, are numerous. The symptom mentioned most often, is the disturbed sleep (82,93). Some patients are affected by sleepiness, while others, by insomnia. The above disturbances were rather rare and mild with out patients.

Very often in literature are mentioned the symptoms of the sexual impotence, or of the diminished sexual potence (14,67,180). Some authors consider this symptom of a pathognomonic value (12,78,131), and Hilpert, classifies this symptom in the group of a possible early sign. Some think that a sexual impotence observed with welders, if it exists at all, should be attributed to the manganese effect (18). Not all authors consider a sexual impotence as an important symptom. Some think it is of small importance (Sourate 166). We did not observe this symptom in an extensive form, but we should not forget that patients incline to hide their sexual impotence. Many other symptoms related to the vegetative nervous system are mentioned: increased function of the fat glands; hypersalivation; increases profuse sweating; then not so often, an occurrence of edemas and cyanosis; a face redness or paleness; an increased dermographism; loosing hair or hair getting gray; loosing teeth; abnormal hungriness or thirstiness; vomiting etc. One of our patients felt a dizziness when changing the body position, similarly to the symptom observed with the postrauma tic encephalitis.

The paroxysmal symptoms are very rare. The epileptic attack affected one of our patients. Also Hilder mentioned, a 69 year old patient with epilepsy. The attack similar to ones of the narcolepsy (loosing tonus), was observed by Baader and Hilpert. One of Baader's patients had a very peculiar attack. During the attack, one arm was shaking and became cold, and cyanotical, while the head was twisted backwards, because of the cramp. The attack usually ended with a very intensive arm tremor. Among the rare paroxysmal symptoms, we can mention the vertigo (82). The psychical disorders usually appear in a mild form. They are considered as significant symptoms of the manganese poisoning, because they are observed in some form with a majority of the patients. They can appear at the initial disease's start and are manifested by neurotic or mental effects. These symptoms are: tiredness, weakness, sleepiness, disturbance and angriness. More often than angriness, is a case of apathy and loss of willingness. These symptoms are often observed by the patient's friends or relatives, but are not felt by the patient himself. These symptoms resemble the begining of mental

illness, as described by Baader (12). Among more frequent and characteristical symptoms, can be included an uncontrollable weeping or laughing. The uncontrollable laugh is more frequent, and is attributed to an euphoric feeling, which these patients often posses. The patient cannot control his emotion and he starts laughing for the smallest reason. One of our patients said; "I cannot get angry any more. Even when I spank my son, I laugh". In case we had a group of patients at the hospital at the same time, an epidemic laugh usually occured among them. It was enough that only one started laughing. One of our patients showed both an uncontrollable laugh and weeping.

There is not conceivable proof that the manganese poisoning can damage intellectual functions. Not many real psychotic cases are registered. Baader (9) described one case in Egypt, where patients showed to be in some state of confusion. One from the same group, showed strong symptoms of Kleptomania. Baader obtained the above data from Egyptian doctors. Ansola, Uiberal and Escudero reported in Chili, 34 cases with psychical changes among a group of 64 patients.

The psychotic disturbances occur particularly in the early disease's stage. Some showed abruptly an affective excitement, the other showed euphoria or ecstasy. The patients recovered in a two to three month period. The presentation of the above authors is considerably different from the other authors opinion about the psychotic symptoms related to the manganese poisoning.

The neurotical reaction toward manganese, was described first by V.Jaksch. Of course, there is a possibility for the neurotical reaction, as it usually occurs everywhere, when a larger group work with substances known to be toxic. We believe that there is not anything specific in it, relating only to manganese. So the V.Jaksch's term "manganophobia" seems to be not adequate.

The cerebrospinal fluid can show the normal results, or slight deviation from normal, particularly regarding the albumin amount and composition. This can cause changes of the collargol reactions. The analysis of results and findings, and a diagnostic value of these results are discussed by some authors (72,77,135). They found the cell number normal. The globin amount was normal or

slightly increased.

The mastic test was normal and the gold reaction showed changed in the middle zone. The authors did not analyze differential diagnostic values of their results with ones particularly obtainable with the cerebrospinal syphilis or with the multiple sclerosis. The multiple sclerosis can give a "paralytic type" of the gold reaction, but it is not a regular result associated with this disease. BRYAN analyzed the fluid on a pathological amount of manganese, and he found sevenfold increased amounts - compared with the normal cased.

We examined the cerebrospinal fluid of eight of our patients. Results are presented in table six, they show that three patients had normal results. Only one patient had an increased number of cells - 26/3 of the small mono-nucleoral cells. The PANDY reaction was positive with two patients and one with three additional patients. One patient had a total albumin slightly increased. Five patients had to some extent changed the collargol readings and these changes varied differently.

TABLE VI

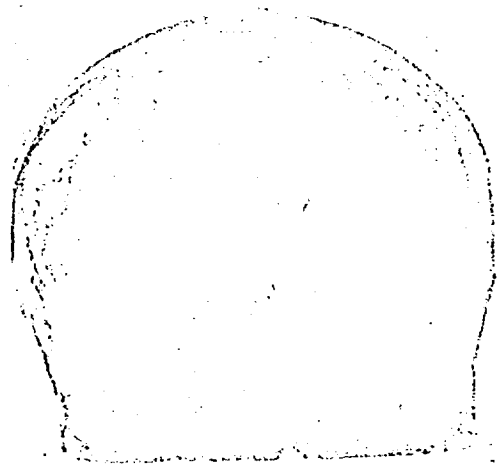
Results of the cerebrospinal liquor of our patients

(eight cases - s.l. abd E.B. are not included)

1 Pretraga	S. J.	S. M.	L. M.	P. I.	M. S.	B. M.	B. J.	V. J.
2 Pandy	±	sl. opal	±	+	opal	±	opal	+
3 Stanice	26/3	4/3	6/3	2/3	2/3	2/3	5/3	8/3
4 Brandberg	0,18%		0,16%	0,25%		0,16%		0,16%
5 Kolargol	1111322444	1111444444			1111424444	1111422444	1111444444	1144434444
6 Mastiks			0000000000	0223100000				
7 WaR	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.

1. Name Initials 2. Subject 3. PANDY 3. Cells 4. BRANDBERG 5. Collargol 6. Mastic 7. WAR

The pneumoencephalograph can show partly the extent of the cerebrae lesion. Because this method is not considered important from the diagnostic point of view, there is not much about this method in literature. We performed the pneumoencephalogram on two patients. One patient showed normal results while the other had the lateral chamber expanded and the forebrain arachnoid were widened diffusely resembling the modest case of the brain atrophy.



Sl. 3. Pneumoencefalogram kod jednog bolesnika

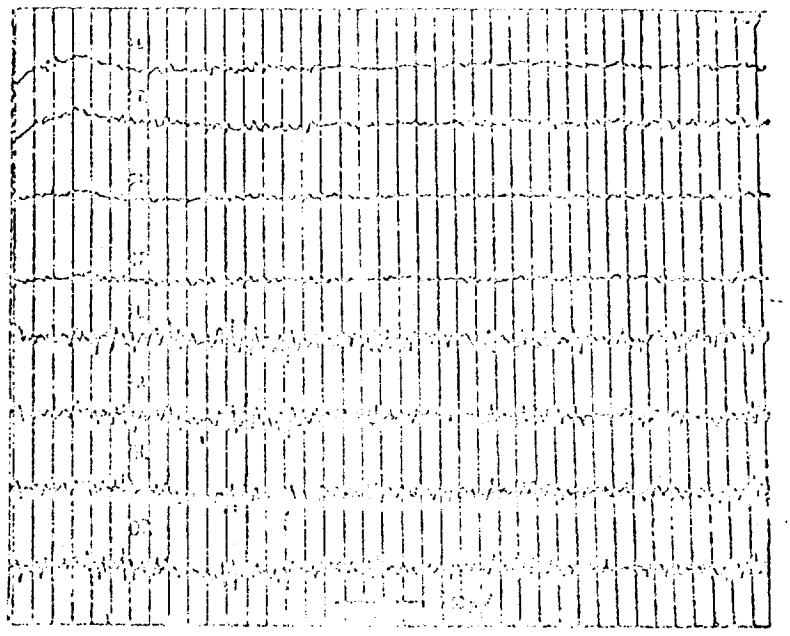
FIG. 3 Pneumoecephalograph of one of our patients.

The electroencelographia was not used on the patients of the manganese poisoning, except by CRITCHLEY. We performed it on six patients. The pathological results were insignificant. No significant activity was observed except of the basic rythm alpha (OCTYPITHAL RYTHM?). One patient showed absence at any rythmic activity during both normal and deep breathing. One patient showed abnormality during the normal breathing, consisting of the periodic activity 4-7 c/s together with the rythm alpha and with the activity 14-20 c/s of low or moderate and even some lighter amplitude. During deep breathing, there occurred activation particularly at the slow activity.

FIG. 4

Electroencelograph of one of our patients

A. Normal breathing

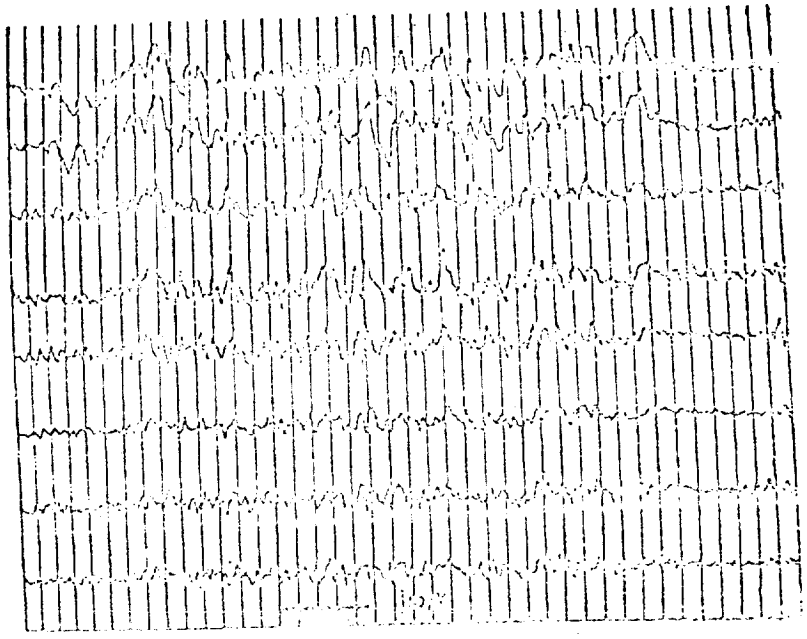


Sl. 4. Elektroencefalogram jednog bolesnika
A. za vreme mirnog disanja

FIG. 4

Electroencelograph of one of our patients

B. Hyperventilation (deep breathing)



Sl. 4. Elektroencefalogram jednog [illegible]
B. za vrijeme hiperventilacije

A professional chromical manganese poisoning does not cause deadly lesions. Because of that, the pathological - anatomic results are little known to date. An autopsy was performed only on a few cases. The first such autopsy of the manganese poisoned person was performed and described by CASAMAJOR. He specially pointed to the widened perivatenlar tissues in the nucleus cularis (caudatus and in the lateral part of the talamus. He also mentioned the liver lesion (ciliar cirrhosis and a accumulation of pigments).

ASHIZAWA described in more detail the pathological - anatomic results of one manganese poisoned person who died of tuberculosis (spondylitis). He found the most degenerative changes on the ganglion cells in the nuculous pallidus and in the nucleous candatus. The number of the ganglion cells in the nucleus pallidus was significantly reduced. Part of the tibies in those regions was damaged. The degenerative changes on other parts of the nervous

system were less distinctive, and for some of them, ACHIZAWA thinks, might have been caused by tuberculosis.

Only light liver damage was found and one author attributes this rather to tuberculosis than to the manganese poisoning. CANAVAN, COBB and DRINKER had performed an autopsy on the 69 - year worker which got sick of the manganese poisoning 15 years before his death and who did not work with manganese for the last 14 years. He died because of the heart trouble. The autopsy was performed on the brain only. An atrophy of the vertex and lateral side were observed microscopically. The lateral chambers of the troutal segments were expanded and the casal ganglions contracted. Some light diffuse changes of the brain core cells were detected. No damage was found of cells of the medulla oblogahta, and only light damage of the PURKIN'S cells in the cerebellar cortex and in nucleus dentatus. The most significant pathological changes were found with the nucleus cavdatus, putamen, nucleus globus pallidus and talamus manifested by degeneration of the ganglious' cells and tatelitosas. Measurement of the Casal ganglious showed that they are reduced in in like much more than expected to occur just because of aging.

The case interesting from the histological point of view of the manganese poisoning was described by STADLER in 1935. The patient got sick whem he was 46 years old and he died in the 56th year of coronary thrombosis. The microscopical findings of the brain were normal, but a microscopical investigation discovered changes on the ganglious' cells and of the mesenchyme, caused by the inflamed centres of a vascular origin. The most intensive changes were in the striatum and pallium. The less intensive changes were found on the cortex. Very small changes were found in the thalamas and the cerebellum. According to the author, because of very diffuse changes it is hard to make conclusions where the process is localized. It can be said only that the situationis striked most, that the nucleus pallidus is changed and that the

supstantia nigra was not significantly affected.

The pathological - anatomic results are not conclusive (25, 182). Two out of three patients died in old age when degenerative and vascular changes are a normal occurence. The old age changes usually occur with the Casal ganglious and particularly in the pallium. The ASHIZAWA patient was sick of tuberculosis which also could have caused the vascular damage of the cerebral substances.

Some more confusions are brought up by two cases described by VOSS (181) where pathological - anatomic results are given by HALLEN VARDEN. The first one had a clinical diagnosis of the amyotrophic lateral sclerosis. There were no symptoms of the manganese poisonings although the patient had been working with manganese. The autopsy showed results expected for the amyotrophic sclerosis and of the bulbar paralysis. Also were detected the cells rarefaction in the neostriatum, and some changes in the hypothalamus.

The second VOSS's patient had a typical clinical result of the manganese poisoning but he died of pneumonia. The autopsy showed no histological changes o.u., the Casal ganglions, some changes were found in the cerebellum. The most marked changes were found in the medulla spinalis and in the peripheral nerves. In the medulla spinalia were found the unilateral degeneration of the pyramidal tract. In both n.n. ischiadia were found the degenerative lesion.

Too, the pathological - anatomic picture of the manganese poisoning stays unexplained, and specific lesions are not localized conclusively. The clinical treatment is still based more on indirect symptoms and results, than on the knowledge obtained by an anatomic investigation.

Thus, an effort was made to solve the problem by experimenting with

with animals. First, it was tried to find out if there is a strict correlation between a definite symptoms and a location of the pathologicd process. In other words and more specifically it was tried to find out if there is a selective affinity of manganese toward the ganglion's cells and fibers.

Many of the earlier tests failed to cause neurological symptoms with animals. The tests performed by V. JANSCHA (1907) with dogs and by CASAMAJORA (1912) with rabbits were unsuccessful. CASAMAJOR thought that initial neurological symptoms are harder to observe with animals than with humans. (for example an abnormal walk or a face expression)LEMCS succeded to accumulate manganese dioxide in the animals' brain by both peroral and respiratory way, but he did not obtain any of neurological symptoms.

HANDOWSKI and others claim that it is possible to provoke with manganese as with other heavy metals, an acute poisoning. "The acute poisoning causes the ascending paralysis of the central nervous system". According to them, the acute poisoning occurs only with a high mangenese concentrations in blood and if it is not obtained, no neurological disorders occur.

LEWY and TEIPENBACH obtained the neurological symtoms with rabbits where food was mixed with pyrolusite. After two or three months, about half of the animals showed an increased tonus of the hind legs, in addition to general weakness abd weight loss. The anatomic changes of the brain were varied. The most damaged was the nucelus caudatus and the nucleus lentiformis, and less damage was found in the brain core, in the cornu Ammonis and in the frontal part of the corpora quadrigemina, great interest was shown for the experiments performed by MELLA (1924) with monkeys Macaccus rhesus. He was poisoning the animals for 18 months by the intraperitoneal injection of manganese chloride. He first observed strange moves in the animals, later the musculature rigidness, then an appearance of a light

tremor in the extremities and finally the contraction of hands. The histological changes of the cortex were light (chromatolysis and vacuolation of the nervous cells). The changes were more marked in the nucleus caudatus. The cells were contracted, many lost their nuclei, sometimes it was observed neuronophagy and other degenerative changes. The fibers were also changed. Similar changes were observed in the glocus pallidus, while findings in the cerebellum were basically negative. In two cases were found changes on the liver, similar to one for an acute Iepatitis and also the imflamation of tissue. MELLA did not find changes on other organs, to be compared with the manganese poisoning of his animals with pathological changes occuring with the WILSON disease. The chemical analysis of various organs showed that manganese concentrations in brains were 10 times, and in liver up to 15 times higher with the poisoned animals compared with the control healthy animals.

GRUNSTEIN and POPOVA published later (in 1922) their results with rabbits. They treated their animals with 2.3 grams of manganese compound daily mixed with food, during a period of six to 16 months.

We haven't mentioned yet, the more recent experimental investigation performed by ROGAERT and DALIEMAGUE (24, 25) in 1943 and 1945. They also tested with monkeys MACACCUS rhesus. The one monkey was exposed to the time dispersed manganese ore dust containing 50% manganese dioxide, for one hour daily during 100 days period. (The intoxication conditions very close to ones in industrial practice). The other monkey was given food containing manganese sulfate; about 10 - 15 mgr. daily for 300 days up to the total manganese dosage of 8.2 grams. The first animal showed neurological cereberall symptoms with small dosages, while the other monkey did not show any sign of poisoning even six months after the experiment was completed in spite the high dosage of manganese sulfate he received. It is interesting to point out, that the chemical analysis of verious organs of the animal

poisoned through the respiratory thract, did not detect any manganese. The histological analysis showed the cerebellum atrophy while the globus pallidus was not affected at all. Also, neither the liver, nor lungs, nor kidneys nor cardivascular system showed photological changes.

As we see, the experimental results are very often contradictory, and can not help much in understanding the clinical aspects of pathophysiology and pathology of the manganese poisoning with humans. Reading the published works, we have feeling that many experiments were performed with some kind of a theoretical prejudice, trying to initiate or create a specific lesion of the extrapyramidal tract, or of liver, or in blood etc. Most of the results were not confirmed by more than one author, so we consider the experimental work of managnese poisoning far to be completed.

The manganese poisoning is a chronical disease, although a full development can occur very fast after appearance of first symptoms. An acute professional poisoning is not known.

There were described a light poisoning where the symptoms disappeared. However, the disease prognosis should be considered with a great caution, because the disease can develope even if a person is not in contact with manganese any more. BAADER, claims that this disease never regrets once it was developed, even after a contact with manganese ended. Similar opinion is shaped with many other authors. MULLER and TISSIÉ described three workers who discontinued work with manganese in 1932 - 1935. These three workers did not have at that time or in earlier periods any objective or subjective signs of the manganese effect. Four years later one had the first light symptoms, the other had fully developed symptoms and the third one did not show any objective signs. Ten years later all three were cheched again. This time, the conditions became worse with two of them and now the

third one showed all symptoms of the manganese poisoning.

It is not explained this post-contact effect of manganese but similar effects are observed with other diseases of the central nervous system. How long manganese can stay within the body is not determined, but known is a case with manganese excretion after seven years since work with manganese ended. HUILER and TISSIÉ do not believe that manganes can have any direct effect after several years since the exposition time. The probable explanation might be that the effected tissue undergoes some sort of a secondary exogenous damage, or within it is initiated a local premature aging, similar to abiotrophy observed with some depenerative disease of the central nervous system. Sometimes some of the symptoms can disappear or get weaker. These symptoms usually are an uncontrolable laugh, motorial disorders and tremor and etc. (72, 93) while the increased muscular tonus does not disappear. This is unfortunate because this symptom is considered one of the worst, having in mind its consequences.

The clinical picture of the manganese poisoning was so developed that its diagnosis should not be a problem. However, we can find in literature, that some patients of manganese poisoning were treated under others diagnosis. The only reason is that many physicians do not pay attention to a working anammesis.

A diagnostic misinterpretation can happen in the beginning of the disease when symptology is still very poor. We had experience with several workers who gave us the typical anamnestic data such as tiredness, movement disorders, cramps, sleepiness difficulities, sexual impotency etc., but we could not observe any of the objective symptoms. A physician's responsibility is great so he has to remove the worker from his job as a first step. Also, there is a possibility of only a neurotic reaction or simulation or aggravation,

because the anemnestic symptons can be learned among workmen. T. and E. RODIER call such cases, with some functional disturbances but with no objective symptoms, "the premanganesism".

The manganese poisoning diagnosis should be based on the work onamnesis and the clinical observation. Relatively a small importance is given to manganese occurence in blood or in the excretes. (72). On one side, many patients come to doctors' attention when it is too late to detectan increased manganese concentration. On the other side, when increased manganese concentrations is found, it does not mean as a proof of intoxication rather that the person was in contact with manganese. The intoxication, as we already mentioned, depends greatly on an individual disposition.

The diagnostic difficulties might occur when only one case in a group appears and symptoms are not typical. The problem is much easier if a group appears with similar symptoms from the same working area.

It is natural that an effort is always made to establish an early diagnosis. At this early time, we are usually lacking a complete clinical picture. If we are faced with only subjective complain~~ments~~ts without objective symptoms then a regular check-up should be established. The first objective symptoms can vary greatly (psychical disorders, loss of face expression, speach difficulties, movements disturbances, tremor, retropulsion, cramps etc.). SCHWARTZ (160, 161) recommended a blood picture control, because an increased globulin can be considered as one of the early signs. Some other authors including BAADER also give some importance to the hyperglobulin in blood but usefulness of this symptom is not really proved in a practice.

The differential diagnosis gives good results when the disease is in a developed stage. Earlier it was emphasized similarity of the manganese poisoning

with the multiple sclerosis, but we think that the similarity is very small except in rare cases when the latter appear with stronger pyramidal symptoms.

The diapustlic difficulties can exist in connection with the extra-pyramidal affection of the PARKINSON'S syndrome. Too the manganese poisoning can be similar to the WILSON type of the Iepatolenticular degeneration. However, this is a very rare disease and coincidence with exposition to manganese is little probable. Besides differences in an anamnesis, the manganese poisoning is not accompanied neither with the liver cirrhosis or with a very characteristic KAYSER - FLEISCHER ring in the eyes. Sometimes the manganese poisoning is mixed with the paralysis agitans c.g. with the postencephalitic parkonisomism.

The clinical observation will help to differ the manganese poisoning from the extrapyramidal effects. The uncontrollable laugh or weeping, sexual impatience, muscular cramps, light pyramidal symptoms, the so called "cock - walk" give a specific coloration to the manganese poisoning. On the other side the manganese poisoning does not show some of the characteristical symptoms of the postencephalitical parkinsonism. Such as are oculsmstor muscular descriptions. Sometimes, one should expect that a clinical picture of the manganese poisoning might not be complectely clear. In that case an anamnesis will be of a great help. The most important is the job anamnesis. Also important, the family anamnesis, because for example the paralysis agitans is a hereditary disease in some cases. Then it is very important to exclude any acute encephalitic symptoms from the anamnesis, but it is not always easy, because they can be very uncharacteristical. There are some other professional intoxications which can give an impression of the PARKINSON'S syndrome. Carbon nonoxide can be present in many industrial plants. However the carbon monoxide intoxication does not cause a chronical symptom of the parkinsonism, rather

acute once what is relatively easy to establish by means of an anamnesis check - up. We have excluded a possibility of the carbon monoxide intoxication in our plant. The furnace smoke contained only 0.1% carbon monoxide so that the job atmosphere could have contained much less than a tolerable amount of corbon dioxide. Someone put a question, whether a presence of carbonmonoxide increases a disposition toward the manganese poisoning. This assumption has not been proven yet. Very rarely the intoxication with carbon disulfide can give a clinical picture of the parkinsonism. In our case there was no such possibility.

Finally, we must not forget a possibility of the combined intoxication with more than one poisoning substances. SALLON and BLANQUE described some cases which might have been the combined poisoning with manganese and arsenic. WILKINSON'S patient showed besides symptoms of ~~the~~ a difficitary deficiency of polyneuritides. So, when we are faced with "an atypical" clinical picture some other poisoning substances should be considered and investigated.

The manganese poisoning therapy is unfortunately still within limits of symptological attempts: there is not an etiological therapy. The earlier authors used to recommend an hydrotherapy, an electrotherapy, potassium 10 date, sodiumthiosulfate but without much effect. CHARIES believed that the liver therapy can be useful but noone except him, obtained any improvement by this therapy. We tried a therapy with "BAL" (2,3 - dimercaptophenol). We treated seven patients following dosages suggested by THOMPSON for the treatment of Hepatolenticular? degeneration. We used "BAL" because it showed good results in cases of poisoning with some other heavy metals. Later we found in literature that the " BAL" treatment showed good results also. In one case of the manganese poisoning manifested by some skin changes (42).

1) The application schere of BAL by THOMPSON

1st	day	4	ml	of	5%	solution	subcutaneously
2nd	"	4	ml	of	5%	"	"
3rd	"	4	ml	of	5%	"	"
4th	"	4	ml	of	5%	"	"
5th	"	4	ml	of	5%	"	"
6th	"	4	ml	of	5%	"	"

The above dosage we have accepted because both hepatolenticular degeneration and ~~the~~ manganese poisoning affect the nervous system chronically. Such a therapy should be repeated up to four times with an interruption period of five to 15 days. We changed the THOMPSON application in such a way that the "BAL" oil - solution was injected intramuscular rather than subcutaneously. One day dosage was split on 2 - 4 individual injections. Some subjective improvements were observed with our patients with the above therapy, but not an objective therapeutical effect. One patient was treated with PAPARNIT without any effect.

In one case we tried pyrodoxine but also without any effect.

BAADER (10) discovered that atropine and scopolamine can have some effect with patients having a stronger rigor.

A physical therapy and rehabilitation techniques can be used in some cases.

2) MANGANESE EFFECT ON THE LIVER

In literature, particularly in one of the earlier dates, often is mentioned as a possibility of a toxic effect of manganese on the liver.

This assumption is based on experimental results with animals and not on the clinical investigation. WICHERT (103) already found in 1860 that

manganese is excreted by means of the bile fluid. The first experiments with animals showed that manganese deposits firstly in the liver. KOBERT, proved in 1883, after having injected manganese citrate into rabbits subcutaneously, that the liver and the kidneys contained the highest manganese concentrations. These findings were confirmed by other authors (32,87, 118) so it was accepted as a fact, that manganese accumulates in the liver and then is excreted by means of the bile fluid and feces.

Manganese is not only deposited in the liver, it also performs some affects. In the first place manganese effects the KUPFER cells (86). It was observed that the antitoxin concentration in the serum of horses immunized against diphtheria is increased by the manganese effect. It was also explained by WALBURN (86) as the manganese effect on the KUPFER cells in the liver. An ability to induce an antibody formation was used to explain some therapeutical effect of manganese in cases of some injection. COUPER mentioned GMELIN'S observation that manganese can cause the liver inflammation in dogs and rabbits. KOBERT found that small dosages of manganese citrate injected subcutaneously can cause vomiting and hepatitis, and later most animals developed the parenchymatous nephritis and partial or complete liver cirrhosis. FINDLAY proved that the manganese chloride subcutaneously injected into rabbits, rats and guineapigs is very toxic for the liver and kidney parenchyma. The liver becomes very expanded, deteriorated, pale yellow with reddish dots, fatty greasy; the KUPFER CELLS swell and die. On the above animals a repeat used of small dosages showed to be particularly toxic. Also it can be developed "a biliary type" of the liver cirrbosis with no fatty greasy degeneration. Observation made by HANDOWSKI, GRÜNSTEIN and POPOV with rabbits, by MATSUMURE with rabbits and dogs (123) and by MELLE with monkeys are in a complete agreement concerning the above findings.

So, by experimental means it was proved that manganese can have a hepatoxic

effect.

Because of the above experience obtained with animals, it was thought that manganese has similar effect with humans. This was particularly accepted after the similarity between the manganese poisoning and the hepatolenticular degeneration was discovered. Experiments were made (94) on animals to learn wether manganese has an etiological role in causing the hepatolenticular degeneration.

The idea was that the cerebral symptoms can be caused indirectly by a damaged liver. Several possibilities were considered:

(a) that the damaged liver retains some dangerous products of metabolism which otherwise would be destroyed or excreted by the undamaged liver;

(b) that the damaged liver can not prevent some exogenous toxic substances to enter blood stream;

(c) that the damaged liver by itself produces some toxic substances as result of an abnormal metabolism in the damaged liver cells;

CHARLES supposed that a normal liver excretes some hormones to protect the central nervous system against endogenous toxins. Accordingly, it was believed that deficiency of this hormone is a common cause for both the hepatolenticular degeneration and the manganese poisoning.

The experimental results of the manganese poisonings by the peroral application looked so convincing that some preffered to consider degenerative changes on the liver as the symptom which appears before pathological changes of the central nervous system (17, 178). So, some recommended a functional test of the liver when manganese poisoning is suspected (17, 110, 178). Also, it was considered (178) that the early functional liver disorders are first signs of manganese poisoning. Even more, some authors thought

that manganese poisoning can be cured by the liver therapy (40).

In connection with manganese poisoning very often were mentioned a disturbed metabolism of carbohydrates. V. TAKOCH already observed a temporary alimentary glycosuria. Recommended tests for levulose and galactose showed an abnormal deviation in some cases of the clinically observed manganese poisoning (17,110, 189).

However, an opposite opinion appeared later. An autopsy of one case of the hepatolenticular degeneration did not show presence of any manganese in the tissues (148). Also, it was not possible to prove any connection between the manganese poisoning poisoning and the hepatolenticular degeneration by experimenting with animals (94). So, the idea of a hepatocerebral lesion with manganese poisoning is abandoned. The rare autopsy results of persons poisoned with manganese do not agree concerning damages observed on the liver.

CASAMAZOR found "a ciliary cirrhosis in a significant extent" and the liver cells with a lot of pigments containing iron. But ACHIZAWA said for his case, that he did not find any marked changes on the liver. Also SEELER did not observe the liver damage with one case of a clinical manganese poisoning. His levulose test was normal. Neither American cases in 1940 (77) or many other cases (138, 150, 186) showed that the liver was damaged. Finally, nobody except CHARLES observed any improvement of manganese poisoning symptoms by means of the liver therapy.

The most recent experiments, particularly ones performed by BOGAERT and DALLEMAGNE did not even show any toxic changes on a liver in animals. It is interesting to point out that experiments with rats (4) showed that manganese has a lipotropic effect similar to choline promoting better utilization of fats. The animals whose food contained manganese had less fat in the

liver than the others fed without manganese.

We also investigated the liver functions with our patients. As results in table 7 shows, all patients had normal results. Also, the galactose test, suggested by BAUER as a very indicative for maganese effect on a liver, showed normal results. The excreted valves are lower in the limit of 3 grams. Even more, the liver biopsy performed with five patients did not show any changes of their livers.

Table 7

Results of the liver functional tests and biopsy of our patients.

1 Proces	2 S. I.	2 S. M.	2 L. M.	2 P. I.	2 M. S.	2 B. M.	2 S. I.	2 B. J.	2 V. J.	2 E. B.
Bilirubin mg %	0,90	0,80	0,64	1,07	0,58	0,58	0,96	1,01	1,07	0,64
3 Kadmijeva reakcija	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	—	—
4 Timol — 5 Mutnoć	1	2	1	1	1	1	1	1	4	4
4 Timol — 6 Flokulacija	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.
Weltmannova koagulacijska zona	7	7	7½	8	7	7	6½	7	—	—
7 Alkalna fosfataza, jedinica (po Kingu)	3,7	5,8	3,7	3,7	3,94	4,5	2,2	5,15	1,75 (B.J)	—
8 Test tolerancije galaktoze (izlučeni grami)	1,25	—	0,63	1,32	1,05	0,75	0,0	0,19	—	—
9 Izlučivanje hipurne kiseline (u gramima)	0,96	—	—	0,55	0,32	—	0,93	0,31	—	—
10 Biopsija jetre	neg.	neg.	—	—	neg.	neg.	—	—	—	pigm.
11										

1. subject 2. patients' initials 3. cadmium reaction 4. Thymoe 5. Turbidity 6. Flocculation 7. Coagulation zone 8. Alkaline phosphatase King's unit 9. Galactose test excreated am. 10. Excretion of hippuric acid (grams) 11. Liver biopsy

There is no data in literature about the liver biopsy. We consider this method very useful for non - lethal cases. A histological liver test "intra vitam" can clarify the real cause of death (a basic disease or a disease which in fact caused death) which is sometimes hard to determine by an autopsy. Our negative results speak against the theory of accumulation

between the liver degeneration changes and the cerebral symptoms of the manganese poisoning. We also think, that one should have in mind the experimental results with animals until a future investigation clarifies discrepancies between those results and clinical observations.

3) MANGANESE EFFECT ON BLOOD

Not much is known about manganese effect on blood. The first assumption is that manganese, as an element which is of some physiological importance, will also play some role in the Hematopoietic system. BUISSON already thought in 1862 (53) that manganese is one of the basic constituents of erythrocetes and he recommended manganese for curing anemia. But, CAHN proved in 1884 that manganese does not cross from blood stream to blood cells. BERTRAND and MEDIGRECEAN found that blood plasma contained twice as much manganese as blood cells. CERVELLO and BARABINI claimed in 1893 (86) that manganese has a hematogenous effect which is twice weaker than one of iron. According to some more recent investigation (178) it might be concluded that manganese helps the erythrocyte's performance and "can replace iron in that function" (120). We will not discuss this assumption further but we will emphasize the fact that manganese is physiologically present in the human blood. According to REIMAN and MINOT there are four to 25 gamma of manganese per 100 grams of blood with an average being 12 gamma per100 grams. According to ALBRITTON'S "The Standard Blood Values", (1952), the manganese amount ia 0 ro 25 gamma for total blood (average 13 gamma), for only erythrocyte up to 48 gamma (average 19 gamma) for plasma only up to 19 gamma (average value 6 gamma). For rats the average value for total blood is 17 gamma. From the toxicological standpoint of view it should be expected that manganese, as a typical poisonous element, will cause more or less pathological effects on blood. Searching literature, we were surprised

how little attention was paid to an investigation of blood affected by manganese compounds. One of the first observations was made in 1921 with two cases of manganese poisoning occured in steel production by VASSERMER'S method. In those cases hyperglobulia was observed. The erythrocyte number was 5.6 and 6.4 million respectively. But, these abnormalities were attributed in both cases "to mild effect of carbon monoxide"not to manganese. SCHWARZ and PAGEL published in 1923 results of their investigation about the effect of manganese dioxide on cats. They used "the gray rock" fromTHURINGIA, which was given to the cats either by a peroral way with food or by a intraheal insulation. During the first stage of poisoning, the authors observed an increase of both hemoglobulin and number of erythrocytes. After a few weeks of testing, the erythrocyte number decreased to about 50 percent of a normal one and hemoglobulin was also reduced. They could have caused the fatal anemia by using a pure manganese dioxide. The similar effects were observed by CASSO in 1932. He was determining glutamine in blood of the poisoned animals and he concluded that manganese compounds first stimulate and then inhibite erythropoiesis. SCHWARZ in 1932 found in one case of the manganese poisoning 6.4 millions erythrocytes and BREZINA found the same year (128) some cases up to 9 millions of etythrocytes. BAADER found with one manganese poisoned patient 6.28 million of erythrocytes and three months later only 4.2 millions, so he concluded that his case is in a good agreement with results obtained by experimenting with animals done by SCHWARZ and PAGELS. He also concluded that hyperglobulia is an early sign of manganese poisoning which disappears later. (10). BÜLTNER and LENZ and LEMOS confirmed the above BAADER'S findings. SCHAWTZ and PAGELS recommended a systematical blood test of all persons working with manganese as a means to detect poisoning in its early stage. BAADER (14) thinks that "hypererythrocytosis" is caused by a damaged diencephalon.

Results obtained in a massive blood test in one pyrolusile mill in

the United States disagree with the above findings (77). The average erythrocyte number was 5.15 millions for a group of workers poisoned with manganese: 5.11 million for a group of workers exposed to manganese byt not poisoned and 5.16 million for the control group of healthy and non-exposed persons. The average hemoglobin was 80.8 % and in the controled group 84.5%. LECLERC, also found some decrease in erythrocytes and globulin. ASHIZAWA recorded also anemia, but his patient was sick with tuberculosis to this finding cannot be considered as valuable. VAN DOGAERT and DALLEMAGNE found anemia with monkeys poisoned by the digestive tract but not other poisoning signs. So, hyperglobulia can by very ofter, but not characteristical occurrence with manganese poisoning. In addition, according to BAADER it is of a temporary character. However there are many more publications which did not mention any occurrence of hyperglobulia. In one of the recent publications from 1946 (74) are given results of a systematical blood test of a large group of workers from the same mill in which was found 11 cases of manganese poisoning in 1940. The author of this publication did not find any significant changes related to hemoglobine or erythrocyte. The same was found for a group of 28 poisoned persons from one mine in Morocco (50) We also did not find with our patients neither increased hemoglobine nor erythrocytes (see Table 8).

Table 8

Pretrage	S.J.	S.M.	L.M.	P.I.	M.S.	B.M.	S.I.	B.J.	V.J.	E.B.
Erythrocyte millipetrum	4,3	4,7	4,4	4,5	4,3	4,3	4,4	4,9	4,6	4,2
Hemoglobin %	75	82	75	80	78	78	78	85	92	84
Sedimentation tiberium	5,2	5,3	6,8	4,2	6,0	4,3	4,7	4,5	8,5	4,7
Seg %	50	46	43	44	36	37	45	47	49	74
Neseg %	2	2	2	4	3	2	3	2	0	2
Mono %	10	9	8	8	9	7	9	5	9	7
Limfo %	30	37	38	35	43	35	39	37	40	16
Eo %	7	5	8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	1
Baza %	1	1	1	4	2	2	0	1	0	0
Pic %	1,7	0,9	1,2	1,6	1,5	1,3	1,2	0,6	—	—
[illegible]	0	0	0	0	0	0	0	0	—	0

The blood picture of our patients.

1. Subject 2. Patients initials 3. Erythrocytes in millions 4. Leukocytes in thousands 5. BpE per million.

In connection with manganese poisoning, less frequently are mentioned cases of leukopenia. In the above mentioned publication (77) about 11 poisoned persons in one mill in the United States , it was reported that lenkopenia was getting more serious as the disease progressed. Leukopenia, defined as less that 5,000 leukocytes per cubic millimeter, had all except one patient. The average number of leukocytes for this group of healthy but exposed workers had average of 7,850 leukocytes and the third controled group consisted of non-expostd persons had average at 7,560 leukocytes.

In our group we also noticed definite leukopenia with 5 out of 10 patients, two showed tendency toward leukopenia, while one shhowed a modest form of leukocytosis.

SCHWARZ in 1932 mentioned a case of lymphocytosis. Also, FLINTZER found lymphocytosis with four of his patients and BAADER in his book in 1943 mentioned lymphocytosis. We did not observe lymphocytosis with our patients even if a percent of lymphocytted was lower than 40% it is considered as an upper limit. SOURATE, mentioned monocytosis (10 -12 %) but even he did not take it as a specific sign. FABRE mentioned leukocytosis with neutrophilia. In literature one can find publications in which is claimed that there is no changes in blood picture connected with manganese poisoning)125, 131, 135, 186, 187, 189). Other blood data was not discussed in literature.

4. MANGANESE EFFECT ON OTHER ORGANS.

BAADER claimed that "the central exophthalmic goitter can appear because of "a damaged diencephalon" (14). VOSS found the increased basal metabolism in two of his patients. RAY and DEYSACH found that when manganese chloride is injected subcutaneously, its accumulation occurs in many organs but most in the thyroid gland, and that the accumulated manganese

in the thyroid gland is proportional to the amount injected. Later, the same authors in 1949 (32) said that accumulation is faster in the beginning, about 70% in the first six hours. The manganese excretion is also a fast process. However, BOBERT and DALLEMAGNE did not find confirmation in their experiments about the thyrotoxic effect of manganese. This effect was also not observed in a clinical practice by many other authors. ECZEMAS are mentioned by many authors (83, 31, 77, 152). Italian CERESA reported one eczematous case. ("dermatitis erythemato - papularis) with one patient who was working on crushing and transportation of the manganese ore. This sporadic case we can not consider as"the professional manganese poisoning" as it was named by the author himself.

FRAISE mentioned the gastrointestinal disturbances among his miners, but he is not sure to consider them as an affect manganese. The author thinks that they were caused by drinking water which contained "colloidal manganese silicate".

According to RODENACKER , "the significant changes of heart" can be occuring with workers processing "the gray rock", which are caused by "the fat degeneration of muscular cells of heart". Among 27 miners in one manganese mine it was found 5 cases of Bradycardia (40-50 per minute) and six among them had extrasystole with a prolonged transfer time. RODENACKER THINKS that above heart disturbances are consequences of the manganese pneumonia. FLIYM on the basis of detailed clinical observation concluded that there was not any increase in cardio vascular diseases in one group of workers who were exposed to the manganese ore dust.

The experimental date about manganese effect on blood pressure indicates that manganese chloride and sulfate caused drop in blood pressure in animals. (103). CALIEBE found that manganese chloride highly diluted has a vasodilatorial

effect; similar observation was made by ESCOBAR. However, there were no such findings with lungs.

ANGOLA claimed that dilatation of blood vessels in the eye bony orbit is caused by manganese.

The experimental results obtained by KOBERT related to the kidney inflammation are not confirmed in a clinical practice to the best of our knowledge. Some cases of hematuria and microhematuria were recorded but it could have not been connected with manganese.

A laboratory test of blood serum of one group of poisoned workers (77) showed a decreased calcium content. The average calcium content in this group was 8.8 mgr per 100 me compared with 9.8 mgr per 100 me found in the control group.

Z. and RODIER found an increased excretion of ~~porfirine~~ porphyrin in urine with some of the poisoned worker. Our investigation did not show any abnormality concerning excretion of ~~porfirine~~ porphyrin..

5. MANGANESE EFFECT ON LUNGS.

An acute conio-pneumonia or otherwise called manganese pneumonia or manganese pneumonitis is of a specific interest in toxicology with heavy metals in general and in manganese toxicology in particular. This acute effect of manganese on the lungs is less frequent than a chronical effect on the central nervous system. This is one of the reason that this disease is very little known and explained.

Manganese pneumonia is more a statistical term than a disease clinically observed and established by epidemiological, histological, toxicological or any other clinical method. The only clinical characteristic of the

manganese pneumonia which differs from non-manganese pneumonia is that the manganese pneumonia is more malignae and with a worse prognosis. For many years up until now there is not a certain proof that manganese pneumonia ia really a disease "sui generis".

Manganese pneumonia was investigated in detail by BAADER and BÜTINER in Germany, by ELSTAD in Norway and LLOYD DAVIES in England.

Pneumonia caused by manganese was first suspected by BREZIN in 1921 (83) He noticed an unusually high percentage of pneumonia in one pyrolusite mill in which five out of 10 workers died in a period of 27 months because of pneumonia. BAKRAKZE (88) published in 1923 his observation at one caecasian mine with more than normal frequence of pnewmonia cases. In a period of 10 years from 1905 to 1914, pneumonia was the most frequent disease among miners and transportation workers and mortality because of pneumonia being four times higher then because of all other diseases taken together. So BAKRADZE classified the manganese pneumonia in 1930 as a typical professional disease. SCHOPPER described two cases of manganese pneumonia from one mine " in which miners seldom recover of pneumonia". He did an autopsy on these two cases, and he found besides expected signs, also manganese deposited in the lung, what he believed caused fibrosis. BUBAREW (88) discovered in 1931, through a sustematic chech-up that 53% of all transportation workers had pnewmonia. The same check-up in Brasil performed by FREISE showed that 61% of all transportation workers had pneumonia. BAADER at that time visited Marocco and discovered that pneumonia is the most frequent cause of mortality among miners. The same year BAADER also found (12) that manganese is often cause of pneumonia in production of the LECLANCHE' cells. DANTIN - GALLEGO published his observation from one manganese mine in Spain in 1936. BÜTLNER and LENZ published results of their investigation in one manganese mine at [illegible]. They found that in a period

of ten years, 57.5% of all death occurred in this area was caused by pneumonia. BRADER describes in 1927 the disease's development and death of one patient who died of manganese pneumonia and who was working in processing pyrolusit in production of the LECLANCHE' cells. An autopsy of their patient showed some sort of black-brown pigment in the patient's lungs which contained manganese. VIGLIANI in Italy, described one fatal case of manganese pneumonia which occurred only 10 days after the worker started his job with manganese dioxide. BRADER (14) concluded on the basis of his own and others' experiences that workers in the manganese mines and mills are inclined to diseases of the respiratory tract such as are pleurisy, bronchopneumonia and lethal pneumonia. In 1939, a great interest was paid to a puzzling endemic pneumonia in a small isolated area in Norway in the city of Sauli which was considered to have a very healthy climate. Mortality rate because of pneumonia in this town, was four times higher than what was the average, for whole Norway. Even more, in a period of 14 years, from 1925 to 1939, pneumonia was the cause of one third of all death casualities (32.3% in Sauli compared with 3.65% in the whole of Norway, or about 10 times higher rate in Sauli). HOLST in 1923 tried to solve "the Saul pneumonia problem" but with no success. His son tried to use a prophylactic vaccine (cit.121) with not much success because many refused to receive a vaccine voluntarily (59). The investigation performed by RIDDERVOLD discredited an explanation that this endemic pneumonia is caused by some sort of highly virulent pneumococci. Finally, ELSTAD working on this problem from 1934 to 1938, presented his opinion that in question is the case of manganese pneumonia (59, 60). He presented his opinion to the 8th Congress of Industrial Medecine in 1938, showing that in the period from 1930 to 1938 the pneumonia frequency was proportional to the production of manganese alloys. In fact,

in Saud in 1923a plant was built up for production of manganese alloys similar to the one we have in Sibenik. During the manganese alloy production a great amount of smoke containing manganese was released from furnaces into atmosphere "hanging over the town". Because of that, the Saud pneumonia case had one specific that is that pneumonia was wide spread among the city population and was not limited to the plant workers only. BÜTLNER presented to the same Congress results of his investigation from one pyrolusite mine in Rine Province. In this area from 1934 to 1937 the pneumonia cases appeared in 17% with 6.3% lethality while the control group had only 0.54% of pneumonia cases. SCHULZ in 1939, also observed an increased lethality caused by pneumonia in one manganese plant. HEINE in Germany during the war published his observation in one manganese alloy plant in the vicinity of Achen. From 1932 to 1938, 41 men had pneumonia and 27 died because of it (about 65%) and only in 1936 20 out of 27 pneumonia cases were lethal (about 73%). After the World War II, AIELLO in Italy wrote about "the manganese fever" and POVOLERI also from Italy wrote about bronchopneumonias in production of ferrsmanganese in AOSTO. These cases occurred with high frequency in a period from 1935 to 1940 with 20 - 32% lethality. Finally, LLOYD DAVIES in Sheffield, England found very frequently the cases of pharyngitis, bronchitis and pneumonia in the potassium permanganate plant with workers exposed to manganese dioxide and higher manganese oxides. The observed frequency was 26% compared with 0.73% of pneumonia in the control group.

So, statistacally the existance of the manganese pneumonia was well documented in areas where work with manganese on manganese compounds was performed.

However, rare cases of an autopsy and histological tests, did not discover anything specifific which would confirm an existance of the

manganese pneumonias in a medical sense. Series of experiments with animals was attempted to explain mysterious pathogenic mechanism of the manganese pneumonias.

Difficulties in an experimental work were that inhalation of any dust (regardless of its chemical nature) can cause pneumonia. So, it was left either to prove histologically the specific manganese effect or to compare toxicity of various kinds of dust.

The first experiments were performed by JÖTTEN and REPLOH in 1938 who succeded to cause bronchopneumonia in rabbits by the manganese dioxide dust. However, SPEIDMANN claimed the next year that he failed to cause the lung's reaction with rabbits, guineapigs and cats after several years of experimenting with inhalation method. The animals inhalated an atmosphere containing 50 - 100 mgr of manganese dioxide per cubic metric. He succeded to cause pneumonia using higher dosage but those pneumonia did not show anything specific. The next year the same authors (98) showed that bronchitis and bronchiolitis can be developed without symptoms of croupous pneumonia.

JÖTTEN and REPLOH, and later REPLOH by himself (94, 147) discovered that besides manganese effect one more factor had to be present to cause the manganese pneumonia in rabbits. These were either inhalation of a suspension of the pneumococci on exposion to extreme climate factors. However, LLOYD DAVIS later showed with very accurate experiments that mice having inhalated the manganese dust did not show an ilncreased sensitivity toward pneumococci or streptococci. HEIM performed experiments with the ferromanganese dust and concluded that with the dust itself can not be caused pneumonia nor animals became more sensitve toward pneumococci. Then LLOYD DAVIS again performed very fine experiments with mice in 1946

and with Rats in 1949 by which he succeeded to cause pneumonia and even more to observe specific changes caused by the manganese effect. caused by the manganese effect. This specific manganese effect is a mononucleus reaction in intersticium and tilling of alveoli with histiocitic cells.

A bacteriological test seems to show presence of pneumococci in most manganese pneumonias. LLOYD DAVIS found some other pathogenic micro organisms and in two cases he did not find pneumococci.

About the manganese pneumonia pathogenesis opinions are rather different. An autopsy of the cases in Saud (Norway) performed by ELSTAD showed an increased content of manganese in the lung. Other authors also found deposed manganese dioxide in lungs (79, 88, 157) and thought that manganese pneumonia is caused by mechanical effect of the precifitated pigment which obstructed normal lymph flow. But KEINE thinks, that the above can cause only bronchopneumonia. JÖTTEN thinks, that mechanical effect of the manganese dust comes from very hard, very fine, very sharp manganese particles which can enter the finest tissue's cavities. This assumption was checked by LLOYD DAVIS, who measured particle size and their number in one plant. He found in the atmosphere of that plant that 90% of particles in the atmosphere was below 1 microne size and 80% below 0.2 micrones size. According to JÖTTEN, the manganese effect is of a local nature because resorption is limited by the very low solubility of manganese dioxide.

In some cases, an autopsy of humans died of manganese pneumonia also showed the symptoms of silicosis (7,90). So, it was thought that lungs damaged by silicosis is one of preconditions for manganese pneumonia. Now it appears that the above vases were insignificant coincidences in both diseases. It happened in areas where manganese ore contained high

silicate content. Also it was thought a possibility of the manganese pneumoconiosis which can decrease resistance of lungs toward other infections. However, the interstitial changes so characteristical for silicosis have not been observed in the case of manganese pneumonias (96).

One theory (30) speaks about a simultaneous inhalation of manganese and sulfur dioxide. According to this theory manganese catalyzes transformation of sulfur dioxide to sulfur trioxide what generates sulfuric acid which by its irritating effect prepares lungs for an infection. Even if this theory is based on a sound assumption it can be applied only for very rare cases.

Finally, LLOYD DAVIES thinks that manganese is simply a toxic substance effecting the whole respiratory tract. It has ability to proliferate and infiltrate the lung tissues and by further effect to cause hydroscopic degeneration and/or death of cells - necrosis. So, he concluded that the primary chance is an alevatous reaction which can occur on the epithelium of any part of the respiratory tract, [illegible] He also proposed a new term "manganese pneumonitis" instead of "manganese pneumonia" to avoid any anatomic localization of the disease.

It is not still explained regarding the correlation between manganese poisoning. The question was; why there are not so many cases of the manganese poisoning in the plants where the manganese pneumonia is a frequent case. Again, LLOYD DAVIES is only one who tried to explain this phenomenon. He explains it ; that the particle size is a crucial factor. Small particle size of manganese in dust does not have sufficient mass to cause a systematic poisoning. On the other side, larger particles with enough mass to cause a systematic poisoning do not posses ability for an intimate contact with the epithelium of the respiratory tract.

KEIHE, during the war, showed that lethality because of manganese pneumonia in one manganese alloy plant dropped from 1.26% in 1936 - 1938 to 0.17% in the period 1939 - 1941. This improvement he attributed to stricter safety rules. FOLOVERI published in 1946 a statistical data which shows that lethality in Aosta declined from 1940 and had a "normal" number in 1943 in spite of the fact that production of ferromanganese was not reduced. FOLOVERI explains it by use of the sulfonamide therapy. Neither LLOYD DAVIS had a lethal case from 1946 to 1948.

There is not much data about happenings in our plant in Sibenik. The Sibenek plant was similar to the ones in Saul, Aachen, and Aosta where the highest frequency of manganese pneumonia was observed. This is probably due to the extreme changes in a microclimate within the plant as well as with pressure of very fine dispersed manganese in the smoke from the thermanganese furnace. So, we could have expected an occurrance of manganese pneumonia. TSLIAJA in 1938 (at 99) observed an increased frequency of manganese pnwumonia. More often (at 99) noticed in one part of town an increased number of a small intiltrate in the lungs, but he did not consider it anything specific. KESIC and SLEZINGER analyzed the causes od mortality in Sibenik before the war II and they did not find anything significant. KARMINSKI believes that in the postwar period, many cases of manganese pneumonia are covered with treatment at home and by using penicilin, but also that there is not a thing to suggest that a problem of pneumonias exists in Libenik.

The last publiction in the modern literature which deals with a therapy of manganese pneumonias is written by LLOYD DAVIES in 1949.

6. A PREVENTITIVE OF MANGANESE POISONING

A preventitive of manganese poisoning does not require anything specific regarding chemical nature of manganese and manganese compounds.

It should be followed and inforced general safety rules and preduction sould be modernized. It means, a process should be closed, a material transportation automatized, a good ventilation should be provided, a wet mining should be performed. The atmosphere in a plant should be controlled and warning issued it manganese concentration sets over a tolerable limit. How much a good ventilation is important should be an example from one plant in Hamburg. In the period from 1921 to 1928 only one case of poisoning was discovered (65), but when ventilation failed 10 new cases appeared (45).

In our opinion, ones who process manganese ores should take care of some ore characteristics shich might be of importance to maintain healthy working conditions (percentage of various manganese oxides, on extent of ore desintegration, content of a hepatotoxic materials in an ore etc.) Because a short exposition can also cause poisoning, it is recommended to use a respiratoru mask on a temporary basis when a working atmosphere might be dangerous.

Workers should be taught or required to keep their bodies and working clothes always clean. A complete physical check - up should be required of any new employee in companies which process or deal with manganese and its compounds. An employment should be refused to any one who has some defects on the respiratory or digestive tracts, who has a damaged liver or who is inclined to have a heavy alcohol drinking intake.

A physical check - up should be sistematic e.g. should be performed periodically. LANGLEZ recommended the first check - up 18 months after

employment started, and then every 12 months. However, we agree more with other authors who recommended a check - up every three months (190) or every four months (20). The workers showing any of the signs of the manganese poisoning (muscular atrophy, tremor) should be transfered to another working area where they can not have any contact with manganese. A medical control should be continued over them. Recommended laboratory tests are: blood test, functional test on a liver, coproportirinurie, "mananemia", manganese test in feces, etc.

Unfortunately, one should have in mind that ther is not a sure laboratory test which would indicate the beginning of the professional manganese poisoning.

APPENDIX

PRESENTATION OF 10 CASES

CASE NO.1: J.J., 48 years old, admitted 3-31-1952

ANAMNESIS: It seems, two years ago he had herpes zoster. This disease started a year ago. First, his friends told him that he now walks differently, that he walks heavy, touching ground with whold feet, particularly with his right foot. Soon later, he could have noticed it. Sometimes it happened to him where he lost balance while walking , resembling a drunk man. Later he started feeling some soakness in both legs, walking made him tired, particularly on up-hill wald. It is hard to lift his leg on his own, on a chair. The walking disturbances did not get worse. The last several months his speech was changed. He speaks in low tone and sometimes keeps repeating words. When he is doing something, his arms shake, He became a slow moving person unwilling to move at all. Usually he is in a good mood, but he thinks that his temper was changed.

Very often he shows an uncontrollable laugh. He can not be seriously angry, rather he starts laughing. He keeps forgetting things easily. A sexual potency decreased during the sickness. A salivation occurs during the night. He smokes and drinks moderately.

WORKING ANAMNESIS: He is a plumber by profession. He was employed in "the factory of ferroalloys and electrodes" before this plant started production of the ferromanganese alloys. From 1931 to 1938 he was working in an auto-garage. From 1938 to 1943 he was working in the plant on installing furnaces and recharging electrodes. On his job, he is heavily exposed to the furnace smoke. During the war he became a professional fisherman and then he entered the army. From 1945 up to now, he is working in

-7t

the same plant performing the same job as in the prewar years. He stopped working on 5-5-1952 because of problems with legs.

CLINICAL RESULTS: Short but strong osteomuscular appearance. A somatic check - up o.k. except the heart is somewhat larger on left side. T: 37.2°C; P: 80, RR: 120/60, CR: 7/14. For the blood and liver tests see table 7 and 8. Urine: o.k. War. MKR and KR: negative. X-ray lungs: o.k.; calcium in serum 11.10 mgr per 100 ml; phosphorous 2.00 mgr per 100 ml; sugar in blood 97 mgr per 100 ml. Iron in serum 150 gamma (10^{-6} gr) per 100 ml, Copper in serum 112 gamma per 100 ml. EKG: Left gram, otherwise o.k.

A neurological test showed a small increase of tonus of the extremities. In the arms can be felt "a gear phenomenon". The patient easily stands up and moves. When he walks he steps on the heels first then quickly on the frontal part producing an acustical effect. This is particularly easy to observe when he walks downstairs. His steps are short. There is fast and fine tremor of the streched arms and tongue. On his face can be observed hiponimia. Deep and surface reflexes are vivid, patelar subclsmic, all are symetrical, only ACHILLES' left is absent. The speach is low voiced and modulatory, when speeding up in speach he keeps repearing syllables or words. Regarding his psychical conditions it can be observed at some degree of confusion and his readiness to smile.

The affective reactions are normal. The psychomotor reactions are rather poor. There is no defect of intellectual functions.

During his admission in the hospital he was treated with "BAL". The observed improvement was very slight. The improvement was of a subjective nature so it can not be said how much the therapy took a part in it.

CASE No 2: M. S. 57 years, admitted 6-25-1952

ANAMNESIS: The disease started 15 months ago with difficulties in walking. He could not walk fast, he pulls legs behind him. He particularly feels difficulites walking up hill. When he sits down he looks like somebody pulls him back. Two or three months later he started to speak harder. He was most bothered that he can not speak so loudly as he used to, and that he keeps repeating the same words or even whole sentances. Then he felt weakness in the arms. The last five to six months, he has many other cramps in the legs, mostly during the night. While he was working he had cramps in the arms. At that time he slept poorly. While he was working, his conditions were getting worse. Since he is on sick - absence from the job, his conditions are unchanged. Until six years ago he smoked heavily. He drinks a little wine and about 4 oz. of brandy daily.

WORKING ANAMNESIS: He started working as an agricultural worker then in 1921 he came to this plant where he was working on mixing line and coal until 1928. At that time tht plant was producing calcium carbide. From 1929 to 1945 he was working in foreign countries. First as a miner in a coal mine in Belgium, then in one coal mill in Germany. Since 11-11-1948 he rejoined "the factory of ferroalloys and electrodes" in Sibenik, first as a transportation worker and later as a stocker of the furnace. He charges the furnace with manganese mixture using shovels. He stopped working on 3-25-1952 because of disturbances in the legs.

CLINICAL RESULTS: Tall with a strong osteomuscular structure. Right scrotal hernia, otherwise somatic check - up o.k.; T: 36.4°C; P: 64, BP: 105/75 SE: 8/15. For the bolld picture and liver tests see table 7 and 8. Urine o.k. WaR, MKR and KR negative; X-ray (lungs): A fibrous process in the right side; EKG: left gram, otherwise o.k.

It is observed hyperkinesis in his neurological status manifested by reduced psychomotorial reflexes, obstructed movements, automatic moves and by hypomimieria. A light rigor exists in his neck and in the lower extremities. While walking he pulls his legs. Light retropulsia. From times to times, there is a tremor in the right leg. Reflexes are o.k. except that the patoral are somewhat vivid. Speech is monotonous, with often habit of repeating himself. There is no obvious psychical changes.

CASE NO. 3: M. L., 42 years, admitted 5-17-1982

ANAMNESIS:

As a 14 year old boy, he had water in the stomach so that medical intervation released 14 liters. He was healthy until 1967, then he got sciatica. A month ago he started feeling a weakness in both legs particularly walking up - or downstairs. He walds up hill hardly, and when going down hill he stumbles. When laying, he has cramps in the legs, under the knees and even in the feet. The cramps are stronger on the right side. Friends of him, told him that his speech is changed but he does not feel it. He is ready for laughing for nothing and it bothers him He smokes about 20 cigarettes and drinks about 1 litre of wine daily

WORKING ANAMNESIS: Until 1987 he was working in "the factory of ferroalloys and electrodes" changing the furnaces but at that time the plant was producing only calcium carbide. The same job performs when the plant plant started production of fettomanganese alloys and he charged the furnaces with manganese ores and others additives. He stopped working 5-8-1982

CLINICAL RESULTS: Medium height with strong osteomuscular structure. The physical check - up of internal organs has not indicate any pathological

changes. T:36.2[illegible]; P: 66; BR: 1[illegible]/[illegible]; [illegible]: 8/11; The blood picture and liver tests are given in tables 7 and 8. Urine: o.k.; WAR, MKR and KR: negative. Feces: eggs of ascarids and trichocephalus. X-rays (lung and heart): o.k. Calcium in serum 11.[illegible] mg per 100 ml; phosphorous 2.[illegible] mg per 100 ml. Sugar in blood 89 mg per 100 ml. Iron in serum 8[illegible] gamma per 100 ml. Copper in serum 100 gamma per 100 ml.

NEUROLOGICAL RESULTS: Mimic and gesture poor. Speach fast and wntangled. While walking he has tendancy to speed up. There is a light propullsion. He does not lift his legs enough while walking; he pulls them, particularly the right one. His right arm is still while he walks. Tonus resembling rigor is increased in the right extremities.

There is "a gear phenomenon" on both upper extremities. A light tremor is observable in the arms, specially when he moves them. Deep and stomach reflexes are vivid. There is no right Achillis reflex. The plantar reflexes o.k. Psychically he appears as a simple and normal person. He has tendency for laughing. He was treated with two sessions of "BAL" injections. Having been treated, he claims he feels stronger in the legs.

CASE NO. 4: I.P.; 45 years; admitted 5-31-1952.

ANAMNESIS: Until this disease, he was always healthy. The first symptoms he observed about two months ago, and they appeared fast and were developed in several days. First he observed that he cannot speak loudly and smoothly, and that he does talking much less than he used to. At the same time he felt weakness in the arms, and even more in the legs particularly when he walks. It is hard for him to walk; there is no strength in the legs, pulls legs, walks slower and tiptoe. While walking he feels sometimes like something is pulling him back, so sometimes he unitentionally walks backwards. He also felt a decrease in a sexual potency. Later, he used

arging without reason. The last month appeared short but painful cramps in the legs, mostly during the night. They occur almost every night. His friends told us that he became a very slow moving and sleepy person. He always claimed that he is a hardworking person but he felt asleep on the job several times. An appetite very changeable lately. Until 1945 he smoked 40 cigarettes daily, then he quit smoking. He drinks about 1 lites of wine and about 4 oz. of brandy daily.

WORKING ANAMNESIS: Until 1941 he was agricultural worker and used to work at the seaport. Since that year, he was working in "the factory of ferroallys and electrodes" in Sibenik as a stocker. He charges using a showel for the furnaces with manganese ore and other additives. He performs this job every 10 - 30 minutes, as required. The job is dusty and smoky. When unfavorable wind blow, the smoke can become so thick that one man can not see another in a close vicinity. He discontinued the job from 1943 to 1948 in the plant, and started again in 1948. He stopped working 4-3-1952 because of disturbances in the legs.

CLINICAL RESULTS: Medium height, strongly built, His behavior is very low temper, with short movements and poor mimic. His jaw is defective and has a marginal gingivitis. There is a hypersalivation. The physical check - up of internal organs is o.k. T: 36.5°C; P: 78; RR: 110/70; SE: 11/25. The bolld picture and liver tests are given in tables 7 and 8. Urine: in the sediment, there were some pale erythrocytes, otherwise o.k. WAR, MKR and KR: negative. X-ray (lungs): a bronchial scheme is slightly distinguished otherwise o.k. Calcium in serum 10.00 mg per 100 ml, phosphorous 2.31 mg per 100 ml. Sugar in blood 114 mg per 100 ml. Copper in serum 200 gamma per 100 ml; EKG: o.k.

Neurological check - up showed no signs of parasis although [illegible]

strength is somewhat lower than expected. His appearance is of a still man, looks like motion impulses are minimized. His mimic is poor. His eye - lids move less frequently than normal. When he is standing up or walking he is inclined forward. The steps are shorter than normal. He pulls his legs; he does not lift them from the ground. He pulls them over tiptoe, so that next step makes first with the foot distal part. He turns around slowly and stiffly. All musells of the extremities and of the neck show during a pasive motion, an increased tonus in form of rigor wit h a very slight "gear phenomenon". There is no hyperkinetic observation except a tremor of the torgue. The ROMBERG TEST showed a slight swaying. There is no other disturbances of a balance or of a coordination. Deep reflexes of the extremities are slightly more vivid, only the left triceps brahii reflex is weakened. The skin reflexes are normal. There is no change of senses. Speach is slowed down, poorly modular, monotonals. Sometimes he has a habit of repeating syllables or words. His answers are in one syllables or words. His answers are in one syllable fashion. The patient does not show any interest for anything; he is apathetic and slightly euphoric. He easily starts crying if he remains himself at his home, or he starts laughing without real reasons. His laugh is unnatural and lasts a long time. The patient is of a primitive education, but appears that his intellectual level is not effected.

The patient was observed during a period of three months and he was treated with the "EKL" therapy. The neurological symptoms did not change by the therapy, and may be only his speach became a little bit more loud. We also have the impression that he became a little bit less sluggish. Leaving the hospital he still had leucopenia (4.5 thousands per 1 cubic millimeter.

CASE NO. 5. S.M., 44 years old; admitted 6-15-1951

ANAMNESIS: The disease started two months ago when he felt a pain in the lower back and a weakness in the legs, specially in the mornings. He walked slower and he could have not walked for a prolonged time. Also, his arms became weak. His speech was changed , he could not talk loudly or yell. Also, while speaking he speeds up and repeats words. He has feeling that something is choking his throat. He smokes 20 cigarette daily, and drinks a little bit occasionally.

WORKING ANAMNESIS: Until 1947 he was working as an agricultural worker, and from 1947 to 1949 in one stone mill. Since 1944 he was working in "the factory of ferroalloys and electrodes". He transports the process mixture to the furnaces and sometimes charges them. Most of the time, while working he spent in the very dusty and smokely transportation tunnel. He has not worked since 6-13-1951.

CLINICAL RESULTS: The somatic check - up :o.k.; T: 36.8: P:80; RR:130/70. The blood picture and the liver tests are given in tables 7 and 8. Urine: o.k. ; SE:9/20; WAR, MKR and KR: negative; X-ray (lung and heart): o.k.; Calcium in serum 11.30 mg per 100 ml. Sugar in bolld 98 mg per 100 ml. Iron in serum 168 gamma per 100ml, Copper in serum 130 gamma per 100 ml: EKG: normal

From the neurological aspects he has hypomimia, he selfon moves his eye lids. There is a general hypokinesis and bradykinesis. When he walks he appears stiffed, inclined llightly forward. He pulls his legs, particularly the left one. The arms' motion during walking are very poor. There is retropulsion and propulsion, so sometimes he suddenly speeds up while walking. A light tremor can be observed with the neck musculaturs and with extremitits, somewhat stronger on the left side. The "gear phenomenon" can be felt sometimes. A light tremor can be observed sometimes when he has his arms streched out. The musculature strength of the extremities

appears awkward, particularly on the left side. The deep reflexes on the left arm and leg are mild than normal, other half are normal. His voice is monotonous without modulation and articulation. He speeds up sometimes while speaking and repeats words.

Psychically, there is a mutual irritative and an impression of brandyfrenzia. There are no affective changes nor intellectual defects.

He was admitted to the hospital for the second time on 5-21-1952. Since the last admission, he did not have a contact with neurosis. His conditions can be considered unchanged. He still had a pain in the lower back and all other symptoms already mentioned. Now, he says that he is very clumsy while he turns around in bed. He also complains, that he is now finding it easy to cry or to laugh. Only his speech is improved slightly. The Objective conclution is: no changes. He was treated with three sessions of "SAL" but no effect was observed.

CASE NO. 6. M.E.; admitted on 5-21-1952

ANAMNESIS: He needed three years to complete the first grade in the elementary school. In the army, he performed only insignificant duties. In 1947 he started working in the factory. In September of 1951 he was transferred from one job to another because of difficulties he felt in the legs. He stopped working on 10-15-1951. Since spring 1951 he had epileptic attacks, total number about 10. At the time when epileptic attacks started he felt some disturbances in the left leg.- contraction of the toes while walking. In the fall 1951 he was treated in the neuro-psychiatrical clinic but his conditions were getting worse since then, except that the epilliptic attacks did not occur anymore. He has problems speaking. He drinks alcohol moderately.

WORKING ANAMNESIS: Until 1947 he was an agricultural worker and then he joined "the factory of ferroalloys and electrodes" in Sibenik. He was working in the factory as furnace stocker most of the time and on one transportation. The working conditions were dusty and smoky.

CLINICAL RESULTS: Medium height, average osteomuscular structure. He has the Lymph knop in both arm - pits. At admittion: T: 36.7°C; P:50, RR: 105/75, SE: 20/45. The blood picture and teh liver tests are given in tables 7 and 8. Urine: o.k., WAR, MKR and KR, negative. X-ray (lung and heart): o.k. Sugar in blood 102 mg per 100 ml, iron in serum 132 gamma per 100 ml, copper 94 gamma per 100 ml, EKG: o.k. except the sinus bradycardia (48). The neurologival results are focused to the changes on the left leg .

There is a strong equinovarus contaction of the foot and toes, which can not be overcome neither by a pasive or inactive motion. There is a light pyramidal spasm in the left knee during a pasive flection. The rigor is slightly expressed on the exticivities. The under knee muscles of the left leg have 2 cm. less diameter that at the right let. The foot extension can not be checked because of a strong contraction. There is no signs of paresis. He walks slowly because he pulls his left leg. He steps on the outside edge at the left foot, with the nictatarcus-phalanges. He has to stop often because of a painful cramp in the left leg and toes; by means of his fingers he streches his toes so he walk some distance more. Sometimes he showed a strong tremor of the streched arms. The arm motion during walk are somewhat obstructed. There is a hyperhydrosis of both legs, toes are desquamated. The reflexes of the upper extremities are are normal and symetrical. The stomach reflexes are a little bit more vivid than normal. The patelar and Achilles reflexes are vivid and more on the right side

than left side. The left plantar reflexes are normal. The right one extensorous (BABINSKI). In the area of the cranial nerves, the mimic is montonous with constant expression of smiling. The stretched tounge shows a strong tremor. He speaks fast and he usually repeats the beginning of a phrase several times very fast. Psychical condition: He is a debilitated person with expressed euphoric emotions. It is hard to obtain an anamnesis data from him He is rather a vivid person in a psychomorial sense.

He was treated with three sessions of "BAL", but not an objective improvement was observed.

CASE NO. 7. I.S., 42 years , admitted 7-15-1952

ANAMNESIS: The disease stated in 1949, stagewise. First, he felt a weakness in the legs and in the arms; starting with the right side and then less extensive on the left side. It bothered him while walking or working. The right leg and arm could have extinguished sometimes, it never happened with the left extremities. In spring 1950, the painful cramps started to occur in musculature of the hip and legs; the crampsare more frequent in the right leg. He still has cramps which occur more often during the night. They last several minutes. He can not stand alcohol as he used to. He has difficulties to speak when he drinks wine which was not a problem earlier. Otherwise he speaks normally. Sometimes he starts laughing without any reason. He shows hyper perspiration during the night and while working. Sometimes he has a double vision which has occuring for about three years. He claims that he has lost much weight since he has been in contact with manganese. His appetite is poor. He smokes little and takes alcohol moderately.

WORKING ANAMNESIS: Until 1938, he was an agricultural worker, a sea-port transportation man and he was working in production of cement in

Dolin. In 1938, he joined "the factory of ferroalloys and electrodes". He usually worked as a stocker of the three-phase furnace. Sometimes he used to work on the ore mixture transportation, but most of the time he spent nearby the furnace, charging it with the process mixture using a shovel.

From 1941 to 1942 he left the factory to rejoin it in 1948 on the same job, charging the furnaces and cleaning the ventilation tunnel. He stopped working 4 - 3 - 1950.

CLINICAL RESULTS: A short height, with less weight than normal. He has a bad breathalveolar pioree. In both armpits he has several lymph knaps. The percutaneous lung test gives a hypersonorant sound. The others o.k. T: 36.2°C; Puls 70; BP: 150/80 SE 5/11. The blood picture and the liver tests are given in tables 7 and 8. WAR, MKR and KR negative. X-rary (lungs and heart) :o.k., sugar in bolld 92mg per 100 cl, iron in serum 168 gamma per 100 ml, copper in serum 84 gamma per 100 ml. EKG: o.k. Neurological results: The face hypomimia is obvious. The eye muscles o.k. but he claims to see double when he looks at extreme left. He twinkles more often with one eye. There is a tremor of the tongue. His steps are short, he pulls his feet particularly the right one. When he walks up-stairs, he makes steps only with the left leg. There is no motion of the right hand while walking. There is, a rigor of the neck musculature, also on the right arm, and somewhat less on the right leg. The left extremities are normal. The musculature strength of the right leg and arm are reduced. The deep reflexes are normal. The stomach reflexes nor poor, the patelar reflexes are normal. The deep senses are normal, the skin senses are decreased on the right extremities and on the whole right half of the body. We have the impression that the damaged senses are of an organic source. Psychically: he is in a condition of a depressive emotion. He was treated

with two sessions of "ZIG". While staying in the hospital he claims that he is feeling better and the cramps occurrence became less frequent. All other symptoms stayed unchanged.

CASE NO. 8.: T. B. 47 years old, admitted 6-15-1951

ANAMNESIS: In 1939 he had malaria. The beginning of this disease is hard to find out. It looks like that the first symptom was when he felt that he could not speak as loudly as he used to. About one month ago he became unstable while walking. He had feeling of dizziness and the possibility of fainting. Sometimes he has a headache several for several days. He feels weakness in the legs and can not walk to long as he used to earlier. Then he felt loosing strenght in the arms. He smokes 20 cigarettes and drinks about one pint of wine daily.

WORKING ANAMNESIS: Until 1948 he was an agricultural worker and that year he was employed by "the factory of ferroalloys and electrodes" in Sibenik. He worked as the furnace stoker and also on the ore transportation to the furnace. He complained that his working area was always smoky.

CLINICAL RESULTS: Normal weight and a strong osteomuscular structure. The internal organs o.k. T:36.7°C; Puls: 90; BP:175/100 SE: 5/17. The blood picture and the liver tests are given in tables 7 and 8. Urine: o.k.' WR, MKR and KR: negative. X-ray lung: a light emphysema otherwise o.k. The eye check - up: sclerosis lentis O.U., fundus: conus myopicus O.U. The veins were slightly sclerotic. Sugar in blood 80mg per 100 ml.

Neurological check - up shows hypomimia and a general hypokinesis. The face expression was poor. There is no sign of an increased tonus or rigor. He keeps his body markedly stiff while walking, this particularly is observable when he turns around. While walking he pulls his legs; some-

times it can be observed propulsion and speeding up. A coordination is o.k. The reflexes are normal. His speech is poorly modular, sometimes mumbling and hard to understand. There is an impression of a light bradyphrenia. The affective reactions are poor.

CASE NO. 9: J. V., 47 years old, admitted 11-15-1949

ANAMNESIS: The disease began in the summer of 1949. He started feeling a pain in the hip and cramps in the legs. He also felt some difficulties breathing, like "his throat was closed". It was hard for him to speak and he stuttered. He kept repeating words. He had difficulties walking. He walks tip-toe. If he is inclined forward, he had to run. If someone pushed him from either side, he would fall down. At one time, he had double vision, but it disappeared since he stopped working. He smokes little, he drinks about one litre of wine daily.

WORKING ANAMNESIS: He was working in "the factory of ferroalloys and electrodes" in Sibenik since February 1948 as the furnace stocker and on the ore transportation.

CLINICAL RESULTS: The patient is medium height and with a strong osteomuscular structure. He has normal weight. The somatic check - up o.k., RR 100/60, SE: 3/8. The blood picture and the liver tests are given in tables 7 and 8. WAR, MKR and KR: negative. Calcium in serum 10 mg per 100 ml, phosphorous 4.75 mg per 100 ml. Fundus oculi: the papilae pale, the bolld veins sclerotical, retinamium perapapilar and paramacular inflamation centres.

Neurological results: His face like a mask in a smiling pose. He twinkles very rarely. The sticked out tongue shows a light tremor. There is a light rigor of all extremities of the extrapyramidal type. When he

walks or sits down he keeps his body in a stiff pose, and his head is inclined forward. The tendon reflexes of all extremities are stronger than normal, the stomach and plantar reflexes are normal. His speech is monotonous, with no melody. When he speaks for for a prolonged time, he is getting lower voiced, hard to understand. He repeats syllables and sometimes whole words. In a psychical sense he is a characteristical person. On the 12-6-1950 he was again admitted to the hospital for a neurological observation. Meanwhile he did not work with manganese. Now, he walks a little bit easier. He does not have a double vision anymore. The arm shaking started five months ago but not often. His speech is unchanged. The objective finding is that his conditions are about same as they were, only the musculature rigor can not be observed anymore.

CASE NO. 16. B.S. 54 years old, admitted 2-7-1953

ANAMNESIS: In his 15th year he twisted left hand in the elbow. This disease is developing for about one year now. First, he felt weakness and cramps in the legs. The cramps occur after the job and after a longer talk, they also occur both during the day and night. He became tiresome very soon after standing or walking. His feeling was like he has lead in his legs. He does not keep his balance well while walking. He can not walk downhill, without running. It is easier for him to go uphill. He has pain in the legs under the knees and in the arms below the elbows. Later, he felt weakness in the arms. Both legs and arms are shaky from time to time. He feels general weakness. He always has a desire to sleep. He has a feeling in his head like he is always drunk. He is completely willingless. He smokes and drinks moderately.

WORKING ANAMNESIS: Until 1946 he was mostly an agricultural worker then he took a job in "the factoru of ferroslloys and electrodes" in Sibenik. He worked as the furnace stocker. He stopped working 1-3-1953 because of pain in the legs and the arms.

CLINICAL RESULTS: He is tall, with an average osteomuscular structure. Except the small fracture in his elbow, his somatic chech - up is o.k. with no pathological symptoms. T: 36.5°C; P: 80; RR:115/75; SE: 6/17. The blood picture and the liver tests are given in tables 7 and 8. Urine: o.k. WAR, MKR and KR: negative. X-ray (lungs and heate): o.k. Sugar in blood 86 mg per 100 ml. Iron in serum 173 gamma per 100 ml. EKG: sinusbradycardia 56. Some increased hypertrophia of the left ventrimlus. Some ventricular extrasystoles.

Neurological observation: In contrast to the poor mimic are normal eye balls movements. The eye-lids twinkle somewhat less frequently than normal. He walks slowly, with a bended upper half of body. His steps are short, he pulls his legs. His arms' motions are poor while he walks. There is no rigor with the arms. Tonus is slightly increased with the legs. The ROMBERG test showed slight unstability. His movements are shaky. With his arms strechad out, a stronger tremor of the right hand is observed. His tongue stuck out shows a very strong tremor. The deep reflexes are vivid and polykimetical, the paternal reflexes are clonical; the stomach and plantar reflexes are normal. His speach is low voiced, monotomous and sometimes with a tendence to speed up. His emotional and other psychical conditions might be considered normal

This patient was checked on seven months ago on his working place and he showed them the same conditions and symptoms.

In the hospital he was complaining about difficulties with walking and speaking, and about a pain in the lower back and in the legs. He claimed that " only his head is feeling better". He was treated 25 days with Pyridoxine but neither subjective nor objective changes were observed.

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SUMMARY

CLINICAL AND INDUSTRIAL HYGIENE ASPECTS OF OCCUPATIONAL MANGANESE POISONING

(with report of ten cases)

Ten cases of occupational manganese poisoning having occurred in a plant producing manganese alloys (ferromanganese, spiegeleisen, silicomanganese and silicospiegel) are reported.

All of these patients were engaged on the same work in which they were highly exposed to the inhalation of manganese ore dust and manganese fumes. The manufacturing method of the plant is briefly described. The total lenght of exposure to manganese varied between 18 months and 12 years.

The most prominent neurological signs corresponded to a mild form of parkinsonian syndrome, in two cases with prevailing unilateral manifestations. The main neurological features were disturbances of gait, present in all cases: patients gave a history of weakness in the legs (and some of them in the arms); the gait was found to be slapping and in one case the so-called »cock-walk« was observed; retropulsion and propulsion, mask-like face, increased muscle tone, inconstant tremor of the hands and of extended tongue, disturbances in speech with palilalic and slow monotous voice, and micrographia were often noted. Nearly all patients complained of muscular cramps. Uncontrollable laughter, less frequently weeping, were also noticed. Symptoms of the affection of the pyramidal tract were also seen in one patient and hemihypaesthesia in another.

In blood picture only a slight leucopenia (4.2–4.7 WBC) in 5 patients was found. Liver function tests revealed no abnormalities. Liver biopsy performed in 5 cases showed no histological changes; in one of these, however, a pigment which did not give positive iron reaction, was discovered in large quantities in liver tissue.

Cerebrospinal fluid, examined in 8 cases, gave normal findings in 3 and slight abnormalities in others; globulin reactions were in one case clearly positive and in subsequent three slightly positive; in five patients there were small changes in mastix and collargol reactions. Pneumoencephalography was performed in two cases: there was a normal finding in one and a slight diffuse cerebral atrophy in the other. Six patients were submitted to electroencephalographic examination: one record was of explicity low voltage, another one exhibited an abnormality consisting of bursts of generalised medium voltage slow and fast activity; all other records were normal.

Treatment with 2,3-dimercaptopropanol (B. A. L.) was tried in seven cases following the dosage which Thompson suggested for the treatment of hepatolenticular degeneration. In another case pyridoxine was also tried. The patients failed to respond to both kinds of therapy.

A detailed account is given of occurrence of manganese in the nature and its occurrence and role in plant and animal tissue. Industrial uses of manganese, its alloys and compounds are listed. The industries and technical processes which give or might give rise to occupational manganese poisoning are described. The sources of occupational poisoning reported in literature up to the present time are reviewed. The importance of a particular disposition towards manganese poisoning as evidenced by numerous examples from literature is emphasized. Opinions are put forward on the toxicity of manganese and its action upon liver, blood, lungs and other organs. An analysis of symptoms and a comparison with the clinical feature of manganese poisoning as described by other authors is made. Some control methods for the prevention of further manganese poisoning in the plant are recommended.

Case histories of patients are added.

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Determination of Manganese in Clinical Material & Normal Blood & Serum Manganese Levels

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Various colorimetric methods available for the determination of manganese have been examined with regard to their suitability for use with clinical material such as blood, serum, cerebrospinal fluid and urine. Although the method using tetrabase for colour formation is the most sensitive, it has not been found suitable for the determination of manganese in blood. The colorimetric method of Wiese and Johnson [*J. biol. Chem.*, Vol. 127 (1939), 203], involving colour reaction with benzidine in acid medium, has been modified and used successfully for the determination of manganese in blood and serum of persons not exposed to an atmosphere containing manganese. The manganese contents of whole blood and serum in normal persons, not exposed to an atmosphere of manganese, have been found to be 29-40 μg. (mean value 33·3 μg.) and 14·0-52 μg. (mean value 37·4 μg.) per 100 g. of blood and serum respectively.

In connection with the work of the Manganese Poisoning Enquiry Committee appointed by the Government of India, the need arose for a sensitive and reliable method for the determination of manganese present in clinical material such as blood, serum and cerebrospinal fluid (C.S.F.). Almost all the colorimetric methods for the determination of manganese described in literature depend on the conversion of the manganese present in the sample into soluble permanganate and then measuring directly the intensity of colour of the permanganate[1-4], or the permanganate formed is utilized to oxidize a colour-forming substance, such as tetrabase[5,6] or benzidine[7-9], to give an intense colouration which is measured. In spite of the availability of such highly sensitive colour reactions, the direct measurement of permanganate colour is still employed for the determination of manganese. It was, therefore, considered necessary to make a study of the different methods and find out their scope and limitations for the problem in hand. The present communication describes the results of determination of manganese levels in the blood and serum of normal persons.

Materials and methods

All the reagents used were chemically pure or of analytical reagent grade and verified to be free from manganese. The containers used for the collection of clinical material and the syringes used for the collection of blood were also tested for extraneous manganese. A Coleman Universal spectrophotometer, model 14, with round cuvettes (19×105 mm.) was employed for carrying out the estimations.

Procedure — Preliminary trials showed that the development of colour with *p*-phenetidine as suggested by Szibelledy and Bartfay[10] was not very reliable as the blanks without manganese also gave colouration and the time required for colour development was long. The method of Gates and Ellis[11], using 4,4'-tetramethyl-diaminotriphenylmethane, also required unusually long time and a close maintenance of temperature for colour development. The following three methods were, therefore, taken up for comparative study: (1) Measuring the intensity of permanganate directly; (2) measuring the intensity of colour formed by the interaction of permanganate with tetrabase; and (3) measuring the intensity of colour formed by the interaction of permanganate with benzidine reagent.

Results

Fig. 1 shows the percentage absorption as a function of wavelength for the above three methods. Even with a comparatively high concentration of manganese (2·0 μg./ml. in the cuvette), only 20 per cent absorption at the peak of the absorption curve was obtained when the colour of permanganate was measured directly. With benzidine reagent the absorption peak is obtained at 420 mμ with 58 per cent absorption for 0·1 μg. manganese per ml. and

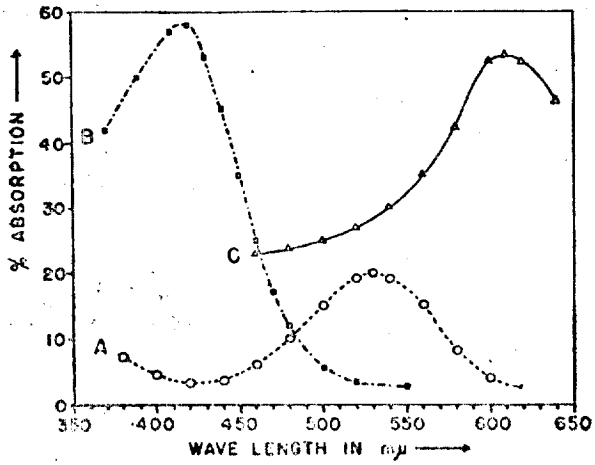

Fig. 1 — Absorption curves for (A) $KMnO_4$ solution (Mn, 2·0 μg./ml.), (B) $KMnO_4$ + benzidine mixture (Mn, 0·1 μg./ml.) and (C) $KMnO_4$ + tetrabase mixture (Mn, 0·04 μg./ml.)

the curve has a good and sharp cut-off. With tetrabase the absorption peak is at 610 mμ with 52 per cent absorption for 0·04 μg. manganese per ml., indicating that this method is almost twice as sensitive as the benzidine method. However, it does not have a sharp cut-off (Fig. 1, curve C) but shows considerable absorption over a wide range of wavelength and as such is not quite satisfactory for the spectrophotometric estimation of manganese.

Since the direct measurement of permanganate colour is not sufficiently sensitive, the tetrabase and the benzidine methods were tested for the estimation of manganese from samples of blood, serum, urine, etc. It was found that apart from the narrow range of *p*H (3-4) required for colour development with tetrabase and its rapid fading, even traces of iron would interfere with the determination, and attempts made to remove the iron present in the samples of blood by treatment with hydrochloric acid and extraction with ether, as suggested by Prodinger[12], would not eliminate the interference. The colour development with benzidine reagent as recommended by Wiese and Johnson[9] also presented some drawbacks. The filtration through asbestos, as recommended by these workers, did not always remove all unreacted bismuthate, and sometimes very fine particles of bismuthate passed through the asbestos pad and gave colouration even with blanks containing no manganese. Direct filtration into a graduated flask already containing benzidine reagent and subsequent making up to volume took much time, and the initially formed colour started to fade. Use of filter paper removed all bismuthate, but the permanganate was found to be completely decolourized. Even traces of oxidizable impurities on the glassware or in the double-distilled water used were found to reduce the permanganate and give low manganese recoveries. The method was, therefore, modified by making up the solution to volume in the presence of bismuthate and filtration through asbestos replaced by centrifugation.

The modified procedure is as follows: A sample containing 1 to 5 μg. manganese (about 3-5 g. of blood, serum, C.S.F. or 10 ml. urine), accurately weighed or pipetted into a porcelain or silica crucible, was heated, first gently to dryness and then vigorously to redness. On cooling, 3 ml. concentrated nitric acid and 5 ml. distilled water were added and the contents evaporated to dryness. After repeating this last step thrice, 3 ml. nitric acid, 10 ml. water and 0·1 g. sodium bismuthate were added and the contents gently heated to boiling and then heating discontinued. The clear solution was quantitatively transferred to a 25 ml. volumetric flask containing 0·2 g. sodium bismuthate and the contents made to volume. The well-mixed contents were kept for about 1·5 hr and thereafter centrifuged for 10 min. at 2000 r.p.m. Ten ml. of the supernatant were pipetted out into a cuvette of spectrophotometer containing two drops of benzidine reagent (1 per cent benzidine in 5 per cent aqueous acetic acid solution), and the absorption of the developed colour measured at 420 mμ against a blank of distilled water subjected to the same treatment.

In the case of plasma, C.S.F. and urine, the colour development was rapid within a few minutes, but with some samples of blood, it was found that the colour development was rather slow, requiring from 1·0 to 1·5 hr. It was, therefore, necessary to take readings of blood samples every 30 min. till a steady and maximum value of the intensity of colour was reached.

TABLE 1 — RECOVERY OF ADDED MANGANESE

Sample	Mn added μg.	Total Mn found μg.	Recovery of added Mn μg.	Recovery %
$MnCl_2$ solution containing 2 μg. of Mn	nil	2·10	—	105·0
$MnCl_2$ solution containing 4 μg. of Mn	nil	4·10	—	102·0
$MnCl_2$ solution containing 6 μg. of Mn	nil	6·06	—	101·0
Urine sample No. 7584 (10 ml.)	nil	0·90	—	—
Urine sample No. 7584 (10 ml.) with added Mn	2·0	2·91	2·01	100·5
Urine sample No. 7255 (10 ml.)	nil	2·29	—	—
Urine sample No. 7255 (10 ml.) with added Mn	2·0	4·20	1·91	95·5

TABLE 2 — INFLUENCE OF TOTAL MANGANESE CONTENT IN PLASMA SAMPLE ON MANGANESE ESTIMATION

Amount of plasma taken g.	Total Mn found µg.	Mn content of plasma µg./100 g.
2·0	0·80	40·0
5·0	2·10	42·0
20·0	8·30	41·5

TABLE 3 — BLOOD AND SERUM MANGANESE LEVELS OF PERSONS NOT EXPOSED TO ATMOSPHERES CONTAINING MANGANESE

Sl No.	Blood Mn level µg./100 g.	Serum Mn level µg./100 g.
1	40·0	—
2	31·0	—
3	32·0	—
4	29·3	44·0
5	39·0	44·6
6	29·0	52·0
7	33·8	50·0
8	32·2	30·0
9	—	43·6
10	—	34·0
11	—	30·0
12	—	24·2
13	—	35·2
14	—	47·0
15	—	14·0
Mean val.	33·3	37·4

A standard manganese curve was prepared employing standard solution of potassium permanganate which was reduced by passing sulphur dioxide gas, and different volumes of this solution, containing known amounts of manganese, were passed through all the stages of ashing, treatment with nitric acid, oxidation with bismuthate and colour development with benzidine reagent. Next, known amounts of manganese were added to samples of urine, blood, etc., whose manganese content was being determined in order to find out whether the added manganese was fully recovered. The results obtained with samples of urine (Table 1) indicate that the recovery of manganese was quantitative.

To see if determination from a small sample (2 to 3 g.) agreed with the results obtained from a much larger sample (20 g.) or whether traces of manganese introduced from apparatus, reagents, etc., would give higher values for smaller samples, different amounts (2, 5 and 20 g.) of a particular sample of blood plasma were taken for determination. The results are given in Table 2.

With the modified method for manganese determination as detailed above, manganese concentrations in the blood and serum of persons not exposed to an atmosphere of manganese were determined. The results are given in Table 3.

Discussion

The values for manganese content of whole blood and serum as determined by the present method range from 29 to 40 µg. of manganese per 100 g. of whole blood (mean value 33·3) and from 14·0 to 52 µg. of manganese per 100 g. of serum (mean value 37·4) in persons not exposed to manganese environment. Bertrand and Medigreceanu[1] found the manganese level in human blood to be 5 µg./100 ml. Reiman and Minot[2] in 1920 obtained values ranging from 5 to 15 µg./100 g. (mean value 11·4). While comparing their values with the much lower values of Bertrand and Medigreceanu[1], Reiman and Minot[2] suggested that this might be due either to the difficulty of recovering all the manganese from the ash of blood by Bertrand's method or that the manganese content of the blood of Bertrand's subjects (France) was actually lower than that of the blood of subjects (U.S.A.) analysed by them. They also mentioned that the amount of manganese in blood can be increased by the ingestion of manganese-containing ores. Sykora[3] in 1955 carried out blood manganese determinations of workers in manganese mines exposed to different levels of manganese and showed that the manganese level increases substantially with the increasing degree of exposure.

In view of our findings that the manganese level in the blood of normal persons is 2-2·5 times that reported by Reiman and Minot in 1920, it would be worth while to investigate the average daily intake of manganese and the manganese concentrations in the different foodstuffs consumed at present.

Acknowledgement

We wish to record our thanks to Dr M. L. Rawal, Chairman of the Manganese Poisoning Enquiry Committee, for suggesting the problem and permitting us to publish the results of this investigation, and to Dr H. I. Jhala, Director, Haffkine Institute, for guidance and encouragement. We are grateful to Prof. Jindrich Sykora, Department of Industrial Health and Occupational Medicine, Kunz, Czechoslovakia, for providing us with the particulars of the work carried out by him on manganese. We are also grateful to Dr N. H. Wadia, Honorary Neurophysician, Sir J. J. Group of Hospitals, for the supply of clinical material and for valuable suggestions, and to Drs S. S. Sabnis and R. T. Shabani of this Institute for the supply of blood samples.

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The Effect of Added Manganese in Feed on Various Mineral Components of Cattle Blood

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THE RELATIONSHIP of calcium and potassium to the disease of cattle commonly referred to as "wheat poisoning" has been investigated and reported in earlier publications.[1,2] The possibility of a correlation between the disease and the manganese (Mn) content of wheat resulted from the work of Stuart, Harbaugh, and Dennis.[3] The concentration of Mn in the winter wheat grazed by beef cattle in west Texas was determined by these authors. Their average value of 77 parts per million (p.p.m.) of Mn is not considered to be excessively high, but they have speculated on the relationship of the Mn to the mineral metabolism of the animals.

The present investigation was initiated in an attempt to investigate the effect of Mn, in varying amounts, on the glucose, calcium, magnesium, iron, and potassium levels in the blood of cattle and to determine whether Mn is an etiological factor in wheat poisoning.

PROCEDURE

Five purebred Hereford cows, representative of the Texas Technological College beef cattle herd, were chosen for the experiment. The animals were maintained in a separate enclosure during the entire experiment, and each animal was confined to a separate stall while feeding, in order to control the amount of feed consumed. The diet consisted of 40 lb. of silage, 2 lb. of alfalfa, and 2 lb. of cottonseed meal per animal per day.

Research was begun in November, 1950, and normal blood values were established for each animal. A determination of the Mn content of each of the three components of the diet was made, and sufficient manganous sulfate added to raise the value to 75 p.p.m. of Mn. The silage was found to contain approximately 22.1 p.p.m. of Mn, the alfalfa averaged 31.5 p.p.m., and the cottonseed meal contained about 15.7 p.p.m. The first Mn level was set at 75 p.p.m. on the basis of the work done by Stuart and associates.[3] Six blood samples were taken from each animal at this level, the time between samples averaging seven days. Subsequently, the Mn content of the feed was raised to 100 p.p.m. and five samples were obtained from each animal. Sampling was continued at levels of 150 and 200 p.p.m. of Mn. The administration of Mn was discontinued in June, 1951, and the experiment was concluded in July of that year, after additional normal blood values were obtained.

Stock solutions of manganous sulfate were prepared such that 30 ml. of solution contained the requisite amount of the salt and elevated the Mn content of the feed to the desired levels. The solutions were transferred directly from 30-ml. containers to the silage each day.

Blood samples were obtained in all cases from the external jugular vein and divided into two portions. One portion, utilized in the determination of potassium, glucose, and iron, approximated 50 ml. in volume. Sodium oxalate was utilized as the anticoagulant. The second sample, necessary for the determination of calcium and magnesium, was collected and allowed to clot in two 15-ml. centrifuge tubes. Each analysis was begun within an hour of sampling. Glucose was determined immediately, and the separation of whole and clotted blood into plasma and serum, respectively, was effected at once in order to prevent alteration of potassium and calcium values. The analytical methods have been enumerated in previous papers.[1,2]

RESULTS AND DISCUSSION

The results of the analyses are shown in tables 1 and 2.

In none of the animals examined were there any visible indications of the onset of symptoms of tetany. The thinness and emaciation noted by Harbaugh and Dennis[1] were absent, as were any indications of abnormal excitability. Beyond a normal reluctance to submit to the insertion of the needle, the animals gave no evidence of hyperexcitability. On the contrary, the animals appeared to derive benefit from the experimental diet. Although no detailed data were collected regarding weight, physical

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The data in this paper have been taken from a thesis presented to the Division of Graduate Studies by Miss Fain in partial fulfillment of the requirements for the degree of master of science.

appearance, etc., examinations as the experiment proceeded indicated the over-all health of the animals to be good.

An examination of table 2 indicates that the concentration of magnesium in the blood serum of the animals exhibited a downward trend during the period in which the Mn level was held at 100 p.p.m. The decreased magnesium concentrations were apparent in the animals for about four weeks. The values increased sharply when the Mn level was raised to 150 p.p.m. and they remained at approximately normal concentrations thereafter. These low values

TABLE 1—Normal Blood Values for the 5 Experimental Animals*

Cow (No.)	Glucose (mg./100 cc. blood serum)	Ca (mg./100 cc. blood serum)	Mg (mg./100 cc. blood serum)	Fe (mg./100 cc. blood)	K (mg./100 cc. blood serum)
97	75.20	10.58	1.91	35.50	16.50
149	72.20	10.92	2.01	33.80	16.20
150	75.70	11.04	1.84	32.60	16.50
152	71.30	11.04	2.01	35.40	15.10
157	75.20	11.20	1.87	35.60	17.20

*The figures given are averages of seven determinations.

can not be considered to be indicative of tetany, since concentrations of the order of 0.145 mg. per 100 cc. of blood serum have been noted in tetany due to hypomagnesia.[5] However, the simultaneous decrease of magnesium in all of the animals at 100 p.p.m. of Mn, and the subsequent rise at 150 p.p.m., are considered noteworthy.

The lowered magnesium values are somewhat similar to results obtained by Blakemore and co-workers.[6] They determined that blood magnesium values fell during the first few weeks that cattle were turned out to pasture. The magnesium concentrations then began to rise and attained normalcy within a short time. They found that the injection of Mn solutions produced like results.

Ratios of calcium to magnesium have been calculated and are shown in table 2. It can be seen that the values at 75, 150, and 200 p.p.m. of Mn are in agreement with the value of 5.6 reported for normal cattle by Sjollema and Seekles.[7] The ratios at 100 p.p.m., although somewhat elevated, do not approach the value of 14.6 noted by the same authors in animals suffering from tetany.

It is possible that a disturbance of magnesium metabolism occurs at Mn concentrations of approximately 100 p.p.m. concentrations to which cattle might be subjected in grazing wheat. The question

TABLE 2—Comparison of the Average Blood Values of the 5 Experimental Animals at Manganese Levels of 75 to 200 Parts per Million*

Cow (No.)	Glucose (mg./100 cc. blood serum)	Ca (mg./100 cc. blood serum)	Mg (mg./100 cc. blood serum)	Fe (mg./100 cc. blood)	K (mg./100 cc. blood serum)	K : Ca	Ca : Mg
75 p.p.m. of Mn							
97	61.80	10.48	2.17	39.40	15.80	1.50	4.83
149	60.60	10.42	1.81	32.20	15.80	1.51	5.66
150	67.10	10.39	2.07	35.70	17.20	1.65	5.02
152	63.70	10.21	1.77	34.80	15.90	1.55	5.76
157	67.90	10.77	2.05	34.50	16.40	1.52	5.25
100 p.p.m. of Mn							
97	65.50	10.40	1.26	34.30	13.90	1.33	8.25
149	62.20	10.40	1.33	32.90	15.20	1.46	7.82
150	67.10	10.46	1.59	35.60	15.90	1.51	6.57
152	66.50	10.81	1.49	34.40	16.80	1.55	7.25
157	64.80	10.43	1.46	35.50	16.50	1.62	7.41
150 p.p.m. of Mn							
97	63.70	10.80	2.41	37.30	14.40	1.33	4.48
149	65.60	11.13	2.15	36.00	18.40	1.61	5.24
150	60.80	10.96	2.01	36.80	17.20	1.56	5.45
152	60.80	11.14	1.96	34.50	16.70	1.49	5.86
157	58.50	11.65	2.02	37.70	16.80	1.44	5.76
200 p.p.m. of Mn							
97	61.70	11.16	1.97	35.30	17.40	1.59	5.66
149	72.50	10.19	1.90	36.80	17.80	1.68	5.52
150	65.10	10.77	2.29	38.10	15.50	1.44	4.71
152	67.10	10.31	1.79	35.70	16.20	1.57	5.76
157	67.40	10.71	2.07	39.80	18.30	1.70	5.17

*The values given above are averages of eighteen determinations.

arises as to whether the low magnesium values would prevail if the Mn level were maintained at 100 p.p.m. over a long period. Lack of finances prevents further investigation of the problem at this time.

Since the Mn content of the feed was determined only once, there is the possibility that the concentration in the feed varied sufficiently from time to time to depress the magnesium values. However, it appears unlikely that such variations would occur only at one level of Mn intake. There is the further possibility that metabolic factors not considered in this investigation had some bearing on the depression of magnesium.

The overall-all depression of calcium metabolism, as reported by Reid and co-workers[8] in their studies of the mineral

metabolism of cattle, was not apparent in this study, although some slight variations in calcium values were obtained from time to time. The concentrations of glucose and iron appeared to remain within normal limits throughout the experiment. Certain increases in the plasma potassium are indicated in table 2. However, no consistent trend is apparent, and the variations can probably be attributed to a temporary release of potassium into the blood stream, such a condition being induced by the nervous reaction to the sampling procedure.

SUMMARY

1) No indications of wheat poisoning are shown by cattle which are fed manganese (Mn) in amounts varying from 75 to 200 parts per million (p.p.m.)

2) A decrease in blood magnesium becomes apparent at Mn levels of 100 p.p.m., but normalcy is attained at 150 p.p.m. The depression of magnesium is not clearly understood, and there is some question as to whether continued intake of Mn at a level of 100 p.p.m. would produce still lower values of magnesium, resulting eventually in tetany.

3) The addition of Mn to the diet, in the amounts given, appears to have no significant effect on the concentration or metabolism of glucose, iron, calcium, or potassium.

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A Si[illegible]ed Rapid Determination of Manganese in Biological Specimens by Neutron Activation Analysis

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The quantitative study of *trace* elements requires a method capable of high sensitivity and good accuracy. Neutron activation analysis (1) fulfills such criteria for a wide list of elements.

Neutron activation analysis procedures for the determination of manganese, involving lengthy, time consuming chemistry have been described (2, 3). However, the study of trace metals in biological research generally necessitates the analysis of many samples. It is clear that a rapid, simplified procedure for the determination of trace manganese in biological materials would be extremely useful.

The procedure to be described involves four basic steps:

1. Irradiation in a thermal neutron flux producing the reaction,

$$^{55}Mn\ (n, \gamma)\ ^{56}Mn \qquad (1)$$

2. Rapid simultaneous dissolution of a number of samples.
3. One step precipitation and separation of MnO_2
4. Quantitation of the ^{56}Mn gamma spectrum by means of two NaI (Tl) crystals and a pulse height analyzer (PHA).

MATERIALS AND APPARATUS

The following analytical grade reagents were used: concentrated nitric acid, 16 N, dilute nitric acid, 2 N, manganese carrier containing 50 mg Mn/ml H_2O, as chloride, 10% w/v H_2O solutions of $Y(NO_3)_3$, $Fe(NO_3)_3$, and $NH_4H_2PO_4$ and a 50% w/v H_2O solution of $NaClO_3$. The counting equipment is shown in Figure 1.

[1] U.S. Army Institute of Dental Research.

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PROCEDURE

Sample Preparation and Irradiation. The tissue samples were placed in preweighed, numbered polyethylene microcentrifuge tubes, dried overnight at 90 °C and reweighed. For small samples, a milligram or less, the weight loss of the microcentrifuge tube itself which was from zero to 0.1 mg and occasionally 0.2 mg, required that overnight drying be done at the lower temperature of 80-85 °C to minimize such weight losses. Use of thin walled quartz tubes eliminated this temperature restriction and was done in some actual analyses at a later time. Teeth were dried overnight at 100 °C, stored in individual polyethylene containers in a desiccator until required and then prepared for analysis and weighed into microcentrifuge tubes. A set of capped tubes containing tissue samples were arranged for irradiation by placing them in a larger polyethylene container along with a set of comparable standards. The standards contained a comparable volume and concentration of manganese in water so as to present a physical arrangement similar to the unknown tissue. While perfect physical similarity is of course impossible, reasonable imitation eliminates uncertainties which could arise when physically

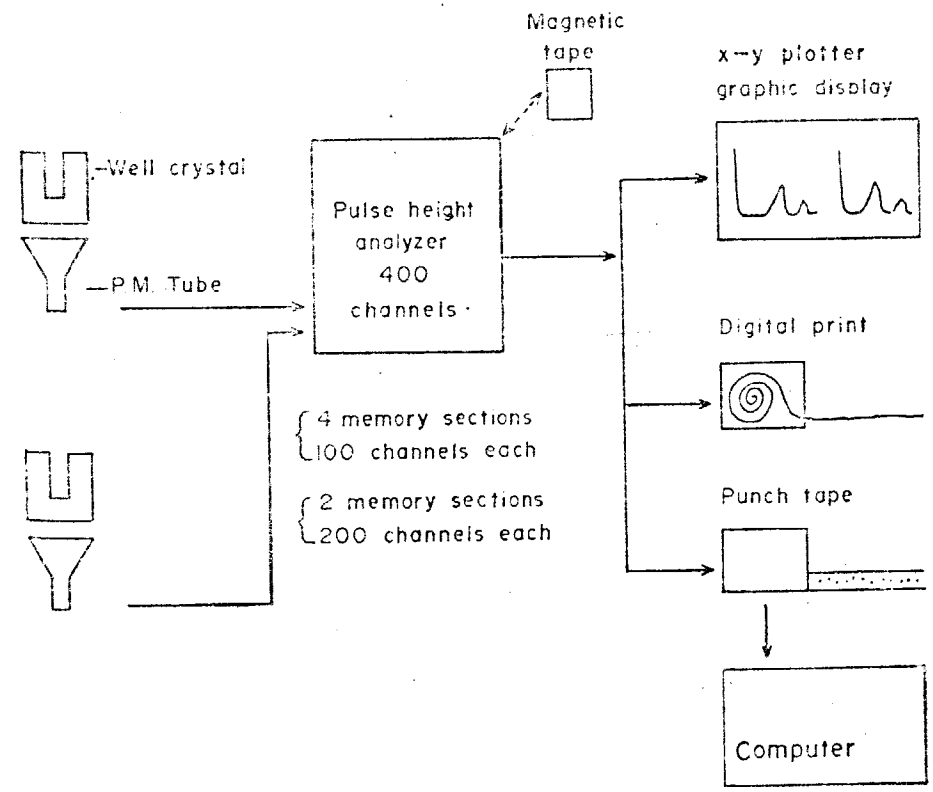

Fig. 1. Dual input, pulse height analysis system with choice of data readout.

differing samples are placed in a reactor with any given neutron flux distribution. Thus, the comparison of a dry powdered standard ($MnCl_2$ salt) with a tissue sample containing manganese in an organic matrix would not be valid (4).

After sufficient irradiation for a given set of samples, generally one half-life of 156 minutes,[1] in a thermal neutron flux of approximately 5×10^{11} n/cm^2 sec, the individual sample tubes were removed from the outer irradiation vessel.

Chemistry. The contents of the polyethylene microcentrifuge tubes were transferred quantitatively to a 50 ml Erlenmeyer flask. The samples were moistened with a few drops of water and 20 mg manganese carrier, 7.5 ml concentrated HNO_3 and three drops each of the other carrier solutions (Cu^{+3}, Y^{+3}, Fe^{+3}, PO_4^{-3}) were added. The Erlenmeyer flask was placed on a sand bath and the solution boiled gently until the sample was dissolved. The resulting clear colorless or brown solution was transferred to a centrifuge tube, combined with an equal volume of water used to rinse the Erlenmeyer, and heated to boiling. One ml 50% $NaClO_3$ was added drop wise to each centrifuge tube which was then set aside until all the resulting MnO_2 precipitations were completed. After centrifugation the precipitate was washed with 2 ml dilute nitric acid, then with 2 ml water and the washes were discarded. The sample was transferred to another preweighed polyethylene microcentrifuge tube for counting according to procedures given in C. After counting, the samples were dried overnight and weighed to establish the chemical yield. The chemical yield generally was about 60-75 per cent. Most of the loss occurred in transferring the MnO_2 precipitate to the small polyethylene tubes for counting and eventual drying and weighing. Had intensity or sample size ever been a problem, more painstaking transfer could have given a yield approximating 100 per cent.

Counting. The standards were taken to the counting room as soon as the outer irradiation vessel had been opened and the individual micro tubes were available. The standards and samples were counted in a two crystal input to a 400 Channel PHA with each crystal signal being stored in half of the memory (200 channels), Figure 1. The duplicate standards were counted alternately in each crystal to afford internal checks on the standards and to evaluate differences in overall crystal and amplifier sensitivity and behavior for the conditions of the particular day's experiment. In addition the use of several standards minimized uncertainties from the positioning of standards and unknowns in the reactor.

A typical plot of the resulting count (Fig. 2) shows the counts observed as a function of energy to be relatively free from interferences. The ^{56}Mn peak is at 0.84 MeV for both the standard and the unknown. The data readout from the PHA memory is recorded graphically by an x,y plotter. The same data could be handled by a computer using punched tape or direct transmission readout. In our

[1] Because of the high sensitivity of this procedure it is entirely feasible to use a much shorter irradiation period, longer counting times and smaller sample sizes. All these options, though readily available, do not change the essential procedure. The possible contribution from [illegible] ^{59}Mn was considered and found to be negligible under the conditions; had the Fe content of samples been relatively high compared to the Mn a correction for this would have [illegible]

work the heights of the peaks were determined manually by [illegible] of an ordinary ruler with millimeter scale. The display value, full scale value, time and duration of the counts were noted. Since each unknown was generally counted at the same time as a standard, no decay correction was needed. A crystal correction determined each day by intercomparison of standards in the two crystals was always used.

Calculation of the manganese content of the unknown was done by use of a simple equation:

$$(2) \quad \frac{\text{ug in}}{\text{unknown}} = \frac{\text{ug in}}{\text{standard}} \times \frac{\text{peak height unknown}}{\text{peak height standard}} \times \frac{\text{crystal correction}}{\text{chemical yield fraction}}$$

The chemistry used proved reproducible and selective enough for manganese[1] quantitation on the pulse height analyzer by simple peak height measurement. Each new material analyzed had its 0.84 MeV peak followed for a period of time

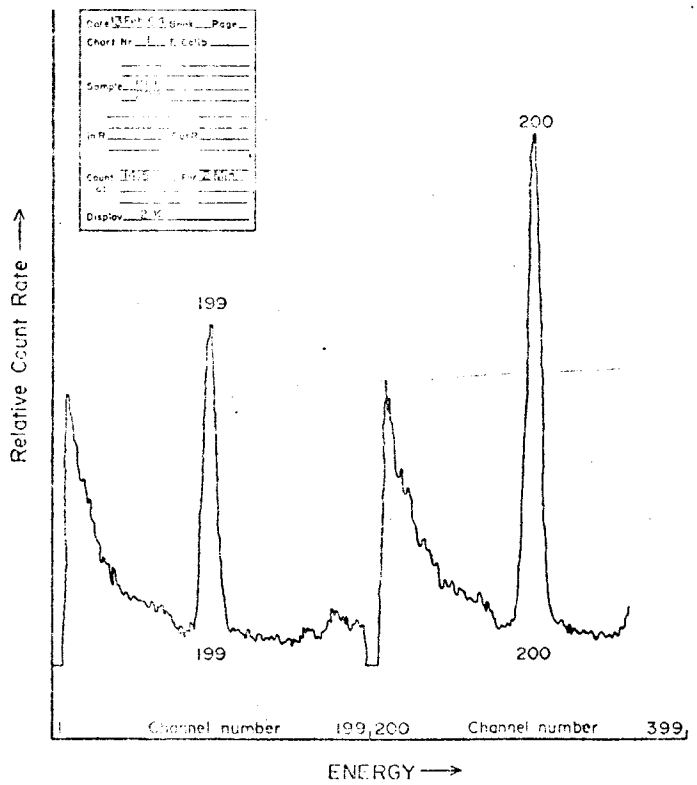

Fig. 2. Spectrum showing *standard* and *unknown* manganese peaks.

[1] More detailed spectrum analysis was unnecessary because of the relatively uncomplicated spectrum obtained.

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to establish by its decay period its nature as manganese-56. This means that a chemical separation, an energy determination, and a half-life determination have contributed to the conclusion that manganese is in fact being measured. After this was done for each new material investigated, the height of the 0.84 MeV peak was sufficient for routine quantitation of manganese.

RESULTS

Test analyses were carried out on two kinds of standard materials.

Soft Biological Materials. Analyses were carried out on soft materials consisting of pooled homogenized dried rat liver, pooled homogenized beef livers and certain other samples, as a function of age of the rat. These analyses were paralleled by separate analyses on aliquots of the same materials carried out by means of atomic absorption.[1] A comparison of the values obtained by the different methods is shown in Table I. Agreement is seen to be good. The accuracy is presumed acceptable since two different methods carried out at two different labor-

TABLE I

COMPARISON OF RESULTS ON POOLED RAT LIVER AND OTHER LIVER SAMPLES

Sample	*Activation Analysis*	*Atomic Absorption**
362**	7.68 ± .75(6)	8.94
363**	7.98 ± .60(6)	8.27
373	5.76	7.8
374	5.60	8.4
375	11.1	8.75
376	9.1	6.66
377	2.64	3.2
378	2.85	
379	6.20	7.4
380	5.94	6.8
381	9.65	8.5
382	9.30	8.3
383**	8.31 ± .02(2)	7.3
384**	7.48 ± .08(2)	7.4

*Atomic absorption data by Gerry Powell, M.D. and Donald B. Cheeck, M.D., Johns Hopkins Hospital. Samples weighed 500-1000 mg.

**Pooled liver samples; mean values and average deviations from mean in a set (6) of samples. All results are ug/g dry weight; samples were approximately 5-25 mg.

[1] We are indebted to Dr. D. B. Cheek and Dr. Geraldine Powell, of the Johns Hopkins Hospital, Baltimore, Maryland, for supplying the standard materials along with the results of their analyses.

TABLE II

PRECISION OF ANALYSIS FOR MN IN DENTIN AND ENAMEL OF CERTAIN HUMAN TEETH

Sample	*Enamel ug/g*	*Dentin ug/g*	*Ratio*
559	.289		
560		.107	2.70
563	.581		
564		.164	3.54
565	.284		
566		.174	1.63
567	.723		
568		.296	2.44
569	.336		
570		.145	2.32
571	.622		
572		.202	3.07
573	.785		
574		.292	2.69
675	.076		
676	.051		
677		.037	2.17
678		.021	
679	.145		
680	.110		
681		.042	2.56
682		.058	
683	.451		
684	.533		
685		.172	3.43
686		.117	
687	.445		
688	.458		
689		.189	2.39
690		.188	

Paired and grouped sample numbers, *i.e.*, 559, 560 and 675, 676, 677, and 678 represent enamel and dentin samples from one tooth.

alicies gave similar results. It is believed this represents a better procedural test than the *recovery* of added manganese (5).

Hard Biological Materials. Some analyses were carried out on normal caries-free human teeth in order to evaluate the importance of sample preparation for hard biological where separation of the desired sample was more difficult. In Table II, columns 2 and 3, are shown the results of analyses of dentin and enamel from single teeth. The duplicate results seem to be in good agreement. These results also have an internal consistency. For a given tooth, the ratio of enamel manganese content to dentin manganese content is approximately 2.5.

These results are in good agreement with the results of one group (6). But these results are in very substantial disagreement with the higher enamel values reported by other workers on the basis of emission spectroscopy (7). It was felt that this disagreement might be attributed, at least in part, to their method of separating enamel from dentin, grinding and cutting. Therefore, tests were run on the analytical preparatory procedures of grinding and cutting versus our own procedure of chipping and crushing with polyethylene covered instruments. Duplicate enamel samples in each case from the same teeth were prepared for analysis by cutting with a tungsten carbide bur or by cutting with a small diamond dust impregnated bur, as opposed to the standard polyethylene protected chipping and crushing procedure. The results of the two kinds of handling procedures when the samples were subjected to the same manganese analysis are shown in Table III, columns 2 and 3. The manganese content of the samples described in the upper sections of columns 2 and 3 of Table [illegible] show a difference which is significant at less than the four per cent level[1], compared to the similarities in columns 2 and 3 of Table II. Manganese is not a major constituent of the tungsten carbide bur used in this test. Tungsten, which is a major constituent of the bur, shows up strongly through the chemical procedure, though not at the same energy as the manganese. The tungsten peaks appear at 0.48 and 0.68 MeV and in the spectrometric measurements used did not interfere with the manganese measurement.

TABLE III

MANGANESE CONTENT OF ENAMEL SEPARATED FROM DENTIN BY GRINDING OR CHIPPING PROCEDURES

Tooth No.	*ug Mn/g Found* — *Method of Separation* — *Tungsten Carbide Bur*	*Polyethylene Protected Chipping*	*Per Cent Increase of Mn in Ground Enamel Over Chipped Enamel*
10.6	0.98	0.72	27
10.9	1.05	0.43	145
10.11	1.05	0.71	45
13.14	0.65	0.46	40
16.24	1.44	0.90	60
16.25	1.38	0.61	128
	Diamond Stone		
15.19	1.35	1.11	22
15.20	1.11	0.84	32
15.22	1.13	0.72	58
16.23	0.88	0.73	21
16.26	0.95	0.62	51

The other pulverizing procedural test in which the cutting of the tooth was by means of a diamond dust impregnated bur gave, once again, results higher in manganese than samples of the same tooth which were crushed in a polyethylene sheet with no farther handling. The sample dust prepared by grinding was collected by gravity on a clean glass surface within a totally inclosed volume consisting of a small beaker with a diaphragm through which the grinding head was introduced. In the work cited (7) the dust collection was by an air suctioning device which collected in the dust in a stream of air.

CONCLUSIONS

The procedure described for activation analysis of trace manganese in biological material is quite simple and rapid. It is capable of good precision and accuracy as determined by comparison to the atomic absorption procedure done on relatively large samples at another laboratory. Thirty analyses could be run in parallel by one technician and all measurements completed in less than one full working day.

When applied to teeth, the accuracy of the method probably suffers from inability to make a standard of a comparable physical arrangement. However, good agreement with results reported by one group of workers was obtained and a reasonable explanation presented for results substantially lower than some other reported data.

[1]We are indebted to Major Miriam K. Ginsberg, ANC, for these statistical analyses.

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Manganese in Rabbit Tissues

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Although many papers have appeared on the manganese contents of the tissues of various animals, in no case has a survey been recorded of the concentrations in all the tissues of a single animal.

Such a survey might provide a better clue to function than comparisons of the manganese contents of tissues from different individuals or species.

EXPERIMENTAL

Animals and materials

The rabbit was chosen for this investigation because it is of a convenient size for obtaining reasonable amounts of small bodies such as the pituitary and pineal glands. Four animals were used; samples of most tissues were obtained from an adult female chinchilla and of the other tissues from the remaining three. Specimens of incompletely separated adrenal cortex and medulla, thymus and thyroid were obtained from a resting adult female chinchilla; specimens of mammary gland, thyroid and lung were obtained from a lactating female albino, and samples of pancreas, thyroid, testes and spinal cord from an adult male chinchilla. Liver samples from each animal were used as controls. The pineal and pituitary glands from the last two animals were examined. The separation of the glandular nucleus and muscularis tissue was only approximate. All dissections were made with carefully cleaned stainless steel instruments.

Using portions of kidney and liver from the first rabbit, homogenates were prepared and dialysed in cellophan bags against changing glass-distilled water for 10 days, toluene being added as preservative. The residue in each bag was divided into two portions and the Mn content of each determined.

Method of analysis

Mn was determined by a modification (Fore & Morton, 1952*a*) of a method described by Kun (1947) based on the catalytic oxidation of dithyloniline by MnO_4^- and periodate to give a coloured product absorbing at 470 mμ. The experimental error is from ±10 to ±20% depending on the size of the sample.

RESULTS

The findings on rabbit tissues are recorded in Table 1. The manganese contents based on fresh tissue weight are listed in decreasing order in Table 2. For comparison, (*a*) the values reported in the literature for each rabbit tissue have been averaged, and (*b*) the values reported for each type of tissue irrespective of species have been averaged. The published figures for the rabbit were obtained from papers by Bertrand & Medigreceanu (1912*a*, *b*; 1913), Ellis, Smith & Gates (1947), Lorenzen & Smith (1947), Lund, Drinker & Shaw (1921), Kay (1938) and Richards (1930). The figures in the right-hand column of Table 2 are averages obtained by attaching the same weight to all the published figures which can be traced. The number of papers consulted is about thirty-five and a full list of references would take up too much space.

The results of the dialyses are given in Table 3, and if the figures 2·0 and 1·2 p.p.m. are accepted for the manganese contents of the liver and kidney homogenates, respectively, it appears that in the liver 74% of the manganese, 72% of the dry matter

Table 1. *Experimental data on rabbit tissues*

Tissue	Fresh tissue wt. (g.)	Dry matter (%)	Ash as % of fresh tissue	Ash as % of dry tissue	Manganese content in p.p.m. in Fresh tissue	Manganese content in p.p.m. in Dried tissue	Manganese content in p.p.m. in Ash
Pituitary	0·0078	12	—	—	3·9	31	—
Pituitary	0·0071	11	—	—	1·4, 2·4	9·7, 17	—
Pituitary	0·0063	24	—	—	1·8	7·6	—
Adrenal	0·713	90	1·5	1·6	0·99	1·1	67
Adrenal	0·816	87	0·56	0·65	0·95	1·1	170
[illegible]	1·32	46	0·72	1·6	0·38	0·82	53
[illegible]	4·97	23	1·1	4·7	0·021	0·10	2·2
[illegible]	1·51	22	1·0	4·5	0·22	0·97	21
[illegible]	0·640	53	0·99	1·8	0·045	0·087	4·7
Bone marrow	0·723	18	1·1	6·0	1·7	9·6	160
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Kidney (one)	7·11	26	1·3	4·9	1·2	4·7	97
[illegible]	1·41	20	1·2	6·1	1·1	5·6	92
Muscle	1·03	18	1·2	6·8	1·6	8·6	130
Muscle	2·22	17	0·91	5·5	0·50	3·1	55
Muscle	1·42	22	1·6	7·1	1·4	6·2	88
Salivary gland	1·75	23	1·1	4·7	1·0	4·4	95
Stomach	1·08	17	0·79	4·7	0·82	4·9	100
[illegible]	1·61	29	1·7	8·5	0·48	2·4	28
[illegible]	1·06	22	1·0	4·5	0·28	1·3	28
Heart	3·84	30	1·6	5·3	2·0, 2·2	6·7, 7·6	130, 140
Heart	3·03	29	1·5	5·3	2·4	7·9	160
Heart	2·79	24	1·2	4·9	2·0	8·1	170
Heart	3·29	25	1·2	4·9	2·2	8·7	180
Pancreas	0·225	74	1·1	1·5	1·61, 1·67	2·11, 6·2	140, 110
Pancreas	0·297	26	1·5	5·8	1·6	4·2	130
Adrenals (two)	0·342	49	1·5	3·1	0·67	1·3	44
Cortex	0·290	42	1·1	2·7	0·56	1·3	50
Medulla	0·027	39	—	—	0·69	1·5	—
Ovaries (two)	0·684	23	1·5	6·3	0·69	2·6	41
Mammary gland	1·88	62	0·31	0·50	0·25	0·41	82
Mammary gland (lactating)	0·738	45	1·2	2·7	2·2	4·8	189
Brain	2·75	22	1·5	6·9	0·36	1·6	24
Muscle	2·07	23	1·2	5·0	0·13	0·56	11
Blood	0·0061	15	—	—	3·8, 0·83	26, 3·3	—
Blood	0·0018	—	—	—	2·3	15	—
Gall bladder	0·431	27	1·7	6·6	0·91	3·4	53
Skin	0·178	11	1·4	3·5	0·086	0·21	6·1
Skin	0·0798	34	1·3	3·7	0·29, 0·21	0·84, 0·76	23, 85
Skin	0·122	35	0·25	0·75	0·24	0·79	12, 17
Skin	0·112	29	1·3	4·7	0·24	0·79	17
Testes (two)	0·0421	32	—	—	0·36	1·2	—
Spermatozoa	0·393	31	1·7	5·5	0·30	1·3	23
Bone	0·068	18	0·99	5·4	0·011	0·061	1·1
Bone	0·018	67	—	—	0·45	0·67	—

[illegible] 52% of the ash was dialysable. In the kidney [illegible] of the manganese, 49% of the dry matter and 88% of the ash was dialysable.

DISCUSSION

Although simple distribution studies of elements or compounds have in some instances provided valuable clues to specialized function the figures given in Table 2 do not indicate a localized role. The ubiquitous occurrence of manganese in the animal body suggests a role in general cell metabolism as [illegible] to metabolic processes characteristic of particular tissues. Such a role for manganese would not of course exclude other roles restricted to those tissues with a specially higher manganese content. Dialysis of kidney and liver homogenates showed that some of the manganese is loosely held and some of it very firmly combined. If it is assumed that in all tissues having somewhat higher manganese contents, a portion of the manganese is dialysable then the already narrow range for total manganese (Table 2) becomes even narrower for the bound manganese. The small range of manganese concentrations is consistent with a general metabolic role. The fact that little or no dialysable manganese is present in ox retinas which contain only 0·13 p.p.m. of manganese (see Fore & Morton, 1952c) supports the idea that it is only those tissues relatively rich in manganese which contain the element in dialysable form.

Indications have been obtained in other work (Fore & Morton, 1952*b*) that some of the manganese in bone and bone marrow is attached to the cell membranes. It is therefore interesting to consider the average proportion of manganese to protein in the tissues. If 0·5 p.p.m. (Table 2) is taken as the approximate tissue content of non-dialysable manganese and 10% as an average for protein in tissue, then the proportion of manganese with respect to the protein is 5 p.p.m. There will thus be 55 parts of manganese to 11 million parts of protein and assuming one (or two) molecules of protein per atom of manganese a protein of very high molecular weight is implied. If the manganese is, however, preferentially attached to the cell-wall protein such protein would need to have a molecular weight of the order 10^6. This figure, though large, is not impossibly so since, for example, the muscle protein myosin has a molecular weight of that order. The concept of manganese attached to the cell membrane is not entirely new. Albert, Rubbo, Goldacre & Raffoni (1947) suggested that oxine inhibited bacterial growth by combining with essential metals in the bacterial surface, and Gale (1949) has shown that oxine inhibits glutamic acid assimilation in bacteria by combination with a metal in the cell, probably manganese.

The relatively high manganese contents of liver and lactating mammary gland could be accounted for, at least in part, by a high arginase content, an

Table 2. *Rabbit tissues arranged in order of decreasing manganese content*

Tissue	Mn content in fresh rabbit tissue (p.p.m.)	Averages of figures recorded in the literature for rabbit	Average of figures recorded in the literature for a wide range of species
Bone (long)		3·5	3·3
Pituitary	2·4	-	2·5
Pineal	2·3	-	-
Mammary gland (lactating)	2·2	-	0·26
Liver	2·1	1·9	2·5
Ileum	1·7		-
Duodenal mucosa	1·6	-	-
Pancreas	1·6	2·3	1·9
Salivary gland (submaxilliary)	1·4	-	1·1
Kidney	1·2	1·1	1·2
Duodenum	1·1	-	1·9
Stomach	1·0	0·90	0·80
Colon	-	1·0	0·75
Hair	0·99	1·2	2·4
Gall bladder	0·91	1·0	1·1
Caecum	0·82	0·43	0·43
Salivary gland (parotid)		—	0·70
Intestine (small)	—	0·70	0·45
Adrenals	0·67	-	0·40
Lymph nodes	—	-	0·65
Ovaries	0·60	-	0·55
Adrenal medulla	0·60	—	-
Adrenal cortex	0·56	-	
Duodenal muscularis	0·50	—	-
Bile	0·48	-	0·90
Thymus	0·45		1·6
Spinal cord	0·39	-	[illegible]
Hide	0·38	0·20	[illegible]
Intestine		0·38	[illegible]
Testes	0·36	0·40	[illegible]
Brain	0·36	0·60	0·40
Uterus		0·35	0·33
Heart	0·28	0·21	0·34
Mammary gland (resting)	0·25	—	0·14
Thyroid	0·24	-	0·55
Spleen	0·22	0·55	0·41
Muscle	0·13	0·14	0·18
Milk		-	0·045
Bone marrow	0·045		0·050
Blood	-	0·03	0·049
Fascia	0·021		-
Lung	0·011	0·47	0·20
Urine	—	0·00	0·001
Parathyroid	—		

Table 3. *Retention of manganese after dialysis of rabbit liver and kidney homogenates*

Wt. of tissue homogenate (g.)	Dialysis residue: Dry wt. (g.)	Dialysis residue: Ash wt. (mg.)	Ash on dry matter (%)	Mn from sample (μmg.)	Mn (p.p.m.) on: Dry wt.	Mn (p.p.m.) on: Ash wt.
13·9 g. liver	0·593	8·6	1·5	4060	6·9	470
	0·583	9·6	1·6	3410	5·8	360
Totals	1·177	18·2	-	7470	-	-
Means	—	-	1·6		6·4	420
5·67 g. kidney	0·342	4·5	1·3	2270	6·6	510
	0·402	4·8	1·2	1750	4·4	360
Totals	0·744	9·3	-	4020	-	-
Means	—	-	1·3	-	5·5	440

the somewhat high manganese content in the tissues of the intestinal tract might similarly be attributed to manganese present as part of the peptidase system.

SUMMARY

1. The manganese contents of rabbit tissues have been determined, and the results compared with those obtained by previous workers.

2. The range covered by the results is narrow, especially as some of the tissues relatively rich in manganese contain much of it in dialysable form.

3. It is suggested that manganese participates in general cell metabolism while attached to the cell-wall protein.

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Microdetermination of Manganese in Biological Material by a Modified Catalytic Method

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In investigating the biochemical roles of manganese it is desirable to employ a very sensitive but reasonably accurate method of estimating the element. Methods based on the detection of manganese lines in emission spectra require the presence of about 50 μmg. of the element in a 10 mg. sample, and even when determinations are carried out in triplicate the accuracy obtainable is not great.

The periodate method, based on oxidation of the manganese to permanganate, which is determined by photoelectric colorimetry or spectrophotometry (Willard & Greathouse, 1917; Richards, 1936; Strickland & Spicer, 1949; Davidson & Capen, 1939; Mehlig, 1939, 1941; Kirk, Rosenfels & Hamilton, 1947; Rowland, 1939) is generally satisfactory. The main disadvantage of the periodate method is that the amount of tissue needed to provide the necessary minimum of manganese is quite often larger than that available. The colorimetric estimation of manganese by means of the Folin-Ciocalteu reagent (Abul-Fadl, 1948) requires at least 10 μg. manganese.

Szebelledy & Bartfai (1936) described a catalytic method for the detection of manganese in amounts less than 1 μmg., and Kun (1947) worked out a quantitative procedure, using photoelectric spectrophotometry. Manganous ions catalyse the oxidation of *NN*-diethylaniline by potassium metaperiodate (KIO_4) in aqueous solution, and the progress of the oxidation can be followed by measuring the increasing absorption of light at 470 mμ. Kun used a medium buffered at pH 7·0 and measured the extinction exactly 200 sec. after mixing the reagents. He found no interference by ions of other metals. Each test required at least 4 μmg. of manganese, but Kun found it necessary to make many readings so as to eliminate the effect of what appeared to be random variations.

The catalytic method appeared to be very promising, but a considerable study of all the variables was necessary before it could be applied with confidence to the problems of determining manganese in very small amounts of tissues. The final procedure adopted will first be described and then discussed.

EXPERIMENTAL

Reagents

[illegible] must be stored in Pyrex vessels.

Glass-distilled water. [illegible] water from the usual type of "[illegible]" is redistilled from all-glass Pyrex apparatus.

Hydrochloric acid. Spray free hydrogen chloride is passed, via a wax-coated funnel, into glass-distilled water in a wax-coated beaker immersed in ice. The acid (approx. 16% w/v) is stored in wax-lined bottles.

[illegible] solution. [illegible] H 7.0. This is prepared by mixing [illegible] 0·1 N and [illegible] (approx. 50 ml.) and [illegible] to 100 ml. Solution *a* consists of citric acid (A.R., 10 g.) and 'pure' conc. HCl (20 ml.) in 2 l. Solution *b* is prepared as follows: approx. 2 N-NaOH (A.R. carbonate-free) is standardized and [illegible] (A.R., 122 g.) is dissolved in exactly 2·5 equiv. of the alkali solution and the whole made up to 2·5 l.

Diethylaniline hydrochloride solution. Diethylaniline (42 g.) is weighed accurately and dissolved in 1·4 equiv. of N-HCl. The solution is made up to 1 l. (0·006 M).

Potassium periodate solution. KIO_4 solution (0·01 M) is prepared by [illegible] g. of the A.R. salt in 800 ml. of hot glass-distilled water and diluting to 1 l. in a volumetric flask.

Manganese solution. A.R. $MnSO_4.4H_2O$ was used for preparing the standard manganese solution.

Method

After cleaning, a Vitreosil crucible is cleaned in the usual way (soaked in conc. HCl, washed in glass-distilled water and heated for 30 min. at 600°). It is cooled in a desiccator, then weighed and after incorporating fresh tissue sample, weighed again. The tissue is dried to constant weight at 110° and is then ashed at not over 650° in a silica-lined muffle furnace. Glass-distilled water may be added to the ash residue to assist removal of the last traces of carbon (this step is optional but effective). The crucible is cooled and weighed, platinum-tipped crucible tongs being used throughout.

A measured volume of the 16% HCl is added to the crucible (0·5–1 ml. for ash from 100 mg. tissue) and left on a steam bath until the HCl has evaporated. Buffer solution (which depends on the amount of Mn in the sample) is added to the crucible which is then placed on the steam bath for 30 min. 2 ml. of buffer is used for samples containing up to 50 μmg. including the Mn contaminating the HCl. With larger samples proportionately more buffer is added. When the amount of Mn is very large the crucible is cooled, a known volume of HCl is added and with buffer and the solution washed into a graduated flask of suitable size. After making up to volume, a portion of the solution is transferred to a carbon crucible, evaporated to dryness and made up in buffer as described above. The beaker is then removed and allowed to cool, protected from dust. With the aid of a small Pyrex funnel and a wash-bottle (Pyrex) with the shortest possible rubber connexion, the contents of the crucible are washed into a graduated flask of 8 or 10 ml. (2·5 times the volume of buffer taken. Pyrex graduated flasks should be used throughout).

For such buffered solutions of ash are made up, together with blank and two graduated buffer alone and a standard consisting of diluted buffer containing 4 μmg. Mn/ml.

These six solutions, the reagents, six 1 ml. pipettes and 36 Pyrex test tubes (9 × 75 mm. with rim), together with other necessary apparatus, are left in the instrument room overnight. Within 24 hr. of making the buffered ash solutions and at a time when the room temperature is fairly stable three 1 ml. portions of each buffered solution are discharged into test tubes and 1 ml. portions of KIO_4 solution into the remaining 18 tubes. Taking the solutions in the order in which they were pipetted (three blanks, four × three buffered ash solutions, then three standards) 0·5 ml. of diethylaniline hydrochloride solution is added from a graduated pipette to the buffered solution. The mixture is immediately poured into the KIO_4 solution, the time at that instant noted on a stop-clock and the reaction mixture mixed by repeatedly pouring from one tube to the other. The solution, in which the colour is now developing, is transferred to a clean dry absorption cell (1·0 cm.) and placed in the photoelectric spectrophotometer. The *E* value of the solution is read exactly 60 sec. after mixing.

The collected using the reaction mixture is rinsed out four to six times with glass-distilled water, three times with redistilled absolute ethanol and three times with redistilled ether. It is then allowed to dry with a current of dust-free air blowing through it and another cell, used in the previous measurement, is used for the next reaction mixture. At the end the cells, pipettes used in the series and flasks are washed out in the manner described for the cells. The test tubes are all cleaned carefully before use. New ones are first cleaned with abrasive soap, but the procedure used for cleaning the tubes for each run is as follows: the tubes are filled with HCl (1 vol. conc. acid + 1 vol. water) and allowed to stand for a few minutes. The acid is poured out and the tubes rinsed three times with tap water, three times with distilled water and three times with glass-distilled water. The tubes are then drained, dried in an electric oven and stored away from dust.

The mean of the three extinction values (*E*) for each buffered solution is obtained. The means for the blank and standard are used to fix the standard straight line from which the Mn concentrations corresponding to the *E* values for the four test solutions are interpolated. From the Mn concentration in the reaction mixture the amount of Mn derived from each sample can be calculated and hence the Mn concentrations in fresh tissue, dried tissue and ash.

RESULTS

The above procedure was developed as a result of an exhaustive investigation of the reaction conditions. The experimental findings can be summarized as follows:

(*a*) A minimum of 1 μmg. of manganese is required for a single determination, but owing to variability in the colour intensity produced, three determinations must be made for each sample examined. In practice the sample should contain at least 5 μmg. of manganese. With the reagents used in this method, the extreme sensitivity (to 0·1 μmg.) claimed by Szebelledy & Bartfai in 1936 for a catalytic method of detecting manganese was not attainable in our hands.

(*b*) The manganese concentrations in the reaction mixture itself could be determined to ± 10%

Table 1. *Comparison of the catalytic and periodate methods*

Wt. of liver taken (g.)	Sample taken	Estimated Mn (μg.)	Total Mn from liver (μg.)	Mn content (p.p.m.)
		Periodate method		
9·98	—	27·2	27·2	2·73
10·26	—	27·3	27·3	2·67
				Mean 2·70 ± 1%
		Catalytic method		
10·24	1/100	0·303	30·3	2·96
	1/100	0·274	27·4	2·67
10·36	1/100	0·262	26·2	2·53
	1/100	0·265	26·5	2·56
				Mean 2·69 ± 10%

(deviation from the mean) over the range 0·4–4·0 μmg./ml. and this permitted similar accuracy in determinations of the manganese contents of moderately large samples of tissue. With smaller samples (containing less than 10 μmg. Mn) reproducibility was not quite so good, possibly owing to contamination, and the accuracy was about ± 20%. From experience the accuracy attainable for the determinations of manganese concentration over the range 0·4–10 μmg./ml. was ± 15%.

(*c*) The rate of the reaction was directly proportional to the manganese concentration under the conditions already described. Kun obtained a logarithmic relationship but his experimental conditions differed considerably from ours.

(*d*) The possibility of interference was studied for the following ions: Cr^{+++}, Co^{++}, Cu^{++}, Fe^{+++}, Ni^{++}, Mg^{++}, K^{+}, Hg^{++}, Sn^{++}, Pb^{++} and Zn^{++}. In each case phosphate ions were present in the buffer. The only effect noticed was that the first three ions exerted an inhibitory effect, but in no case was it shown at a concentration small enough to have relevance to the determinations on tissues.

(*e*) To obtain satisfactory results the pH must be kept constant to within 0·1 unit. The reagents need to be kept overnight in the instrument room and the actual determinations on the spectrophotometer must only be made when the room temperature is steady.

(*f*) By using diethylaniline hydrochloride solution containing excess hydrochloric acid, it was found that the reagent need not be freshly prepared, but should not be more than a fortnight old.

(*g*) It was important to adhere exactly to the procedure described for mixing the solutions, otherwise the intensities of absorption and the shape of the standard curve varied markedly.

(*h*) The addition of hydrochloric acid to the ash and subsequent evaporation to dryness is an essential step in ensuring that the ash is soluble in the buffer. It also serves to convert any Mn^{++} or Mn^{+++} present in the ash to Mn^{+}. The hydrochloric acid contained a trace of manganese, even when prepared as described (p. 595), and allowance had to be made for this in working out the results.

(*i*) Excellent agreement was obtained when the manganese content of ox liver was determined by both the catalytic method and the periodate method using large samples. The results of such an experiment are given in Table 1.

Table 2. *Comparison of the catalytic and periodate methods using small samples for the former on silica or platinum crucibles for both*

Wt. of liver (g.)	Estimated Mn (μg.)	Mn content (p.p.m.)
	Periodate method (silica crucibles)	
3·05	6·5	2·1
2·90	5·0	1·7
2·18	4·0	1·8
		Mean 1·9 ± 11%
	Periodate method (platinum crucibles)	
1·90	4·5	2·4
2·11	4·7	2·2
		Mean 2·3 ± 4%
	Catalytic method (silica crucibles)	
(mg.)	(μmg.)	
2·17	6·2	2·5*
1·24	5·1	4·1*
1·18	0·6	0·5*
1·23	1·8	1·5*
		Mean 2·2 ± 77%*
	Catalytic method (platinum crucibles)	
1·14	9·9	8·7
1·12	8·6	6·8
		Mean 7·8 ± 13%

* These results show that the method is only semi-quantitative for amounts of material as small as about 1 mg.

(*j*) Samples ashed below 650° in silica crucibles lost some manganese by combination with the silica and were therefore expected to give low results. When very small samples for determination by the catalytic method were ashed in platinum

crucibles, however, the results obtained were higher than those obtained by the periodate method using [illegible] samples also ashed in platinum crucibles. The results obtained by the catalytic method using very small samples ashed in silica crucibles agreed well with those (presumably the true ones) obtained by the periodate method using platinum crucibles. This was presumably due to a fortuitous compensation for the loss due to combination with the silica by some unknown factor. Table 2 illustrates this point with the results obtained when the method was applied to a protein powder prepared from ox liver.

(*m*) When the sample examined (e.g. blood) contained a large excess of iron the reaction mixture was coloured green and the readings obtained at 470 mμ. were below those for the blank. Thus determination of manganese in such material by the catalytic method was not possible.

Mechanism of the reaction

No direct attempt has been made to elucidate the mechanism of the catalytic process, but the development of the method yielded some relevant information.

Table 3. *Results of experiments with added manganese*

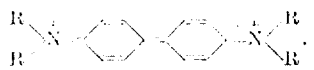
Sample of tissue (mg.)	Mn added (μmg.)	Mn estimated (μmg.)	Mn content (p.p.m.) Estimated	Calculated
54·2	Nil	11·7	0·22	
54·2	Nil	10·7	0·20	
			Mean 0·21	
54·2	10·0	21·1	0·39	
54·2	10·0	25·2	0·46	
			Mean 0·43	0·39
54·2	20·0	29·8	0·55	
54·2	20·0	36·2	0·67	
			Mean 0·64	0·58
54·2	30·0	41·8	0·77	
54·2	30·0	38·6	0·71	
			Mean 0·74	0·76

This table also shows that, for samples containing [illegible] 5 μmg. of manganese, the deviation of the results from the mean is no longer within $\pm 20\%$. Kun observed good agreement between his catalytic method and a method based on the oxidation of manganese to MnO_4^-, but in making the comparison he used relatively large samples only.

(*k*) A puzzling phenomenon was observed in some calibration experiments. The usual procedure of adding known amounts of manganese to the ash of a sample and determining the manganese present was followed. The individual differences between calculated and observed manganese content bore no relation to the relative amount of added manganese and the observed values were, on the average, 10% higher. Table 3 gives the results of one such experiment in which eight samples of an acid solution of an ox eye-tissue (sclera) ash were evaporated in [illegible] crucibles, known amounts of manganese being added to six of them. The manganese in all the crucibles was then determined.

(*l*) It was hoped that by omitting the phosphate constituent of Kun's buffer the catalytic method might be adapted to the determination of manganese in bone but interference by the large excesses of calcium and phosphate ions proved an insurmountable obstacle.

It appears that the potassium periodate oxidizes Mn^{++} to MnO_4^- probably by the mechanism studied by Strickland & Spicer in 1949. The MnO_4^- then oxidizes the diethylaniline to a 1:4-quinonoid compound, possibly a diquinone of the type



The manganese is then reoxidized to MnO_4^- by the periodate. Under conditions where there is excess of periodate and diethylaniline, as in the early stages of the reaction, a direct relationship between manganese concentration and rate of reaction would be expected when only a trace of the catalyst is present.

SUMMARY

1. A procedure for the determination of amounts of manganese of the order of 5 μmg. is described. Precise adherence to the instructions is essential for the success of the method.

2. The method cannot be applied to the determination of manganese in bone, nor, without further study, to manganese in blood.

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The Effects of Inorganic Ions on the Activity of Serum Cholinesterase

II. In vivo Studies with the Guinea Pig

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In a preceding work (Frommel, Herschberg and Piquet [1]) we studied modifications of the activity of horse serum cholinesterase in vitro. In this way we have ascertained that a large number of inorganic ions inhibit this enzyme's activity and that others encourage enzymatic activation.

This study performed outside the living organism can only give partial results. We have judged it necessary to complete our experiments with in vivo studies, in order to be able to develop more generalised statements.

We consider these studies to be of more practical value, because in this case we can not only understand the direct toxic effect on serum cholinesterase, but also the total effect on the whole organism with its defense and equilibration faculties still intact.

We can also make more precise conclusions about the pharmacodynamics of inorganic ions and the pathogenic mechanism of certain vagotonic symptoms from intoxication by these ions.

Another point deserves to be noted in this experimental series, it is that the serums of the guinea pig and the horse do not always behave in the same way with respect to certain inhibitors. We have been able

to show recently (2) that histamine inhibits the hydrolytic capacity of guinea pig serum, while horse serum cholinesterase is not influenced by this amine, at whatever dosage used.

Is the behavior of these different types of enzymes similar toward inorganic ions? That is likewise the question which we seek to resolve.

Method

We used injections in guinea pigs, of various inorganic salts, and we studied the concomitant changes in the serum cholinesterase.

Serum cholinesterase is very variable in the guinea pig from one animal to another, but in the same individual is practically constant.

This is why we rejected the method of statistical comparison. Our guinea pigs were tested before and after the experiment. We only attach significance to the variations which were able to be verified in the same animal.

Next we established the average individual variations, in order to ascertain the total effect of the injected medicine.

We have shown in a preceding study (3) that the level of cholinesterase in the guinea pig is subject to seasonal variations: This level is high in the spring and in summer, and low in winter. We attributed this variation to a deficiency in winter in the Vitamin C content of their food. It also appears that cold likewise plays a part. For this reason the animals were kept at constant temperature and received an identical diet. Our experiments only lasted a maximum of 8-10 days for each series, an insufficient period to bring about a variation in cholinesterase level due to external circumstances.

Blood samples were taken by a cardiac tap. They rarely exceeded 1-15 cm of blood and were spaced apart at least 48 hours. We know in fact that blood letting lowers the cholinesterase level, but recuperation takes place in less than 48 hours. The blood is allowed to coagulate at ambiant temperature and waits for the clot to shrink before putting the blood in the refrigerator. The determination was never made much later than 24 hours after taking a sample.

The determination was carried out, as with the horse serum, by the Hall and Lucas method (4).

The injected solutions were administered subcutaneously and rarely intramuscularly. We never used the intravenous route.

The injection of some drugs gave rise to small cutaneous scabs, incapable of altering the serum cholinesterase level.

Results

In order to avoid repetition, we give our experimental results in the form of a synopsis table that we discuss afterward.

As one can see from the table, the general reactions of serum cholinesterase in the guinea pig take place in the same way as in our in vitro experiments on horse serum.

On the other hand, one sees a definite quantitative difference. These differences furthermore can be explained by rather simple facts. A number of organs are involved in the regulation of serum cholinesterase. The most important of these organs is the liver. Certain authors [Antopol, 5; Butt, 6; Hawes and Alles, 7; McArdle, 8; Vincent, 9] in fact have shown that in cirrhosis of the liver, hepatitis and jaundice, the activity of cholinesterase in the serum was lowered.

In addition, thyroid deficiency (Antopol, Tuchman and Shifrin, 10; Werle and Stuttgen, 11), likewise entails a slowing down of enzymatic hydrolysis of acetylcholine.

Beyond doubt, in the toxicity by inorganic ions that we have produced in the guinea pig, we have strongly disturbed the various functions of the glandular parenchyme and have created anatomical problems.

To the direct action of the ion on cholinesterase, which we have established in vitro, is now added the modification of this enzyme from the organic injury of the viscera.

On the other hand, in other cases, the living organism reacts by neutralising the toxic action of certain ions by a mechanism that we scarcely understand. One could postulate a neoformation of enzyme having the reestablishment of the initial enzyme level as its purpose.

Finally we should mention some particular cases.

1. Divergences with in vitro experiments

NaBr. If we compare the results obtained in the in vitro experiment on horse serum with the in vivo studies in the guinea pig, we find a definite difference. NaBr is in fact a strong inhibitor of cholinesterase in horse serum in vitro, while it accelerates that of the guinea pig in vivo.

It is the same for the double tartrate of Sb and Na. A slight inhibitor in vitro, it is practically inactive on the serum cholinesterase of the guinea pig in vivo.

An inverse action is found with $CaCl_2$ which is an activator in vitro and is an inhibitor in vivo. The same for calcium hyposulfite.

An analogous discrepancy is seen with injections of K_2SO_4 a slight inhibitor in vitro, inactive in vivo.

Table

Ions	Guinea pig	Weight	*Dose received in g/kg	CHE before	CHE after	Result in %	Average
Sodium hyposulfite	f	480	5 x 0,50	2,77	3,45	+24%	
	m	430	5 x 0,50	1,84	2,93	+58%	+41%
Magnesium chloride	m	410	4 x 1,0	2,40	3,60	+33%	
	m	430	5 x 1 g	2,05	2,75	+34%	+34.5%
	m	570	2 x 1 g	1,40	1,90	+36%	
Manganese chloride	m	505	4 x 0,01	2,01	1,10	-45%	
	m	400	4 x 0,01	1,55	1,10	-29%	-37%
Aluminum sulfate	m	540	2 x 0,01	2,45	2,44		
	m	480	3 x 0,01	2,60	2,05	-21%	
	m	500	4 x 0,01	2,93	2,51	-15%	

*The injected dose was calculated according to Aberhalden (Handbook of Biological Work Methods 1, 7) to obtain the chronic toxicity for the ion studied.

Similarly manganese chloride does not seem to be active in vitro on horse serum, but in vivo inhibits cholinesterase in guinea pig serum.

These various discrepancies are interesting. It is possible that introducing certain ions in a living organism releases a number of compensating mechanisms. One of the mechanisms which can be invoked in the case of injections of Ca salts, is a liberation of K ions, which is a cholinesterase inhibitor. Does this reaction exceed its purpose? That is what we allow ourselves to surmise.

The inverse is produced in the case of injections of K salts.

But another hypothesis should be examined: our experiments in vitro were carried out on the cholinesterase of horse serum; those in vivo, on the cholinesterase of guinea pig serum. But a number of previous studies showed different cholinesterase reactions in various animal species. In particular, there certainly exists a difference in behavior between horse serum cholinesterase and that of the guinea pig, the more so because the horse, contrary to the guinea pig, possesses a strong vagus tonus.

Consequently, we have redone the study of the action of ions on guinea pig serum. Guinea pig serum being extremely difficult to obtain in a sufficient amount, we limited this verification to only two ions, Br and Sb, because if we can accept equilibrium mechanisms for normal ions present in serum (Ca, K), it is more difficult to picture analogous reactions for ions foreign to serum (at least in considerable amounts).

The study of cholinesterase in guinea pig serum gave us the following results.

Bromide and sodium exert a pronounced inhibition on guinea pig cholinesterase, just as that with horse serum. The difference found between

the in vivo and in vitro studies does not reside therefore in a different reactivity of the cholinesterases of dissimilar species, but must be sought in a compensatory mechanism which occurs at the moment the bromide is introduced into the living organism.

As for manganese chloride, neutral with the cholinesterase of horse serum and inhibiting when injected in the guinea pig, it induces a positive acceleration of cholinesterase when put in direct contact with guinea pig serum.

Here, besides the compensatory phenomenon, it is possible that the difference in the origin of the serums comes into play.

These facts limit still further the general statements that we have made in this study, in the sense that the results obtained cannot be extended to other species than those which we have studied.

2. Experiments in reactivation. Nachmansohn and Lederer have pointed out what we have already said repeatedly, that Ca^{2+} ion reactivates cholinesterase.

Seeking to determine the mechanism of the desensitization action of some compounds used, for therapeutic purposes, we have been able to show that ascorbic acid, for example (Frommel, Herschberg and Piquet, 3), inactive with whole cholinesterase, returns its activity to cholinesterase inhibited by various toxins. It is the same for Ca. We have been able to show (15, d) that if one lowers the activity of guinea pig serum cholinesterase by injections of Myosalvarsan (0.08 g/kg) one gets an enzyme fall of about 70%. If one treats this guinea pig with simultaneous injections of calcium gluconate (0.40 g/kg) this lowering is only 35% on the average.

Calcium is therefore a reactivator of cholinesterase in vivo, while at the same time in vitro, it returns its activity to cholinesterase destroyed by dialysis.

Additionally, one sees that a cholinesterase relay exists in the desensitization effect of calcium.

3. Compounds studied uniquely in vivo or in vitro. Our experimentation on the guinea pig was carried out with a certain number of ions not studied in vitro. The reason for this is that one can inject oily solutions in the animal (phosphoric oil, bismuth oleate) which does not lend itself to in vitro studies, which require colorless, aqueous solution. Contrarily, some ions cannot be injected in the guinea pig because of their great causticity, such as silver nitrate, iron sulfate, etc. For all these compounds we were obliged to settle for in vitro experiments.

Conclusions

The experiments for which we have just given detailed results have apart from their purely theoretical interest, a practical interest, even clinical in the sense that they appear to be able to give us the key to the pathology of the symptoms of certain intoxications.

It is curious, for example, to show by analysing the manifestations of lead poisoning, the major part of the clinical disturbances are the consequences of a strong hypervagotonia (colic, spasms of the smooth musculature, muscular problems). Now we have just shown that Pb is one of the strongest inhibitors of cholinesterase, both in vivo and in vitro. It is easy to imagine that the symptoms of vagotonia could appear when acetylcholine is no longer sufficiently destroyed by an inhibited cholinesterase.

Moreover, hasn't clinical therapy anticipated our experimental demonstration by using atropine or adrenaline against lead poisoning symptoms?

It is the same with antimony poisoning. The animal poisoned with massive doses of Sb shows copious salivation, nausea, vomiting, diarrhea, asthmatic dysprea, and finally muscular asthenia terminating in death with violent convulsions.

Paralleling this clinical picture, the heart is strongly depressed and the pressure falls. The heart ceases by stopping in diastole.

But, this syndrome, which is that of an acute hypervagotonia, point by point agrees with that produced by a massive intravenous injection of acetylcholine.

On the other hand, if one takes the group of drugs called "desensitizers" which are the hyposulfites of Na, Ca or Mg and the salts of Ca or Mg, we see that the entire group are cholinesterase activators as much in vitro as in vivo. It is that finding which seems interesting to us, and which contributes to the pharmacodynamics of these ionic mineral medicines.

We recall that these activating ions, all of which have a considerable effect on intact cholinesterase, exert a powerful effect above all on cholinesterase which has been partially destroyed by a poison. We developed these ideas in a preceding study (12).

In that way, we came upon ascorbic acid another cholinesterase reactivator. We showed in fact that ascorbic acid, probably a coenzyme of cholinesterase, does not act on intact cholinesterase in vivo or in vitro, but restores its former activity to previously inhibited cholinesterase.

It is in this way that we have been able to show that Ca^{2+} counteracts the inhibition of cholinesterase produced by neoarsphenamine. The protective action of ascorbic acid against arspehamine symptoms can be similarly interpreted.

Summary

Having established in a previous study that horse serum cholinesterase is subject to functional modifications under the influence of certain inorganic ions, we attempted to study the effect of these ions on cholinesterase in vivo.

To this end, we injected guinea pigs with inorganic salts, and we measured the enzymatic activity of the serum (drawn off by successive cardiac punctures), by the titrimetric method of Hall and Lucas, before and after the injections.

Most of the ions studied inhibited the serum cholinesterase of the guinea pig. The inhibition varied according to the ion or the reactions of each individual.

Other ions were activators of intact cholinesterase, some finally restored the activity of a previously inhibited enzyme.

In general, the results obtained paralleled those of the first experimental series on horse serum in vitro.

On the other hand, we have found some discrepancies which we have attempted to interpret either as a difference in the origin of the serum or by a compensatory phenomenon produced in a living organism.

The practical interest in this group of experiments is to contribute some explanations for the pathogenesis of certain vagotonic symptoms of acute or chronic poisoning from the therapeutic use of various inorganic ions.

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Institut de Thérapeutique de l'Université de Genève

Effets des ions inorganiques sur l'activité de la cholinestérase sérique

II. Études, in vivo, sur le cobaye

Par Ed. Frommel, A. D. Herschberg et J. Piquet

Reçu le 29 janvier 1944

Dans un précédent travail (*Frommel, Herschberg* et *Piquet* [1]) nous avons étudié les modifications de l'activité de la cholinestérase du sérum de cheval in vitro. Nous avons ainsi pu constater qu'un grand nombre d'ions inorganiques inhibent l'action de ce ferment et que d'autres ions entraînent une activation enzymatique.

Cette étude faite hors de l'organisme vivant ne pouvait que donner des résultats partiels. Nous avons jugé nécessaire, afin de pouvoir tendre vers des constatations plus générales, de compléter nos expériences par des essais in vivo.

Nous attribuons à ces essais une plus grande valeur pratique qu'à la première série, car dans ce cas nous pouvons connaître non seulement l'effet direct du toxique sur la cholinestérase sérique, mais encore l'effet global de ce toxique sur un organisme conservant intactes toutes ses facultés de défense et d'équilibration.

Nous pouvons également tirer des conclusions plus précises sur la pharmacodynamie des ions inorganiques et le mécanisme pathogénique de certains symptomes vagotoniques des intoxications par ces ions.

Un autre point mérite d'être signalé dans cette série expérimentale, c'est que les sérums de cobaye et de cheval ne se comportent pas toujours de la même manière vis-à-vis de certains inhibiteurs. Nous avons pu montrer récemment (2) que l'histamine inhibe le pouvoir hydrolytique du sérum de cobaye, alors que la cholinestérase sérique du cheval n'est pas influencée par cette amine, quelles qu'en soient les doses utilisées. Le comportement de ces enzymes d'espèces différentes est-il identique envers les ions inorganiques? C'est également la question que nous avons cherché à résoudre.

Méthode

Nous avons pratiqué des injections de divers sels inorganiques à des cobayes et nous avons étudié les variations concomitantes de la cholinestérase sérique.

La cholinestérase du sérum de cobaye est très variable d'un animal à l'autre, mais est pratiquement constante chez un même individu.

C'est pourquoi, nous rejetons la méthode de travail par comparaison statistique. Nos cobayes ont été testés avant et après l'expérience: nous n'attachons d'importance qu'aux variations pouvant être constatées chez un même animal.

Puis nous avons établi les moyennes des variations individuelles, afin de constater l'action globale du médicament injecté.

Nous avons montré dans un travail précédent (3) que le taux de la cholinestérase chez le cobaye subit des variations saisonnières: ce taux est haut au printemps et en été, il est bas en hiver. Nous avons attribué cette variation à une déficience hivernale en vitamine C alimentaire. Il semble que le froid joue également un certain rôle. Ce sont les raisons pour lesquelles les animaux furent gardés à une température constante et reçurent une nourriture identique: nos expériences ne durèrent pour chaque série qu'un maximum de 8–10 jours, période insuffisante pour entraîner une variation du taux cholinestérasique due à des circonstances extérieures.

Les prises de sang furent faites par ponctions cardiaques. Elles ne dépassaient que rarement 1–1,5 ccm de sang et étaient espacées d'au moins 48 heures. Nous savons en effet que la saignée baisse le taux de la cholinestérase sérique, mais que la récupération se fait en moins de 24 heures. On laisse le sang se coaguler à la température ambiante et on attend que le caillot se rétracte avant de mettre le sang à la glacière. Le dosage n'a jamais été fait plus tard que 24 heures après son prélèvement.

Le dosage fut pratiqué, comme pour le sérum de cheval, par la méthode de *Hall* et *Lucas* (4).

Les solutions injectées furent administrées par voie sous-cutanée, rarement par voie intra-musculaire. Nous n'avons jamais utilisé la voie intra-veineuse.

L'injection de certaines drogues a donné lieu à de petites eschares cutanées, incapables de modifier le taux de la cholinestérase sérique.

Résultats

Afin d'éviter les répétitions, nous donnons nos résultats expérimentaux sous forme d'un tableau synoptique, que nous commenterons ensuite.

Ions	Cobaye	Poids	Dose reçue*) en g/kg	CHE avant	CHE après	Résultat en %	Moyenne
			Anions				
Bromure de sodium	m	340	5 × 0,50	2,30	2,67	+12%	+17%
	m	355	5 × 0,50	2,50	3,10	+24%	
	m	480	5 × 0,50	2,36	2,70	+14%	
Iodure de sodium	m	540	4 × 0,20	2,14	1,52	—29%	—28%
	m	525	4 × 0,20	2,16	1,58	—27%	
Hyposulfite de sodium	f	480	5 × 0,50	2,77	3,45	+24%	+41%
	m	430	5 × 0,50	1,84	2,93	+58%	

*) Les doses injectées furent calculées d'après *Abderhalden* (Handbuch der biologischen Arbeitsmethoden, 7, 7), pour obtenir une intoxication chronique par l'ion étudié.

Ions	Cobaye	Poids	Dose reçue*) en g/kg	CHE avant	CHE après	Résultat en %	Moyenne
Myosalvarsan[1])	m	520	4 × 0,04	2,95	1,30	—56%	—55%
	m	530	4 × 0,04	3,44	1,60	—54%	
	m	520	6 × 0,04	3,15	0,84	—77%	—69%
	m	560	6 × 0,04	3,40	1,27	—61%	
Tartrate double de Sb et K	m	590	3 × 0,003	1,85	1,30	—30%	—16%
	m	480	3 × 0,003	1,90	1,85	— 2%	
Tartrate double de Sb et Na	m	640	6 × 0,02	1,94	1,10	+ 3%	+ 1%
	m	460	6 × 0,02	1,95	1,03	— 1%	
Huile phosphorée[2])	f	610	2 × 1 ccm	2,58	1,59	—39,5%	—46%
	f	550	2 × 1 ccm	2,85	1,38	—52%	
Borate de sodium	m	360	2 × 0,30	3,99	3,50	—12,5%	
	m	420	3 × 0,30	3,62	2,39	—34%	
	m	380	4 × 0,30	3,95	2,75	—31%	
			Cations				
Chlorure d'ammonium[3])	m	480	4 × 0,05	1,85	2,33	+26%	+18%
	m	340	4 × 0,05	2,56	2,82	+10%	
Sulfate de lithium	m	610	4 × 0,05	1,60	1,33	—17%	—17%
	m	680	4 × 0,05	1,43	1,20	—17%	
Sulfate de potassium	m	350	2 × 0,30	2,93	2,80	— 5%	
	f	430	3 × 0,30	3,00	3,10	+ 3%	
	f	380	4 × 0,030	2,60	2,74	+ 5%	
Hyposulfite de Calcium	m	580	3 × 0,20	0,87	0,86	0	— 8%
	m	540	3 × 0,20	1,28	1,08	—15%	
	m	400	3 × 0,20	1,20	1,05	—10%	
Galactogluconate de Calcium	f	540	5 × 0,40	3,10	3,27	+ 5%	+ 5%
	f	510	5 × 0,40	2,70	2,87	+ 5%	
Chlorure de Calcium	m	480	4 × 0,10	2,57	1,65	—36%	—35,5%
	m	535	4 × 0,10	1,89	1,24	—35%	

*) Les doses injectées furent calculées d'après *Abderhalden* (Handbuch der biologischen Arbeitsmethoden, 7, 7), pour obtenir une intoxication chronique par l'ion étudié.

[1]) Le Myosalvarsan est une variante du Novarsénobenzol, injectable par voie intramusculaire.

[2]) L'huile phosphorée du Codex, que nous avons employée, est une huile de vaseline fluide contenant, en solution, 0,475–0,525% de phosphore.

[3]) Le pH du sérum de cobayes traités par les injections de chlorure d'ammonium ne dépassait pas celui d'animaux témoins.

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Ions	Cobaye	Poids	Dose reçue*) en g/kg	CHE avant	CHE après	Résultat en %	Moyenne
Chlorure de Magnesium	m	410	4 × 1,0	2,40	3,60	+33%	+34,5%
	m	430	5 × 1 g	2,05	2,75	+34%	
	m	570	2 × 1 g	1,40	1,90	+36%	
Hyposulfite de magnesium	m	410	3 × 1,0	1,94	1,91	— 1%	+ 5%
	m	640	3 × 1,0	1,66	1,85	+11%	
Chlorure de baryum	m	380	4 × 0,01	2,37	1,70	—29%	—19%
	m	440	3 × 0,01	1,82	1,71	— 9%	
Sulfate de zinc	f	425	2 × 0,075	3,80	3,51	— 8%	
	f	350	3 × 0,075	3,55	2,54	—19%	
	f	520	4 × 0,07	3,35	1,79	—46%	
Chlorure de manganèse	m	505	4 × 0,01	2,01	1,10	—45%	—37%
	m	400	4 × 0,01	1,55	1,10	—29%	
Acetate de nickel	m	230	2 × 0,01	2,93	2,68	— 8%	
	m	400	3 × 0,01	2,90	2,95	+ 2%	
	m	350	4 × 0,01	4,93	3,00	+ 2%	
Chlorure stanneux	f	430	2 × 0,01	3,62	3,05	—16%	
	f	500	3 × 0,01	3,30	2,75	—18%	
	f	380	4 × 0,01	4,00	1,99	—50%	
Sulfate d'aluminium	m	540	2 × 0,01	2,45	2,44		
	m	480	3 × 0,01	2,60	2,05	—21%	
	m	500	4 × 0,01	2,93	2,51	—15%	
Protéinate d'argent	f	560	2 × 0,05	1,86	1,03	—45%	
	f	340	3 × 0,05	3,77	2,30	—39%	
	f	610	4 × 0,05	3,80	1,59	—59%	
Myoral[4])	m	565	5 × 0,015	4,12	2,06	—50%	—40%
	m	575	5 × 0,015	3,22	2,69	—16%	
	m	550	5 × 0,015	4,30	2,06	—55%	
Acetate de Pb-triéthyle	f	720	2 × 0,01	3,37	1,50	—56%	—49%
	f	560	2 × 0,01	2,80	1,35	—52%	
	f	440	2 × 0,01	3,20	2,00	—38%	
Sulfate de cuivre	m	480	4 × 0,01	1,42	0,95	—31%	—19%
	m	370	4 × 0,01	1,36	1,28	— 6%	

*) Les doses injectées furent calculées d'après *Abderhalden* (Handbuch der biologischen Arbeitsmethoden, 7, 7), pour obtenir une intoxication chronique par l'ion étudié.

[4]) Le Myoral est un composé aurique en solution huileuse injectable par voie intramusculaire. Sa formule chimique est: aurothiogiycolate de Ca, et sa teneur en Au: 64,16%.

Ions	Cobaye	Poids	Dose reçue*) en g/kg	CHE avant	CHE après	Résultat en %	Moyenne
Sublimé	f	590	2 × 0,02	2,20	1,83	—17%	—46%
	f	590	2 × 0,02	3,44	0,88	—75%	
	m	630	3 × 0,02	2,33	mort		
	m	530	3 × 0,02	2,20	mort		
Merfen[5])	m	470	3 × 1 ccm	1,24	0,74	—41%	—32%
	m	560	3 × 1 ccm	1,79	1,40	—22,5%	
Oléo-Bi[6])	f	600	5 × 1 ccm	2,94	1,48	—55%	—45%
	f	680	8 × 1 ccm	2,58	1,70	—35%	
Eserine	m	350	2 × 0,0005	3,55	0,85	—76%	—68%
	m	420	3 × 0,0005	3,84	1,48	—62%	

*) Les doses injectées furent calculées d'après *Abderhalden* (Handbuch der biologischen Arbeitsmethoden, 7, 7), pour obtenir une intoxication chronique par l'ion étudié.

[5]) Le «Merfen» est un produit désinfectant du commerce, dont la formule est: borate de phényl-hydrargyre. Nous avons utilisé ce produit afin de comparer l'action d'un Hg organique avec celle d'un Hg minéral (sublimé). Sol. aq. à 2‰.

[6]) L'oléo-Bi est un oléate de bismuth, titrant 0,012 g par ccm.

Discussion

Comme on peut le voir sur le tableau, les réactions générales de la cholinestérase sérique du cobaye se font dans le même sens que dans nos expériences in vitro sur le sérum de cheval.

Par contre l'on assiste à de nettes *différences quantitatives*. Ces différences quantitatives peuvent d'ailleurs être expliquées par des faits assez simples. Nombre d'organes interviennent dans la régulation de la cholinestérase sérique. Le plus important de ces organes est le foie. Certains auteurs (*Antopol* [5], *Bott* [6], *Hawes* et *Alles* [7], *McArdle* [8], *Vincent* [9]) ont en effet constaté que dans les cirrhoses du foie, les hépatites et les ictères, il y avait une baisse d'activité cholinestérasique dans le sérum.

Par ailleurs, les insuffisances thyroïdiennes (*Antopol*, *Tuchman* et *Shifrin* [10], *Werle* et *Stöttgen* [11]), entraînent également un ralentissement de l'hydrolyse enzymatique de l'acétylcholine.

Dans l'intoxication par ions inorganiques que nous avons produite chez le cobaye, il est hors de doute que nous avons perturbé fortement le fonctionnement des divers parenchymes glandulaires et que nous avons créé des troubles anatomiques.

A l'action directe du ion sur la cholinestérase, que nous avons constatée in vitro, vient s'ajouter donc la modification de ce ferment par lésion organique des viscères.

Par contre, l'organisme vivant réagit, dans d'autres cas, en neutralisant par des mécanismes que nous ne connaissons que mal l'action toxique de certains ions. On pourrait supposer une néo-formation de ferment, ayant pour but de rétablir le taux enzymatique initial.

Il nous faut enfin mentionner quelques cas particuliers:

1. *Divergences avec les expériences in vitro*

BrNa. Si nous comparons les résultats obtenus dans l'expérience in vitro sur le sérum de cheval avec ceux des essais in vivo sur cobayes, nous constatons une nette différence. En effet, le BrNa est un inhibiteur puissant de la cholinestérase du sérum de cheval in vitro, alors qu'il accélère celle du cobaye in vivo.

Il en est de même pour le *tartrate double de Sb et Na.* Léger inhibiteur in vitro, il est pratiquement inactif sur la cholinestérase du sérum de cobaye in vivo.

Une action inverse peut être constatée avec le Cl_2Ca, qui, activateur in vitro, est inhibiteur in vivo. Idem pour l'*hyposulfite de Ca.*

Une discordance analogue se révèle avec les injections de SO_4K_2, inhibiteur léger in vitro, inactif in vivo.

De même, le *chlorure manganeux* ne paraît pas avoir d'action in vitro sur le sérum de cheval, mais inhibe, in vivo, la cholinestérase du sérum de cobaye.

Ces quelques divergences sont intéressantes. Il est possible que l'introduction de certains ions dans un organisme vivant déclenche de nombreux mécanismes compensateurs. Un de ces mécanismes, que l'on peut invoquer dans le cas des injections de sels de Ca, est une libération de ions K, qui est un inhibiteur de la cholinestérase. La réaction dépasse-t-elle le but? C'est ce qu'il nous est permis de supposer.

L'inverse se produirait en cas d'injections de sels de K.

Mais une autre hypothèse doit être examinée: nos expériences in vitro portent sur une cholinestérase de sérum de cheval; celles in vivo, sur une cholinestérase de sérum de cobaye. Or, de nombreux travaux antérieurs montrent les différentes réactions des cholinestérases de diverses espèces animales. En particulier, il existe certainement une différence de comportement entre les cholinestérases sériques de cheval et de cobaye, d'autant plus que le cheval, contrairement au cobaye, possède un fort tonus du vague.

Nous avons par conséquent refait l'étude de l'action ionique sur le sérum de cobaye in vitro. Le sérum de cobaye étant fort difficile à obtenir en quantité suffisante, nous avons dû limiter cette vérification à deux ions seulement: le Br et le Sb, car si nous pouvons admettre des mécanismes équilibrateurs pour des ions normalement présents dans le sérum (Ca, K), il est plus difficile de s'imaginer des réactions analogues pour des ions étrangers au sérum (du moins en quantité notable).

L'étude de la cholinestérase du sérum de cobaye nous a donné les résultats suivants:

Le *bromure de sodium* exerce une inhibition prononcée sur la cholinestérase du sérum de cobaye, tout comme sur celle du sérum de cheval. La différence constatée entre les essais in vivo et in vitro ne réside donc pas en une différente réactivité des cholinestérases d'espèces éloignées, mais doit être recherchée dans un mécanisme compensateur qui se produirait lors de l'introduction de bromure dans un organisme vivant.

Quant au *chlorure de manganèse*, indifférent sur la cholinestérase du sérum de cheval et inhibiteur en injection au cobaye, il provoque une certaine accélération de la cholinestérase quand il est mis en contact direct avec le sérum de cobaye.

Là, entre les phénomènes compensateurs, il est possible que la différence d'origine des sérums entre en jeu.

Ces faits limitent encore davantage les constatations générales que nous avons faites dans ce travail, dans le sens que les résultats obtenus ne peuvent être étendus sans autre aux espèces autres que celles que nous avons étudiées.

2. *Expériences de réactivation*

Nachmansohn et *Lederer* ont indiqué, comme nous l'avons déjà dit à plusieurs reprises, que l'ion Ca^{++} est un réactivateur de la cholinestérase.

Cherchant à déterminer le mécanisme de l'action désensibilisante de certains corps utilisés dans ce but en thérapeutique, nous avons pu montrer que l'acide ascorbique par exemple (*Frommel, Herschberg* et *Piquet* [3]), inactif sur une cholinestérase intacte, rendait son activité à une cholinestérase inhibée par divers toxiques. Il en est de même pour le Ca. Nous avons pu constater (15, d) que si l'on baisse l'activité de la cholinestérase sérique du cobaye par des injections de Myosalvarsan (0,08 g/kg) on obtient une chute fermentaire de 70% environ. Si l'on traite ces cobayes par des injections simultanées de gluconate de calcium (0,40 g/kg) cette baisse n'est plus que de 35% en moyenne.

Le calcium est donc un réactivateur de la cholinestérase in vivo, tout comme il rend, in vitro, son activité à une cholinestérase détruite par dialyse.

Accessoirement, on voit qu'il existe un relai cholinestérasique dans l'action désensibilisante du calcium.

3. *Corps étudiés uniquement in vivo ou in vitro*

Notre expérimentation sur cobayes porte sur un certain nombre d'ions non étudiés in vitro. La raison de ce fait est que chez l'animal on peut injecter des solutions huileuses (huile phosphorée, oléate de bismuth) qui ne se prêtent pas à l'étude in vitro, qui nécessitent des solutions aqueuses inodores. Inversément, certains ions n'ont pas pu être injectés aux cobayes

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à cause de leur grande causticité, comme le nitrate d'argent, le sulfate de fer, etc. Pour tous ces corps, nous avons dû nous contenter des expériences in vitro.

Conclusions

Les expériences dont nous venons de donner les résultats détaillés ont, en dehors de l'intérêt purement théorique, un intérêt pratique et même clinique dans le sens qu'elles semblent devoir nous donner la clé de la pathogénie des symptômes de certaines intoxications.

Il est curieux, par exemple, de constater en analysant les manifestations de l'intoxication saturnine, que la majeure partie des troubles cliniques sont des conséquences d'une forte hypervagotonie (coliques, spasmes de la musculature lisse, troubles musculaires). Or, nous venons de montrer que le Pb est un des inhibiteurs les plus puissants de la cholinestérase, tant in vivo qu'in vitro. On imagine facilement que les symptômes vagotoniques puissent apparaître, lorsque l'acétylcholine n'est plus suffisamment détruite par une cholinestérase inhibée.

D'ailleurs, la thérapeutique clinique n'a-t-elle pas devancé notre démonstration expérimentale en utilisant contre les accidents saturnins l'atropine ou l'adrénaline?

Il en est de même dans l'intoxication stibiée: l'animal intoxiqué par des doses massives de Sb présente une salivation abondante, des nausées, des vomissements, de la diarrhée, une dyspnée asthmatiforme, enfin une asthénie musculaire aboutissant à la mort dans de violentes convulsions.

Parallèlement à ce tableau clinique, le cœur est fortement déprimé, la tension tombe. Le cœur finit par s'arrêter en diastole.

Or, ce syndrome, celui d'une hypervagotonie aiguë, se superpose point par point à celui produit par une injection massive intra-veineuse d'acétylcholine.

D'autre part, si l'on prend la série des médicaments dits «désensibilisants», qui sont l'hyposulfite de Na, de Ca ou de Mg, les sels de Ca ou de Mg, on voit que toute la série est activatrice de la cholinestérase tant in vitro qu'in vivo. C'est là une donnée qui nous paraît intéressante et qui contribue à la pharmacodynamie de ces médicaments ioniques minéraux.

Nous rappelons que ces ions activateurs, tout en ayant une action notable sur la cholinestérase intacte, exercent une action puissante surtout sur une cholinestérase partiellement démolie par un toxique. Nous avons développé ces notions dans un travail précédent (12).

Par là, ils s'approchent d'un autre réactivateur de la cholinestérase: l'acide ascorbique. Nous avons en effet montré que l'acide ascorbique, ce ferment probable de la cholinestérase, n'agit pas sur la cholinestérase intacte, in vivo et in vitro, mais rend l'activité première à une cholinestérase préalablement inhibée.

C'est ainsi que nous avons pu montrer que le Ca^{++} s'oppose à l'inhibition de la cholinestérase produite par le Novarsénobenzol. L'action protectrice de l'acide ascorbique contre les accidents salvarsaniques peut être interprétée de la même manière.

Résumé

Ayant constaté, dans un précédent travail, que la cholinestérase sérique du cheval subit des modifications fonctionnelles sous l'influence de certains ions inorganiques, nous avons cherché à étudier l'effet de ces ions sur une cholinestérase in vivo.

A cette fin, nous avons injecté des sels inorganiques à des cobayes, et nous avons mesuré l'activité enzymatique du sérum (prélevé par ponctions cardiaques successives), par la méthode titrimétrique de *Hall* et *Lucas*, avant et après les injections.

La plupart des ions cités donnent une inhibition de la cholinestérase sérique du cobaye, inhibition variable suivant les ions et suivant les réactions de chaque individu.

D'autres ions sont activateurs de la cholinestérase intacte, quelques uns enfin rétablissent l'activité d'un ferment préalablement inhibé.

En général, les résultats obtenus sont parallèles à ceux de la première série d'expériences, sur le sérum de cheval in vitro.

Par contre nous avons constaté certaines divergences que nous nous sommes efforcés d'interpréter, soit par la différence d'origine des sérums, soit par des phénomènes compensateurs se produisant dans un organisme vivant.

L'intérêt pratique de cette série expérimentale est d'apporter quelques éclaircissements à la pathogénie de certains symptomes vagotoniques des intoxications aiguës ou chroniques par divers ions inorganiques employés en thérapeutique.

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The Amount of Manganese Required to Prevent Perosis in the Chick[1]

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PEROSIS has recently been recognized as a deficiency disease affecting the development of bone. Up to the present time it has been observed in several species of domesticated and game fowl but has not been observed in other animals. The most characteristic symptoms of the disease are enlargement of the tibio-metatarsal joint, bending of the distal end of the tibia and the proximal end of the metatarsus, and displacement of the Achilles tendon. Locomotion is impaired and in severe cases the animal is unable to stand. These outward manifestations of injury have given rise to the descriptive terms of "slipped tendon" and "enlarged hock" as names for the deformity. Titus (1932) first proposed the name perosis.

The disease is prevalent among rapidly growing chicks confined in battery brooders. Diets high in calcium and phosphorus aggravate the condition according to Wilgus (1931). That the disease is distinct from rickets, *in sensu stricto*, was first demonstrated by Hogan, Shrewsbury, and Kempster (1928) who found the ash content of the bones and the inorganic calcium and phosphorus of the blood of chicks suffering with perosis to be within the normal range. Furthermore, the course of the disease is unaltered by adequate dietary supplements of vitamin D. Further proof that the disease differs from other known bone disorders has been offered by Hunter and Funk (1930), by Hall and King (1931), and by Titus (1932) in studies of perosis by means of chemical, x-ray, and histological methods. In the above investigations the principal changes found in the bone were anatomical.

Titus and Ginn (1931) announced that rice bran contains a dietary factor which prevents perosis. Heller and Penquite (1936) reported that the protective factor was present in a water-soluble extract of the bran and that it was heat-stable. They were of the opinion that the potency of the extract was not due to its mineral content.

Sherwood and Fraps (1936), however, found a protective factor to be present in the ash of wheat gray shorts. Thus, a controversy arose as to whether these protective factors, if identical, were inorganic or organic in nature. The matter was almost immediately settled in favor of an inorganic deficiency upon the discovery of Wilgus, Norris, and Heuser (1936, 1937a, 1937b) that manganese prevents perosis and that the preventive properties of feeds are roughly in proportion to their manganese content.

In order to determine more definitely the function of manganese in the prevention

[1] The research work reported in this paper was done in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Cornell University and was part of an experiment station project under the direction of L. C. Norris. A preliminary report of the work was presented at the 31st annual meeting of the American Society of Biological Chemists at Memphis, April 21-24, 1937.

[2] On sabbatical leave of absence from the Oklahoma Agricultural Experiment Station, 1936-37.

of perosis it seemed necessary first to attempt confirmation of the results of the latter investigators by using a variety of manganese salts and then to establish, if possible, the minimum preventive amount of manganese.

During the time this investigation was in progress (Gallup and Norris, 1937), Heller and Penquite (1937) confirmed the findings of Wilgus and others by showing that $MnCO_3$ (0.02 percent of the diet), prevents perosis. They found the ash of rice bran to be preventive when fed at a level equivalent to 70 percent of rice bran. They were of the opinion, however, that factors other than manganese were involved in the prevention of perosis since they observed that ". . . some of the best growth and protection came from rations having no manganese addition. . . ." More recently Lyons, Insko, and Martin (1938) have reported that intraperitoneal injections of $MnSO_4$ are effective in preventing slipped tendon. Wiese *et al.* (1938) found oral administration of this salt to be effective in some diets but ineffective in a diet which contained excessive amounts of calcium and phosphorus. They pointed out that the effectiveness of the manganese was enhanced by rice bran and concluded that rice bran contains some labile substance ". . . which either alone or in association with manganese is operative in preventing perosis on the ration used."

It is evident from the foregoing that the qualitative as well as the quantitative aspects of the relationship between dietary manganese and perosis have not been satisfactorily settled. It appears likely that the complete prevention of perosis is dependent upon a satisfactory balance between certain dietary constituents involving manganese, and possibly some unknown factor.

EXPERIMENTAL

Day-old New Hampshire chicks were distributed as uniformly as possible in groups of 25 each and kept on wire floors in electrically heated battery brooders. The brooders and feeders which were of metal construction were lacquered to eliminate metal contamination. Unless otherwise noted, the experimental periods were of six weeks' duration.

The chicks were given constant access to a basal diet of the following composition:

Diet No. 3.000

	Parts
Ground yellow corn	63.5
Dehydrated alfalfa meal	5.0
Dried skimmilk	20.0
Menhaden fish meal	10.0
Iodized salt	0.5
Reënforced cod liver oil	0.25
Cottonseed oil	0.75
Bone meal	1.00

The diet contained 19.55 percent of protein, 1.52 percent of calcium, 0.96 percent of phosphorus, and 0.0010 percent (10 p.p.m.) of manganese. Water was provided either in glass or glazed earthenware containers. This diet and the same general procedure were used throughout this investigation.

The effectiveness of chemically pure $MnCl_2.4H_2O$, $MnSO_4.4H_2O$, $KMnO_4$, $MnCO_3$, and MnO_2[3] in preventing perosis was determined. These salts were added to the basal diet in such amounts as to supply manganese at the rate of 50 p.p.m. of diet. The additions were made by intimately mixing the finely ground salt with 2 pounds of ground corn and combining this with the proper amounts of the other ingredients.

Manganese carbonate was used to determine the minimum amount of manganese required to prevent perosis. This salt was added to the basal diet of seven groups of chicks in amounts that increased from 20

[3] The authors are indebted to Merck and Company, Rahway, New Jersey, for the supply of c.p. Manganese Salts.

to 1,000 p.p.m. of diet. The two groups of chicks which received 500 and 1,000 p.p.m. and their controls were retained until 20 weeks of age to determine whether or not these larger amounts of manganese would be toxic.

Manganese was supplied to other groups of chicks by means of $MnCl_2$ and $KMnO_4$ dissolved in equivalent amounts in the drinking water. This procedure prevented possible dietary changes brought about by the incorporation of manganese salts in the dry diet.

developed by Wilgus, Norris, and Heuser (1937).

$$\frac{\text{Total H} + \text{total T} + \text{total S}}{\text{Number of chicks} \times 18} \times 100 = \text{Severity index.}$$

H indicates hock enlargement, T bending of the tibia and metatarsus, and S slipping of the tendon.

The severity of these conditions was graded by the use of the numbers 1, 2, or 3. According to this method of grading maximum severity for any one chick would be 9 for each leg or a total score of 18. The course of development of perosis was observed

TABLE 1.—*Perosis-preventive properties of different manganese salts*

Group	Chicks per group	Addition to basal diet	Manganese in diet	Av. wt. of chicks at 6 weeks	Cases of perosis	Severity of perosis
	no.		p.p.m.	gm.	per cent	
1	27	None	10	383	81.5	38.8
2	25	$MnCl_2.4H_2O$	50	547	4.0	2.0
3	23	$MnSO_4.4H_2O$	50	591	13.0	6.0
4	25	$KMnO_4$	50	558	0.0	0.0
5	25	$MnCO_3$	50	557	4.0	2.6
6	24	MnO_2, 85%	50	560	4.1	1.8
7	25	$MnSO_4.4H_2O$	100	574	4.0	2.1

All chicks were scored for perosis and weighed each week and a record was made of feed consumption. The average weight for each group was obtained by adding the average weights of males and females and dividing by 2. The severity of perosis for each group was determined by the formula

daily and x-ray pictures of the leg bones of a selected number were taken at frequent intervals.

RESULTS AND DISCUSSION

The results of the first two experiments which are presented in Tables 1 and 2 give

TABLE 2.—*Amount of manganese required to prevent perosis in chicks on diet 3,000*

Group	Chicks per group	Manganese[1] content of diet	Av. wt. of chicks at 6 weeks	Cases of perosis	Severity of perosis
	no.	p.p.m.	gm.	per cent	
1	23	10	386	74.0	36.6
2	25	20	523	56.0	21.5
3	25	30	518	16.0	8.0
4	26	40	536	11.0	2.8
5	25	50	535	4.0	2.0
6	24	70	547	8.2	3.7
7	25	500	538	4.0	1.5
8	24	1,000	541	4.1	2.0

[1] Manganese provided by means of $MnCO_3$.

convincing evidence of the effectiveness of various salts of manganese in preventing perosis. When supplied in amounts yielding equivalent amounts of manganese at the rate of 50 p.p.m. of diet, $MnCO_3$ and the relatively insoluble MnO_2 were as effective as the readily soluble salts, $MnCl_2$ and $MnSO_4$. No perosis developed in the chicks that received the highly oxidized form of manganese, $KMnO_4$, in which manganese acts as a negative radical (MnO_4^-). The results of later studies, however, have given no indication that this form of manganese is superior to the other forms. The average gains made by the two groups of chicks that received $MnSO_4$ were slightly greater than those made by any of the other groups. In view of the similarity of $MnSO_4$ and $MnCl_2$ and the doubtful nutritional importance of the SO_4^{--} radical, further work would be necessary before attaching significance to this result.

The results presented in Table 2 show that the minimum amount of manganese required to prevent perosis is close to 50 p.p.m. of diet although complete prevention (less than 4 percent of perosis) was not possible even when the manganese was increased to 500 and 1,000 p.p.m. These unpreventable cases developed during the first 10 or 14 days of the experiment, indicating that perosis in its initial stages, or the conditions that produce it, appeared during embryonic growth. If it is assumed that perosis is due solely or in large part to a deficiency of manganese, then the possibility of a manganese deficiency in the maternal diet immediately suggests itself. Inability of certain embryos to metabolize the manganese in the egg or of chicks to assimilate sufficient amounts during early growth were other possibilities suggested by these results.

Table 3 contains the results of experiments in which manganese was added to the drinking water of chicks as a means of insuring an abundant intake of manganese in a readily available form. Groups 12 and 13 served as negative and positive controls, respectively. Excessive amounts of manganese, 0.15 mg. per ml., were supplied in the water for group 17 during the first two weeks. The comparative value of $MnCl_2$ and $KMnO_4$ was determined with groups 18 and 19. As in the preceding experiments, there was some perosis in all groups at the end of six weeks. The number of cases was decreased from 74 percent in the basal group to 4 percent in the other groups. The latter cases developed during the first two weeks in chicks that were average or above average in weight, indicating that in these

TABLE 3.—*Perosis-preventive value of manganese when supplied in the drinking water*

Group	Manganese content of diet	Manganese content of water	Average daily manganese intake during—		Av. wt. of chicks at 6 weeks	Cases of perosis	Severity of perosis
			1st week	2nd week			
	p.p.m.	mg. per ml.	mg.	mg.	gm.	per cent	
12	10	0	0.08	0.16	538	74	32.4
13	40	0	0.43	0.76	606	4	0.4
17	10	0.15[1]	3.86	5.81	582	4	1.8
18	10	0.015[2]	0.30	0.70	606	4	1.3
19	10	0.015[3]	0.35	0.68	556	4	2.0

[1] Manganese supplied in the water as $MnCl_2$ during the first 2 weeks only. Thereafter the chicks received manganese in the diet, 40 p.p.m. as $MnCO_3$.
[2] Manganese supplied by $KMnO_4$.
[3] Manganese supplied by $MnCl_2$.

instances low manganese intake was not the causative factor. The possibility of low retention is discussed in later experiments. During the first two weeks the manganese intake of chicks in group 17 was approximately 10 times that of the other groups, yet complete prevention was not obtained. $MnCO_3$ when added to the diet was as effective as the dissolved salts, $KMnO_4$ and $MnCl_2$.

In the foregoing experiments, the average weight of chicks in each group at six weeks of age was obtained from the individual weights of all chicks irrespective of whether or not they developed perosis. The deformities which resulted from perosis were in many cases so severe toward the close of the experimental period that the chicks were unable to move about freely, and loss of weight resulted from fasting. The average six-week gain made by chicks which developed slipped tendon on the low-manganese diet (Mn, 10 p.p.m.) was 396 gm.; and that in which the tendon remained in position was 438 gm., whereas that of the chicks which received adequate manganese (50 p.p.m.) was 519 gm. These comparisons afford evidence of the favorable effect of manganese on growth. Van der Hoorn, Branion and Graham Jr. (1938) have also reported results which they interpreted as indicating that manganese is essential for the growth of chicks.

Confirmation of this growth effect was obtained in the results of experiments to determine whether or not manganese would be toxic when fed at relatively high levels over prolonged periods. Chicks which from hatching until five months old received manganese as $MnCO_3$ at levels of 50, 500, and 1,000 p.p.m. of diet made gains of 1,821, 1,794 and 1,861 gm., respectively. Those which received the basal diet (Mn, 10 p.p.m.) from 6 weeks to 20 weeks of age gained 1,662 gm. No evidence of toxicity was obtained with the chicks receiving the high levels of manganese. In fact, egg production began early among these pullets and at seven months of age those receiving manganese at a level of 1,000 p.p.m. had an egg production record of 66.4 percent for the last month.

In view of the report of Serfontein and Payne (1934) that perosis may be inherited, experiments were conducted with New Hampshire chicks of another source and with chicks of another breed, White Leghorns. The latter breed is reported to be less susceptible to perosis. The results of these experiments, conducted in the same manner as the first ones described in this paper, are presented in Table 4.

The results indicate that chicks of the White Leghorn breed have a low-manganese requirement. Complete prevention of perosis among the chicks of this breed was readily accomplished with a level of manganese equivalent to 30 p.p.m. of diet. The

TABLE 4.—*Variations in the response of chicks to graded amounts of manganese*

Manganese content of diet	White Leghorns		N. H.[1] source B		N. H.[1] source G	
	Number	Cases of perosis	Number	Cases of perosis	Number	Cases of perosis
p.p.m.		per cent		per cent		per cent
10	63	46	23	70	70	87
20	24	21	25	60	—	—
30	51	0	25	16	65	35
40	—	—	26	6	—	—
50	27	0	25	7	33	18
100	—	—	24	4	—	—

[1] New Hampshires

high percentage of perosis that developed among the New Hampshire chicks of source G even when receiving 50 p.p.m. of manganese suggests strain differences in susceptibility. The results as a whole emphasize the importance of uniformity of test animals for studying manganese requirements and perosis.

The chicks which developed perosis in the foregoing experiments showed by gross observations the same general symptoms. About the fourteenth day the metatarsus of one or both legs appeared bent at the tibio-metatarsal joint in such a way as to give the chick the appearance of being "knock-kneed." Bending in the opposite direction to give the appearance of bowed-legs was much less frequent. Only occasionally did the joint become swollen. By the third week, the amount of bending at the joint had increased and the tendon was now being forced from its normal position through muscular activity. Further displacement of the tendon occurring during the next few days resulted in a definite crippling. At this time the joint became swollen due to bruising and to the unnatural strain placed upon it by the chick in moving about. Maximum severity was usually reached by the fourth week. Only occasionally did the initial stages of perosis appear after the fourth week.

These observations do not agree entirely with those of earlier investigators who reported on the symptoms of perosis in chicks fed on diets of high calcium and phosphorus content. It was observed by them that swelling of the tibio-metatarsal joint was most frequently the first indication of perosis. Loose tendons were also frequently reported. Such variability in response of chicks to low manganese diets is attributed to breed, rate of growth, and composition of basal diet employed.

SUMMARY

Perosis is a nutritional disease involving a deficiency of manganese. Approximately 80 percent of a group of New Hampshire chicks developed perosis when placed on a basal diet of low manganese content (Mn, 10 p.p.m.). When the manganese content of the diet was increased to 50 p.p.m. by means of $MnCl_2.4H_2O$, $MnSO_4.4H_2O$, $KMnO_4$, $MnCO_3$ and MnO_2, the incidence of perosis was reduced to about 4 percent. In quantitative studies using c.p. $MnCO_3$ as the source of manganese the minimum amount of manganese required to prevent perosis in this breed was found to be approximately 50 p.p.m.

Additional amounts of manganese dissolved in the drinking water or incorporated in the diet up to 1,000 p.p.m. were ineffective in preventing cases of perosis which developed early. Apparently perosis in its initial stages or the conditions that produce it appeared during the embryonic development of the chicks.

Manganese at a level of 50 p.p.m. of diet reduced the incidence of perosis from 87 percent to 18 percent in a strain of New Hampshire chicks that were very susceptible to the disease. Manganese at a level of 30 p.p.m. completely prevented perosis in White Leghorn chicks. The results indicate breed and strain differences in manganese requirements and susceptibility to perosis.

Manganese deficiency not only results in perosis but prevents optimum growth of chicks and pullets. Large quantities of manganese, 1,000 p.p.m. of diet, were not found to be toxic.

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A MICROCOLORIMETRIC METHOD FOR THE DETERMINATION OF MANGANESE IN BIOLOGICAL MATERIALS WITH 4,4′-TETRAMETHYLDIAMINOTRIPHENYLMETHANE

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In studies of manganese deficiency in the rabbit,[1] a method for the determination of this element in small samples of blood serum and other tissues was needed. The most promising of the organic reagents suitable for manganese appeared to be 4,4′-tetramethyldiaminodiphenylmethane, which has been used by Harry (1) in a colorimetric procedure and by Mellan (2) as a spot test reagent. Preliminary tests showed that 4,4′-tetramethyldiaminotriphenylmethane is approximately 10 times as sensitive as the diphenyl compound; consequently this investigation was limited to the triphenyl compound.

The method consists of a periodate oxidation of manganese to permanganate with subsequent development of a yellow color by the reaction of permanganate and 4,4′-tetramethyldiaminotriphenylmethane.

Factors influencing the reactions involved were found to be the kind and concentration of acid used, the concentration of periodate, and the temperature and time of reaction between the reagent and permanganate. An acid mixture of 0.6 N in HNO_3 and 3.2 N in H_3PO_4 was found to be satisfactory. The concentration of periodate is kept sufficiently constant by measuring the required amount of sodium periodate with a bowl-shaped glass scoop.

Oxidation of the reagent by periodate is appreciable at a temperature of 90–100° and is slight by permanganate at temperatures of 20–40°. If the reagent is added to the permanganate-periodate solution at 75–85°, the reaction with the periodate is not appreciable, while the reaction with permanganate is sufficient to yield a good color. A temperature of 80° was therefore chosen as most suitable.

The measurement of the final color is made by means of a photoelectric colorimeter of the Evelyn type (3) with matched test-tubes 18 mm. in diameter. The course of the reaction is followed by noting the galvanometer reading, and the lowest per cent transmittance is taken as the final reading.

[1] Ellis, G. H., Smith, S. E., and Gates, E. M., unpublished work. Smith, S. E., and Ellis, G. H., unpublished work.

Method

Reagents—

Water. Redistil water from an all-Pyrex still.

Nitric acid. Redistil a 1:1 mixture of C.P. concentrated HNO_3 and water from an all-Pyrex still.

Phosphoric acid. C.P., 85 per cent. Different bottles of the same lot as well as different lots of phosphoric acid from the same company have been found to vary considerably in their manganese content. Choose a supply giving a low blank.

Acid mixture. To 600 ml. of 1 N HNO_3 (redistilled), add 75 ml. of 85 per cent H_3PO_4 and make to 1 liter with redistilled water.

Sodium periodate. The grade supplied by the Eastman Kodak Company is satisfactory.

Color reagent. Make a 0.1 per cent solution of 4,4′-tetramethyldiaminotriphenylmethane in 5 per cent, by weight, H_3PO_4. The reagent, as obtained from Eastman, can be used without further purification. This solution should be prepared daily and must be absolutely colorless.

Standard manganese solution. Dissolve 0.144 gm. of C.P. potassium permanganate in about 100 ml. of redistilled water and reduce with sulfur dioxide, as suggested by Richards (4). Boil off the excess sulfur dioxide and, after cooling, dilute to 1 liter. 1 ml. of this solution contains 0.05 mg. of manganese, and a suitable working standard is prepared weekly from this stock solution.

Preparation of Samples. Bones—Ash in a covered porcelain or silica dish for 18 hours at 600°. Add sufficient 1:1 HNO_3 (redistilled) to dissolve the ash and evaporate to dryness on a hot plate. Reash at 600° for another 18 hours. Repeat if necessary to get a white ash.

Blood or Blood Serum—Dry the sample in a porcelain or silica dish in a drying oven or on a steam plate. Start with a cool furnace, raise the temperature gradually to 600°, and hold for 18 hours. Add 3 ml. of 1:1 HNO_3, take to dryness, and reash at 600° for a few hours.

Liver—It is usually difficult to ash liver, and a few ml. of 20 per cent magnesium nitrate added to the sample prior to ashing are helpful. Ash at 600°, as above. It is sometimes necessary to resort to three HNO_3 treatments before a white ash is obtained.

Procedure

Dissolve the ash in the least possible volume of the nitric-phosphoric acid mixture, warming gently to hasten solution. Transfer an aliquot of this solution containing 0.05 to 0.5 γ of manganese to a 20 ml. glass-stoppered test-tube, make to 10 ml. volume with the acid mixture, and add 50 mg. of $NaIO_4$ (a glass measuring scoop is sufficiently accurate). Heat for 1.5 hours in a boiling water bath. Transfer two 3 ml. aliquots to color-

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meter tubes, cover with an inverted micro beaker, and place in a water bath held at 80 ± 1.0° for at least 10 minutes. Remove a tube from the bath, immediately add 3 drops of the 4,4′-tetramethyldiaminotriphenylmethane solution, shake gently for 5 seconds, place in the colorimeter, and note the minimum galvanometer reading. The filter used is made up of Corning standard thickness Filters 3387 and 3962 and half standard thickness Filter 5113. A complete set of standards must be run simultaneously. Reagent blanks should read 99 per cent transmittance or higher.

DISCUSSION

The determination is most conveniently carried out by two persons, one to operate the colorimeter and record the results and the other to prepare the samples for the colorimeter.

Contamination is a big factor in reproducibility; samples should be covered at all times to be protected from dust. During ashing Pyrex watch-glasses may be used. All glassware should be cleaned just prior to use with hot concentrated HNO_3 and rinsed with distilled water, followed by a small amount of redistilled water. Before the initial reading and between subsequent readings the colorimeter tubes should be cleaned by rinsing with distilled water, followed by hot acid-mixture to which $NaIO_4$ has been added. After draining, the tubes are ready for use.

If samples are too concentrated, it is unnecessary to repeat the entire periodate oxidation if the sample is diluted with acid-mixture which has been boiled with $NaIO_4$. The diluted sample is then heated for 10 minutes in the boiling water bath before aliquoting.

Upon addition of the reagent, the rate of development and of fading of the yellow color depends upon temperature, light intensity, and concentration of manganese. When the reaction is carried out according to the procedure described above, maximum color of the blanks develops in about 3 minutes, upon addition of the reagent, and then remains constant for about 5 more minutes. Standards containing 0.5 γ of Mn per 10 ml. reach a maximum color in about 30 seconds and remain constant for about 45 seconds more. The rate of color development and fading is the same with standard manganese solutions or with samples of bone, liver, Canada field pea hay, blood serum, and milk, indicating that interfering substances are absent from these materials.

Since the color reaction is influenced by strong light, this work was carried out in a darkened room with the light covered by yellow cellophane.[2] After fading for a half-hour or so, the yellow color remaining is stable for at least 20 hours. This stable color, however, did not prove as satisfactory for quantitative work as the maximum color.

[2] No. 300 PRC tango, du Pont.

Various organic solvents listed in Table I will extract the yellow color and yield a blue solution in the non-aqueous phase, or, in the case of some

TABLE I

Color Reactions with Organic Solvents

An equal volume of solvent added to a deep yellow-brown solution produced by the reaction of permanganate and 4,4′-tetramethyldiaminotriphenylmethane.

Solvent	Reaction	
Single phase		
Ethanol	Colorless	
Acetone	No change	
1,4-Dioxane	Clear blue	
Pyridine	" "	
Isopropyl alcohol	" "	
Double phase	Aqueous phase	Solvent phase
Shellysolve	Yellow	Colorless
Xylene	"	"
Carbon tetrachloride	"	"
Diethyl ether	"	"
Isoamyl acetate	"	"
Cyclohexane	"	"
Benzene	Deep yellow	Faint blue
Chloroform	Faint "	Blue
Isobutyl alcohol	Colorless	"
Cyclohexanol	Yellow	"
Toluene	Deep yellow	Faint blue
Ethylene dichloride	Colorless	Blue

TABLE II

Comparison of Manganese Values on Various Materials by Periodate and 4,4′-Tetramethyldiaminotriphenylmethane Methods

Sample	Periodate method	4,4′-Tetramethyldiaminotriphenylmethane method
	γ per gm.	γ per gm.
Soy bean hay	69.0	70.0
	57.5	59.8
	55.5	56.4
Liver	0.375	0.385
Bones	3.13	3.43
	2.77	3.35
	16.4	16.4
	7.88	8.72
	11.8	12.8
	14.4	14.4
	6.71	6.71
	11.6	10.6

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water-miscible solvents such as isopropyl alcohol and 1,4-dioxane, a blue homogeneous solution is produced. The intensity of the blue color is

TABLE III

Recoveries of Added Manganese

Sample	10 ml. solution	Added	Found	Recovery
	γ	γ	γ	*per cent*
Liver	0.094	0.050	0.142	96.0
			0.145	102.0
			0.150	112.0
Soy bean hay	0.215	0.200	0.405	95.0
		0.050	0.260	90.0
Bone	0.170	0.200	0.350	90.0

TABLE IV

Comparison of Manganese Values on Bone Samples Analyzed on Different Dates and with Different Aliquots

Bone No.	Apr. 15, 1946		Apr. 18, 1946	
	Aliquot analyzed	Mn per 10 ml.	Aliquot analyzed	Mn per 10 ml.
	ml.	γ	*ml.*	γ
1	5	0.134	10	0.137
6	3	2.190	1	2.630
2	5	0.100	10	0.110
7	3	1.720	1	1.670
9	5	0.116	10	0.115
12	3	0.790	3	0.783
16	5	0.118	10	0.109
19	3	1.425	3	1.265
20	5	0.168	10	0.169
23	3	1.465	3	1.483
35	5	0.160	10	0.155
37	3	Over 2.7	1	3.560
45	5	0.118	10	0.128
48	3	1.400	3	1.435
47	5	0.150	10	0.148
49	3	2.310	1	2.810
50	5	0.062	10	0.086
51	3	1.345	3	1.355
53	5	0.088	10	0.086
54	3	1.000	3	0.980

roughly proportional to that of the yellow color and is somewhat more intense. If the need should arise for a more sensitive method, the use of such organic solvents offers a possible solution.

Results

The validity of the method was tested by a comparison with the periodate method (5), as shown in Table II. Since results by the two methods were

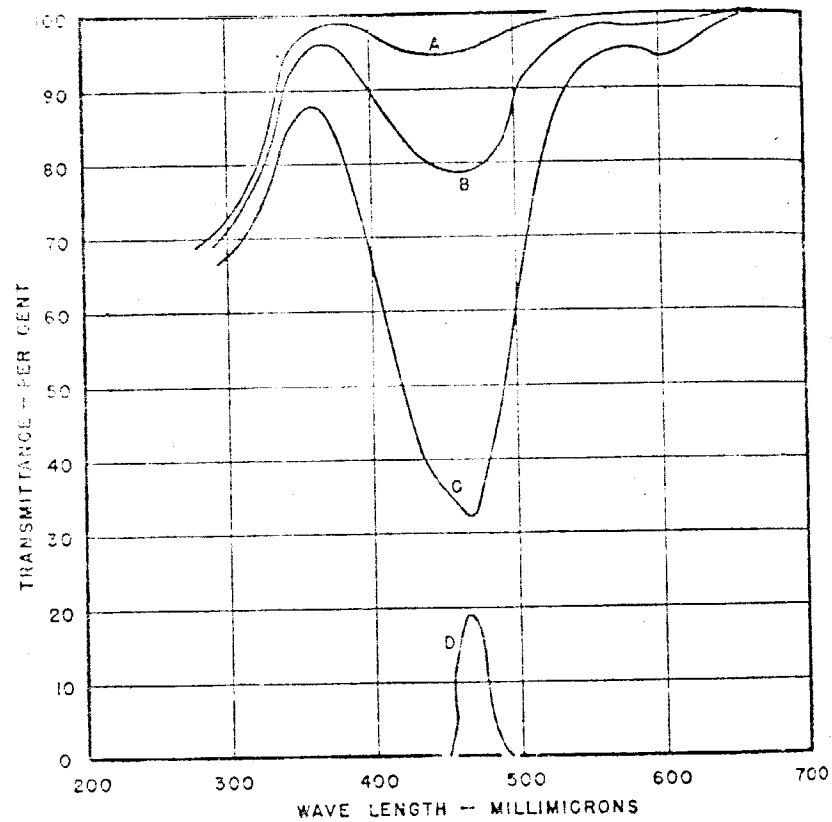

FIG. 1. Spectral transmittance curves for 4,4′-tetramethyldiaminotriphenylmethane with manganese and the filter combination used in the determination of manganese. Curves were determined on the stable color with the Beckman spectrophotometer with 1 cm. cells. Curve A, reagent blank; Curve B, 0.2γ of manganese per 10 ml.; Curve C, 0.5γ of manganese per 10 ml.; and Curve D, light transmitted by combination of Corning filter Nos. 5113 (0.5 standard thickness), 3387, and 3962 (standard thickness).

in good agreement, no extensive tests for interfering substances were made. However, negative results were obtained with the following substances: iron, lithium oxalate, lead acetate and nitrate, copper acetate and sulfate, ammonium hydroxide and sulfate, magnesium sulfate, and silver nitrate.

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The following substances gave a yellow color: chromium trioxide, ammonium vanadate, ceric ammonium sulfate, and ceric sulfate. The chloride ion also interferes and all samples containing chloride must be evaporated to dryness with 1:1 HNO_3. Cerium and vanadium are not likely to be present in biological material to any appreciable extent, but care must be taken not to use glassware cleaned in chromic-sulfuric acid cleaning solution.

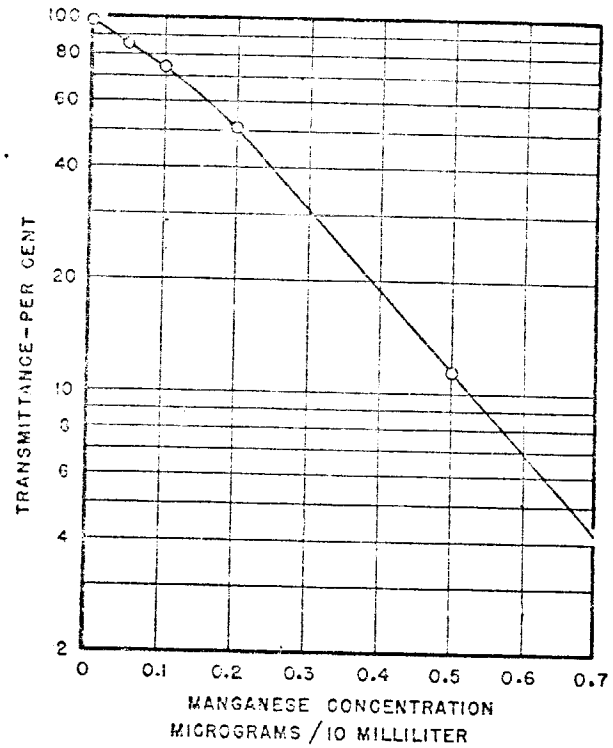

FIG. 2. Typical manganese standard curve

Recoveries of manganese added to various materials ranged from 90 to 112 per cent (Table III). The data of Table IV show the reproducibility of the method carried out on different days with different sized aliquots of bone samples.

The manganese method of Wiese and Johnson (6) was found by the authors to give erratic results when applied to samples containing less than a few micrograms of manganese. This is believed to be due in part to the difficulty found in obtaining samples of sodium bismuthate giving

a sufficiently low blank and in part due to contamination of the sample during filtration. Of six lots of sodium bismuthate obtained from various sources, only one gave a reasonably low blank, but even in this case the blank was considerably greater than that obtainable in the present procedure.

The absorption curve for the yellow color, as determined with a Beckman spectrophotometer, is shown in Fig. 1. The transmission of light by the colorimeter filter combination is also shown in Fig. 1.

The standard curve in Fig. 2 shows that Beer's law is not followed.

SUMMARY

A microcolorimetric method for the determination of manganese in biological materials with 4,4'-tetramethyldiaminotriphenylmethane is presented. Results obtained by this method are comparable with those obtained by the periodate method, and satisfactory recoveries of added manganese are shown. The method is sensitive from 0.02 to 0.5 γ of Mn per 10 ml. of solution, and results are reproducible on different days.

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MANGANESE, COPPER, AND IRON CONTENT OF SERVING PORTIONS OF COMMON FOODS*

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The last five years have witnessed the development of a marked interest in the distribution of certain mineral elements that occur in only small quantities in our foodstuffs and a rather definite belief that several of these elements play a much more important rôle in nutrition than has hitherto been assigned to them. This widespread interest is evidenced by an increasing number of papers dealing with the analysis of our common foods and by numerous investigations to determine the function of these elements in the animal body. The French investigator, Bertrand (1–3), has been a conspicuous pioneer in focusing public attention on such elements as manganese, zinc, cobalt, and nickel. McHargue (4–6), in this country, has published many papers during the last decade regarding the distribution of these minor elements, and has made numerous suggestions regarding their importance in biological processes. Public attention was sharply focused on one of these elements by the brilliant discovery of Hart, Steenbock and associates (7, 8) that copper possesses the property of supplementing iron in hemoglobin formation in anemic rats. These investigators (9, 10) demonstrated that nutritional anemia in other animals also, namely, chickens and pigs, could be corrected by the addition of copper to the ration. While some investigators (11–16) do not agree with the Wisconsin group as to the specificity of copper for hemoglobin formation, the concensus of opinion is that copper occupies a unique and important position in this respect. Mills (17) has recently published a paper showing the remarkable benefits derived from ingestion of copper by humans in cases of idiopathic anemia.

Although recent work has not assigned to manganese as striking a rôle as that given to copper, there is considerable evidence of important functions performed by manganese. McCarrison (18), Richet, Gardner and Goodbody (19), and Levine and Sohm (20), show that additions

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of manganese to the ration improve the growth of animals, and several investigators (11–14, 21) believe that manganese as well as copper plays a part in the regeneration of hemoglobin. Faludi (21) reported that manganese administered orally in the form of an albuminate exerted a marked regenerating action on the blood, and was of great value in clinical cases of both secondary and pernicious anemia.

Since iron has been recognized for many years as an indispensable element in nutrition, no review of its functions is necessary.

MANGANESE, COPPER, AND IRON CONTENT OF COOKED FOOD

In view of the recognized importance of these elements in nutrition it becomes a matter of some consequence to know the copper and manganese content of our common foods. It is also desirable to have the mineral content expressed on a more readily usable basis than the customary fresh or dried basis. It is not easy to calculate from the usual analytical table the mineral content of the food as it is eaten. Expression of the mineral content in a unit of cooked food, or per serving, translates into usable form figures which are virtually meaningless to the average individual. While it may seem that a serving of food is an indefinite unit, the plus and minus errors due to the use of this unit would probably compensate one another in the intake of food for a day. Also, because of the great variations in the mineral content of different samples of the same food—variations which may be one hundred per cent or more—it is doubtful whether there is anything to be gained by expressing the figures on a more definite basis. Since a fifty per cent margin of safety is to be adopted in all cases, the serving unit is probably sufficiently accurate. Therefore this paper presents figures for the manganese, copper, and iron content of foods as eaten, and shows how such figures may be used to estimate the approximate daily intake of these elements by various individuals.

In table 1 are given the data for 120 different foods. The portions are based largely on Rose's *Laboratory Handbook for Dietetics* (22), while the figures used in the computations are taken mainly from published and unpublished papers from this laboratory (23–27). It will be noted that the iron content exceeds both manganese and copper in all but twelve of the foods, and that the manganese exceeds the copper in approximately one-half of the materials. The manganese varies from 0.005 mg. in butter to 3.64 mg. in wheat bran. Ten other servings high in manganese are blueberries, whole wheat, split peas, beets, navy beans, chocolate,

TABLE 1

*Manganese, copper, and iron content of serving portions of foods**

MATERIALS	MEASURE: One serving	MEASURE: Weight E.P. (fresh or undried)	MANGANESE	COPPER	IRON
		gms.	mg.	mg.	mg.
Almonds	12–15 nuts	15	0.27	0.18	0.61
Apples, E.P.	1 medium	100	0.04	0.10	0.60
Apricots, dried	3 large halves	25	0.07	0.09	1.82
Artichoke, E.P.	1 medium	75	0.27	0.23	1.42
Asparagus	5 large stalks, 8″ long	100	0.10	0.14	0.79
Bacon	2 slices	25	0.02	0.13	0.63
Bananas, E.P.	1 medium	100	0.82	0.21	1.76
Barley	¼ c. cooked	14	0.21	0.09	0.50
Beans, kidney	½ c. cooked	50	0.82	0.33	3.46
Beans, Lima	½ c. cooked	50	0.54	0.43	5.83
Beans, navy	½ c. cooked	50	1.27	0.35	4.76
Beans, string	½ c. cooked	70	0.14	0.07	0.65
Beef, lean	Slice 4″ x 1½″ x ¾″	114	0.02	0.11	4.45
Beef, liver	Slice 4½″ x 2½″ x ½″	100	0.25	2.15	8.30
Beet greens	½ c. cooked	100	0.90	0.09	2.98
Beets	⅔ c. cooked	100	1.35	0.19	2.36
Blackberries	¾ c.	100	0.59	0.16	1.00
Blueberries	¾ c.	100	2.29	0.11	0.41
Brazil nuts	2 nuts	14	0.13	0.20	0.55
Bread, Graham	1 med. slice	25	0.79	0.08	0.63
Bread, rye	1 med. slice	25	0.32	0.07	0.48
Bread, white	1 med. slice	25	0.08	0.09	0.23
Brussels sprouts	4–5 med.	75	0.20	0.08	1.67
Butter	2 squares, 1¼″ x 1¼″ x ¼″	13	0.005		0.03
Butternuts	4–5 nuts	15		0.18	1.03
Cabbage	½ c. raw shredded	42	0.03	0.02	0.14
Calf liver	Slice 4½″ x 2½″ x ½″	100	0.32	4.41	5.40
Cantaloupe, E.P.	½ cantaloupe	128	0.05	0.08	0.52
Carrots	⅔ c. cooked	100	0.06	0.08	1.07
Cauliflower	⅔ c. cooked	100	0.17	0.14	1.43
Celery	3 stalks	30	0.05	0.003	0.23
Celery cabbage	1 slice 1″ thick 2½″ diam.	50	0.06	0.03	0.29
Chard	½ c. cooked	100	0.80	0.11	4.02
Cheese	Piece 1⅓″ x 2¼″ x 1⅓″	46	0.05	0.07	0.64
Cherries, E.P.	¾ c.	100	0.03	0.14	0.49
Chicken	3 slices 4½″ x 2½″ x ¼″	120		0.39	0.99
Chocolate, bitter	1 square	28	0.90	0.75	0.88

* Abbreviations used have the following meanings: E.P. = edible portion; c. = cup; T = tablespoon; ″ = inches; med. = medium; diam. = diameter.

TABLE 1—*Continued*

MATERIALS	MEASURE: One serving	MEASURE: Weight E.P. (fresh or undried)	MANGANESE	COPPER	IRON
		gms.	mg.	mg.	mg.
Cocoa	1 T	8	0.28	0.27	0.25
Cocoanut, fresh	¼ c.	22	0.29	0.15	0.59
Corn flakes	½ c.	20	0.009	0.04	0.56
Cornmeal	⅔ c. cooked	27	0.06	0.05	0.35
Corn, sweet	½ c. or 2—6″ ears	100	0.15	0.06	0.80
Cranberries	⅓ c.	50	0.15	0.05	0.30
Cream of wheat	½ c. cooked	18	0.08	0.05	0.15
Cucumbers, E.P.	⅓ cucumber 9″ long	50	0.08	0.03	0.18
Currants	¾ c.	100	0.31	1.12	0.70
Dates, E.P.	¾ c. stoned	45	0.07	0.17	2.28
Duck	3 slices 4½″ x 2½″ x ¼″	120	0.04	0.49	2.05
Eggs	1 egg	50	0.02	0.12	1.26
Egg yolk	1 yolk	16	0.02	0.06	1.22
Egg plant, E.P.	1⅔ slices 4″ diam. ½″ thick	100	0.11	0.10	0.61
Figs, dried	2½ large	50	0.17	0.16	1.98
Fish, E.P.	Piece 3¾″ x 2½″ x ¾″	110	0.02	0.27	0.63
Flour, buckwheat	1 T	11	0.23	0.08	0.13
Flour, Graham	1 T	9	0.39	0.04	0.33
Flour, patent	1 T	7	0.03	0.01	0.06
Flour, rye	1 T	9	0.18	0.04	0.26
Goose	3 slices 4½″ x 2½″ x ¼″	120	0.06	0.40	2.42
Gooseberries	¾ c.	100	0.04	0.08	0.47
Grapes, E.P.	1 medium bunch	100	0.08	0.06	0.67
Grapefruit, E.P.	½ large	213	0.03	0.06	0.58
Hominy	½ c. cooked	18	0.02	0.03	0.10
Honey	1 T	31	0.01	0.06	0.36
Kohlrabi	⅔ c. cooked	100	0.11	0.14	0.68
Lamb chops, E.P.	2 small chops	96	0.04	0.40	3.20
Lemon, E.P.	1 large	76	0.03	0.03	0.11
Lettuce, head	¼ head	50	0.40	0.02	0.21
Lobster	½ c.	80	0.03	0.58	0.35
Milk	1 c.	240	0.005	0.04	0.58
Molasses	1 T	23	0.10	0.44	1.83
Mushrooms	½ c. or 8–10, 1″ diam.	100	0.05	1.79	3.14
Oatmeal	½ c. cooked	25	0.70	0.13	0.95
Olives, green, E.P.	6 olives	40		0.14	0.85
Onions	1 medium	100	0.05	0.08	0.30
Orange, E.P.	1 large	196	0.05	0.16	1.84
Oyster	⅓ c. or 4–8 oysters	110	0.24	3.38	3.45
Oyster plant	⅔ c. cooked	100	0.35	0.27	1.24
Parsley	3 sprigs	5	0.05	0.01	0.96
Parsnips	4 pieces 3½″ x 1½″ x ½″	100	0.04	0.12	1.07

TABLE 1—*Concluded*

MATERIALS	MEASURE		MANGANESE	COPPER	IRON
	One serving	Weight E.P. (fresh or undried)			
		gms.	*mg.*	*mg.*	*mg.*
Peaches, dried	2 large halves	25	0.17	0.07	1.52
Peanuts, E.P.	1 T chopped	9	0.14	0.09	0.21
Pears, E.P.	2 large halves	100	0.64	0.10	0.46
Peas, green, shelled	⅔ c.	100	0.42	0.24	1.77
Peas, split	⅔ c. cooked	50	1.39	0.70	2.85
Pecans	12 meats	14	0.49	0.19	0.36
Peppers, green	1 pepper 3½″ long	75	0.11	0.08	0.31
Pineapple	1 slice	65	0.70	0.05	0.21
Pistachio nuts	30–35 nuts	15	0.10	0.18	1.19
Plums, E.P.	3 large	90	0.07	0.14	0.70
Pork, chops, E.P.	1 large chop	54	0.03	0.17	0.82
Pork, liver	Slice, 4½″ x 2½″ x ½″	100	0.38	0.65	25.00
Potatoes, E.P.	1 small	100	0.10	0.17	0.85
Potatoes, sweet, E.P.	½ large	100	0.15	0.15	0.92
Prunes, E.P.	4 medium	33	0.06	0.14	1.71
Puffed rice	½ c.	10	0.07	0.06	0.11
Puffed wheat	½ c.	7	0.19	0.05	0.29
Pumpkin	⅔ c. cooked	100	0.04	0.03	1.10
Quince	3 small halves	100	0.04	0.14	1.02
Radishes	6 red button	57	0.03	0.09	0.78
Raisins, seeded	¼ c.	29	0.09	0.08	2.03
Raisins, seedless	¼ c.	38	0.20	0.12	2.40
Raspberries	¾ c.	100	0.51	0.13	0.99
Rhubarb	⅔ c.	50	0.08	0.03	0.43
Rice, polished, cooked	½ c.	20	0.22	0.04	0.21
Rutabagas, E.P.	⅔ c. cooked	100	0.13	0.15	1.07
Shredded wheat	1 biscuit	27	0.65	0.17	1.22
Spinach	½ c. cooked.	100	0.70	0.12	6.60
Squash	½ c. cooked	100	0.16	0.04	0.72
Strawberries	⅔ c.	100	0.06	0.02	0.66
Tangerines, E.P.	2 medium	100	0.04	0.09	0.61
Tomatoes	1 small	100	0.14	0.06	0.60
Turkey	2⅓ slices 4″ x 2½″ x ¼″	120	0.04	0.20	1.72
Turnips	⅔ c. cooked	100	0.04	0.09	0.70
Veal chops, E.P.	1 large chop	80	0.02	0.20	0.32
Walnuts, black	8–16 meats	14	0.45		0.84
Walnuts, English	8–16 meats	14	0.25	0.14	0.30
Watercress	1 bunch 3″ x 2″	50	0.26	0.02	3.61
Watermelon, E.P.	1 slice 6″ diam. 1¾″ thick	300	0.05	0.21	0.69
Wheat, whole	½ c. cooked	25	0.86		0.91
Wheat bran	½ c.	40	3.64	0.47	3.41

beet greens, bananas, kidney beans, and chard. The copper content varies from 0.003 mg. in celery to 4.41 mg. in calf liver. Foods containing more than 0.5 mg. per serving are oysters, beef liver, mushrooms, currants, chocolate, split peas, pork liver, and lobster. Iron varies from 0.03 mg. in butter to 25 mg. in pork liver. Ten other foods high in iron are beef liver, spinach, Lima beans, calf liver, navy beans, lean beef, chard, watercress, kidney beans, and oysters.

TABLE 2

Daily intake of manganese, copper, and iron of various types of individuals

(Based on menus in *Feeding the Family* by Mary Swartz Rose)

INDIVIDUALS	MANGANESE	COPPER	IRON
	mg.	*mg.*	*mg.*
Child one and one-half years (p. 156)	1.16	0.69	7.00
Child three to four years (p. 166)	.80	.63	4.50
Child five to six years (p. 178)	3.52	1.73	16.69
Child ten years (p. 193)	3.43	1.86	13.86
Boy sixteen years (p. 202)	7.46	3.01	21.17
Girl sixteen years (p. 204)	2.86	1.74	13.45
Sedentary man (p. 55)	3.79	1.89	13.56
Sedentary woman (p. 97)	2.03	1.39	15.73
Working man (p. 61)	8.41	4.81	36.12
Active woman (p. 94)	1.91	1.76	13.31
Family, summer, No. 2 (p. 242)	1.29	1.73	12.06
Family, summer, No. 4 (p. 242)	2.32	2.14	9.88
Family, summer, No. 6 (p. 243)	3.00	1.85	13.39
Family, winter, No. 1 (p. 244)	2.51	1.86	21.72
Family, winter, No. 2 (p. 244)	2.29	1.68	13.60
Family, winter, No. 6 (p. 245)	2.94	4.27	16.97
Family, average	2.39	2.26	15.44

DAILY INTAKE OF MANGANESE, COPPER, AND IRON

The usefulness of table 1 is illustrated in the calculation of the daily intake of these mineral elements. Table 2 gives the figures for diets that have been recommended for various types of individuals. The menus were taken from Rose's *Feeding the Family* (28) and the recipes used in the preparation of the food are those given in the above book or in Farmer's *Boston Cooking School Cook Book* (29). From the table it is evident that the manganese, copper, and iron content of any diet depends on the amount and kind of food eaten. The manganese varies from 0.80 mg. in the menu for the three to four-year-old child, to 8.41 mg. in

that for the working man. The adolescent boy with a calorie intake practically equal to that of the working man has almost the same quantity of manganese in the day's diet. If the boy's diet is compared with that of the adolescent girl, it is found that the manganese ratio of the two (2.6) is considerably higher than the calorie ratio (1.5). The average manganese content per person of six family menus is 2.39 mg. It will also be noted that the intake is lower for the summer menus, 2.20 mg., than for the winter menus, 2.58 mg.

Copper varies in the different menus from 0.63 mg. for the three to four-year-old child, to 4.81 mg. for the working man. The maximum figure is due to the high calorie intake, i.e. quantity of food in the menu. One of the family winter menus is next highest with 4.27 mg. This is caused by the presence of one serving of oysters, a food which is very high in copper. The average copper intake of all the family menus is 2.26 mg. The intake of copper is also lower in summer, being 1.91 mg. as compared with 2.60 mg. in winter.

The variation for iron is from 4.50 mg. for the three to four-year-old child, to 36.12 mg. for the working man. From the figures for iron it appears that many of these menus could be improved by the use of more foods rich in this element; out of twelve adult menus, six are below the dietary standard for iron given by Sherman (30)—15 mg. per day—and one of these is strikingly deficient in this element. The average iron intake of all the family menus is 15.44 mg.

Because of the greater abundance of vegetables in summer it would be expected that the summer menus should be higher in iron than the winter menus. The figures, however, show an average for the two seasonal menus of 11.78 mg. and 19.10 mg. respectively. The winter menus owe their high content of iron to a liberal use of dried foods, meats, and eggs.

DISTRIBUTION OF MINERAL INTAKE AMONG VARIOUS CLASSES OF FOODS

Table 3 shows the contribution of various classes of foods to the manganese, copper, and iron content of ten of the diets discussed under table 2. In making these calculations the mineral content of the several constituents in the food as eaten has been assigned, so far as possible, to the appropriate classes of foods. From the table it will be observed that cereals contribute the largest proportion of manganese and copper to the diets, and that vegetables are second in this respect. Cereals and vegetables supply about the same proportion of iron. Milk, which is ordinarily considered as a poor source of iron, is shown to be the largest contributor of this element to the diet of the young child. The reason for this is, of course, the large quantity of milk consumed at this age.

TABLE 3

Contribution of various classes of foods to the manganese, copper, and iron content of the diets considered in table 2 (Percentage of total intake)

INDIVIDUALS	VEGETABLES	FRUITS	CEREALS	MILK	EGGS	MEATS	OTHERS
Manganese:							
Child 1½ years	15	2	80	1.6	1.4	0	0
Child 3–4 years	5	2	90	2.2	0.2	0	0.6
Child 5–6 years	45	3	51	0.4	0	0	0.6
Child 10 years	16	3	78	0.5	0.1	0.2	2.2
Boy 16 years	5	18	71	0.3	0.1	0.1	5.5
Girl 16 years	13	17	57	0.4	0.2	0.5	11.9
Sedentary man	34	2	56	0.2	0.7	1	6.1
Working man	17	16	61	0.1	0.02	0.3	5.6
Family, summer, No. 6	15	21	42	0.8	1.3	0.7	19.2
Family, winter, No. 6	13	43	33	0.7	0.2	6.6	3.5
Average	18	13	62	0.7	0.4	0.9	5
Copper:							
Child 1½ years	14	7	49	20.3	9.7	0	0
Child 3–4 years	12	7	58	20.6	2.4	0	0
Child 5–6 years	37	12	45	6	0	0	0
Child 10 years	21	15	38	6	2	2	16
Boy 16 years	11	15	40	4.7	1	2.1	26.2
Girl 16 years	20	19	34	3.8	2.6	5.4	15.2
Sedentary man	31	7	26	3.4	10.2	14.7	7.7
Working man	21	9	28	1.8	0.3	3.3	36.6
Family, summer, No. 6	19	11	21	7	15.2	6.3	20.5
Family, winter, No. 6	7	8	9	3.3	1.1	63.3	8.3
Average	19	11	35	7.7	4.5	9.7	13.1
Iron:							
Child 1½ years	24	6	21	31.7	17.3	0	0
Child 3–4 years	8	14	28	46.3	3.5	0	0.2
Child 5–6 years	59	12	18	10.2	0	0	0.8
Child 10 years	20	20	24	13	3	10.4	9.6
Boy 16 years	10	17	34	10.6	1.5	11.4	15.5
Girl 16 years	14	24	21	9.6	3.8	22.1	5.5
Sedentary man	38	7	22	7.4	15.1	6.4	4.1
Working man	24	11	22	3.9	0.5	17.3	21.3
Family, summer, No. 6	27	11	13	15.3	23.2	4.2	6.3
Family, winter, No. 6	14	15	12	13	2.3	34.1	9.6
Average	24	14	21	16.1	7	10.6	7.3

Meats furnish a large share of the iron and ordinarily little manganese or copper. It is also evident that eggs contribute only a small part of

the total manganese, copper, and iron in the diet of older children and adults.

As a whole, Rose's (22) menus, in comparison with those given by McCollum and Simmonds (31), appear to be high in cereals and low in vegetables. Six family summer and winter menus given by McCollum and Simmonds contain thirty-four servings of vegetables and twenty-six servings of cereals, as compared with twenty-three servings of vegetables and thirty-three servings of cereals in Rose's menus. While the latter are undoubtedly adequate from the standpoint of calories, they could probably be improved from the mineral point of view by the inclusion of more vegetables.

SUMMARY

The manganese, copper, and iron content of serving portions of 120 different foods are given. The manganese varies from 0.005 mg. in butter to 3.64 mg. in wheat bran; copper from 0.003 mg. in celery to 4.41 mg. in calf liver; iron from 0.03 mg. in butter to 25 mg. in pork liver. Food portions richest in manganese are wheat bran, blueberries, whole wheat, split peas, beets, and navy beans. Those richest in copper are calf liver, oysters, beef liver, mushrooms, currants, and chocolate. Those richest in iron are pork liver, beef liver, spinach, Lima beans, calf liver, and navy beans.

The daily intake of manganese, copper, and iron has been calculated for sixteen different menus given by Rose for children, adolescents, men, and women. The manganese varies from 0.80 mg. for a child three to four years old, to 8.41 mg. for a working man; copper from 0.63 mg. for the young child, to 4.81 mg. for the working man; iron from 4.50 mg. for the child, to 36.12 mg. for the man. The average daily intake based on six family menus given by the same author is as follows: manganese, 2.39 mg.; copper, 2.26 mg.; iron, 15.44 mg.

Cereals supply the largest proportion of manganese and copper in these diets. Vegetables and cereals are the chief contributors of iron. Fruits are an important source of all three elements. Because of the large quantity consumed, milk furnishes a large proportion of the total intake of iron in some of these diets.

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"Mineral elements in fresh vegetables from different geographic areas."

The concentration of ten elements in nine different fresh vegetables collected in the Washington, D. C., wholesale market is reported. Marked divergencies between different producing areas were found for the sodium content of lettuce and onions, and for manganese in carrots and celery, among others. Copper and manganese in sweet corn showed statistically significant differences between means for individual lots grown on the same [illegible] and illustrate the problems inherent in reporting averages for highly variable elements for a given producing area.

Hopkins + Eison

With the introduction of new varieties and the use of different cultural practices in crop production during the last decade, there is interest among nutritionists and others regarding the nutrient content of present-day foods (*1, 10, 12*). This paper reports on the mineral composition of nine fresh vegetables grown in widely different sections of the United States during 1956 and 1957.

Materials and Methods

Among the 27 or more commercial fresh vegetables available to consumers in this country, nine were chosen for study: asparagus, snap beans, cabbage, carrots, parsal celery, yellow sweet corn, iceberg lettuce, dry yellow onions, and tomatoes. The selection was based on data from the Department of Agriculture's 1955 food consumption survey (*19*) which show these to be the vegetables, other than potatoes, consumed in the largest quantities by households in the United States. Market grades of all vegetables studied were designated as U. S. No. 1 or better.

Three or more lots of each vegetable were collected equally from major producing areas represented in the Washington, D. C., wholesale market during 1956 and 1957. By contacting as many as six wholesale merchants sufficiently in advance, it was possible to locate those who could furnish the needed vegetables. The producing areas for each vegetable studied in 1957 were chosen to account for 90% or more of the volume reaching the Washington wholesale market, except for sweet corn and tomatoes (*16*).

Most of the vegetables chosen for this study had a seasonal delivery pattern in the local market (*16*). Because of this, not more than five different vegetables made up each set collected in any one year. Difficulties in collection arose even with this small number, particularly for vegetables coming from a large number of producing areas.

Each lot collected came from a large population with respect to volume shipped into the Washington market. The smallest carrot population encountered in the wholesale market during 1957, among five carrot shipping areas, was 100,000 pounds shipped from Arizona, while the largest carrot population was 675,000 pounds shipped from Texas (*16, 18*).

In cases where the same shipper's label appeared during successive weeks of collecting the replicate lots, the wholesaler would make available other railway car or truck shipments from which it was possible to collect another lot. The shipper's name, railway car or truck number, name of wholesaler, date of collection, shipper's label on the carton, and other pertinent information were recorded on the receipt of each lot of vegetables. During 1957, much of this information was transferred to a postcard questionnaire and mailed to the various shippers and growers requesting more information on the origin of the samples.

Upon arrival of the vegetables in the laboratory, the entire sample was weighed to obtain the "as purchased" weight. Lot size for each vegetable corresponded to units normally found in the wholesale market, such as crates, cartons, or bushels. The entire lot or portion thereof was trimmed to the "edible portion," rinsed in demineralized water, and blotted with clean filter

paper. Rinsing was not done on sweet corn. The discarded portion was weighed, and the percentage of edible yield was calculated. In handling the lots of lettuce, cabbage, tomato, and onion each head, fruit, or bulb was quartered and opposite quarters were allocated to four sublots. With celery, one stalk was severed at a time, starting on the outside of the bunch and allocating it to one of the four sublots, until the entire cluster had been dismembered. With snap beans the entire lot was thoroughly mixed on a large tray and one fourth of the entire lot was used.

All samples were blended in a stainless steel blender. One portion of the blend was removed for determination of the percentage of total solids, and another portion, calculated to give about 100 grams of dry weight, was spread on a [illegible] stainless steel tray and dried under a battery of heat lamps. The dried sample was then ground in a Wiley mill fitted with a 20-mesh stainless steel screen and thoroughly mixed. For each vegetable lot, 2 grams of the dried sample were weighed into tared, borosilicate glass beakers for charring in a muffle furnace at 500° C. Another 2-gram portion was weighed into a tared [illegible] crucible, quickly ashed at 475° to 500° C. in a muffle furnace lined with a quartz liner, and used for potassium determination in the flame photometer. A third portion was removed for determination of the percentage of residual moisture. The charred sample was cooled, thoroughly ground in an agate mortar, and used for spectrographic determinations. None of the steps from the time the vegetables were received in the laboratory contributed any measurable contamination.

Charring was used to partially concentrate the samples, because the magnesium, manganese, copper, and aluminum levels were often low. A 10-mg. portion of the charred sample was weighed with a torsion-type microbalance, transferred to the crater of an electrode, and ashed in a furnace fitted with a quartz liner at a temperature of 475° to 500° C. over a 90-minute period. Ash residues in the electrodes were taken up in approximately 0.1 *N* hydrochloric acid after waterproofing the electrodes with a paraffin-kerosine mixture (*19*).

The initial evaluation of variability of minerals in fresh vegetables was made during the summer of 1956. The experimental design involved five vegetables, three producing areas per vegetable, and three lots from within each producing area. Nine lots for each of five vegetables, snap beans, domestic round cabbage, pascal celery, iceberg lettuce, and dry yellow corn, were collected during the peak of the delivery cycle from the principal producing areas represented, at that time in the wholesale market at Washington, D. C. The experimental design used for each vegetable was a randomized block plan (*12*) consisting of four plates—blocks—for each of the nine lots.

Samples with four levels of standard solutions for all elements were included on each 4 × 10 inch spectrographic plate. Up to 16 spectra constituted one of the four replicates for each vegetable. To increase the accuracy of spectrographic determinations, the 16 samples were arced on four different days, making a total of 64 arcings for each vegetable (*20*). The analytical system employed a total spectral energy technique, and a sufficient range of known standard concentrations to bracket unknown samples on each plate. It was thus possible to construct analytical curves relating intensities of the spectral lines to known concentrations of the respective elements in the standard solutions. From these analytical curves the concentrations of the elements in the unknown samples were read. As the spectra of the standard solutions were photographed on every plate, the emulsion response characteristics were easily established, making it possible to eliminate calculations necessary in plate calibrations (*15*). Statistical evaluation of the spectrographic data was accomplished by analysis of variance on logarithmically transformed data (*12*). The spectrographic assay system is known to be subject to a number of random variations (*2, 3, 8*). In a study designed to measure the frequency distribution of the spectrographic error employing the direct current arc, the arcing error was found to be normally distributed (*8*). Therefore, tests of significance based on the normality hypothesis are applicable to investigations employing the direct current arc.

The spectrographic analytical system used in these assays was similar to that described by Specht *et al.* (*15*), with the following modifications: A medium quartz spectrograph was used and the motor generator provided an excitation current of 225 volts and 20 amperes, with an average of 40 volts across the arc during the burn. Nine elements determined simultaneously on each sample were: boron, 2496.8 A., phosphorus, 2535.6 or 2553.3 A., magnesium, 2781.4 A., manganese, 2801.1 A., iron, 3020.6 A., aluminum, 3082.2 A., calcium 2997.3 or 3158.9 A., copper, 3247.5 or 3273.9 A., and sodium, 3302.3 A. Both sodium and potassium were determined on a Beckman DU flame photometer equipped with a photomultiplier attachment. Sodium values determined by the two instruments agreed well. Prior to analysis of the experimental samples, check runs were made on a number of plant samples previously analyzed by 10 other laboratories using both the spectrograph and the flame photometer. Results obtained on the medium spectrograph were in satisfactory agreement with the one reported by the other laboratories (*1*).

Investigations in 1956. Mean values and the upper and lower 95% confidence limits for nine elements in the five vegetables studied in 1956 are shown in Table I. The variance of a producing area mean was regarded as a linear function of the variance components for lots, plates, and plate-lot interaction. In most cases Satterthwaite's method of determining the degrees of freedom associated with estimates of variance components was used (*10*).

Figure 1 shows the ratio between the average element content of six vegetables from different geographic areas. Among the nine elements studied, the sodium content of lettuce and onion, and the manganese content of celery exhibited the greatest divergence in values between producing areas. In each instance the divergence occurred between an eastern and a western area.

Results of the analyses of variance revealed significant differences between lot means for certain elements in lettuce, celery, cabbage, and snap beans grown within the same general area. In most cases, there were significant differences between the mean values for producing areas included in the [illegible] study. This implies that chemical average for all producing areas should be reported separately. In the statistical analysis, variation was eliminated from differences between lot and area means.

Investigations in 1957. Results from the work in 1956 pointed to the need for collection of more than three lots of vegetables from known areas. In 1957, the plan was adopted of collecting six lots equally from producing areas delivering vegetables during the period April through October. The vegetables included asparagus, carrots, corn, tomatoes, and lettuce. Sample origins were checked and verified. Adherence to the plan of collecting six lots equally from each area was not always possible, however. Spring floods in Texas shortened what usually is a fairly long delivery season for carrots. Poor market conditions eliminated Virginia tomatoes earlier than expected, and North Carolina shipments of corn were limited because of bad weather and market conditions. These unforeseen difficulties, compounded with complications associated with the simultaneous collection of the different vegetables, illustrate some of the problems in carrying out a plan involving simultaneous collection of several vegetables.

Table I shows average fresh weight values and the upper and lower 95% confidence limits for the mean values for 10 elements in asparagus, carrots, corn, lettuce, and tomatoes collected in 1957. A twofold or greater spread occurred between certain areas in the [illegible] sodium, and manganese in carrots,

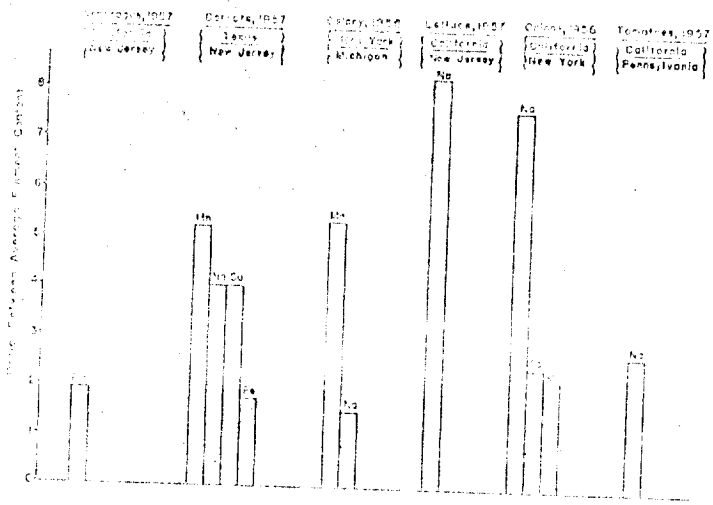

Figure 1. Ratio between average element content of six vegetables from different geographic areas

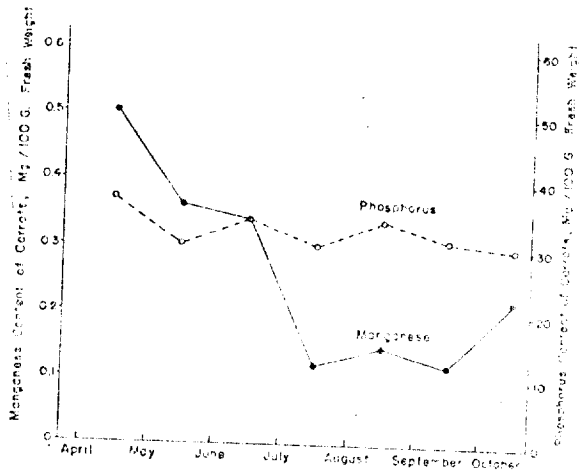

Figure 2. Average manganese and phosphorus content of fresh carrots received in Washington, D. C., market from different geographic areas according to season, 1957

manganese and sodium in tomatoes; and sodium in asparagus (Figure 1) and Table I. The sodium content of California lettuce, collected in Washington, D. C., in 1957 was about tenfold higher than that from New York or New Jersey, [illegible] the 1956 results. [illegible] in the element content of lettuce were relatively small. Statistically significant differences were observed between lot means for sodium, manganese, iron, copper, magnesium, and calcium in practically all five vegetables.

Discussion and Conclusions

Coefficients of variation for most of the elements were less than 10%. There were 11 instances in which the coefficient of variation was equal to or greater than 20%. In several of these cases—copper in lettuce from New York (1956), copper in carrots from New Jersey, copper in onions from Michigan, copper in sweet corn from central Florida, eastern Maryland, and northeastern North Carolina, and boron in sweet corn from New York—the estimated variance component for lots was considerably greater than that for plates. This suggests that better precision could have been obtained by increasing the number of lots, thereby permitting a decrease in the number of spectrographic plates for each lot.

The coefficient of variation for copper in onions from Michigan can be reduced from 31 to 21% by increasing the number of lots from three to eight and using four plates for each lot.

In the other four cases in which the coefficient of variation was equal to or greater than 20%—copper in onions from New York, boron in sweet corn from central Maryland and Delaware, and manganese in sweet corn from southeast North Carolina—the estimated variance component for plates was larger than that for lots. This implies that the coefficients of variation can be reduced by increasing the number of spectrographic plates for each lot, thereby permitting a decrease in the number of lots. The coefficient of variation for boron in sweet corn from Delaware can be reduced from 23 to 19% if four lots and eight plates for each lot are used instead of five lots and four plates for each lot.

Area or regional effects are evident for the sodium content of onions and lettuce from western and southwestern areas of the United States. This condition is probably associated with the higher sodium content in irrigation water and soils characteristic of these areas. However, because lettuce is low in sodium when compared to other foods in the diet, a tenfold increase in the average sodium content of lettuce and onions from western areas would not constitute an alarming situation for those on low sodium diets (*7*).

Within limits, carrots and celery appear to be in a class apart with respect to their high sodium content. Both these vegetables belong to the same botanical family. The existence of physiological processes of selective exclusion of certain inorganic elements, by plant parts, is seen by the essential absence of sodium and calcium in sweet corn. These observations emphasize that, among the vegetables analyzed in this study, the existence of element binding specificity residing in different plant parts and between different species is probably more decisive than typical environment factors *per se* with respect to the relative inorganic composition of foods. This does not imply that environmental factors do not exert strong effects, for it has been shown that such effects are important (*13, 14*).

The variation of certain elements in the different lots coming from the same specific area should not be ignored. The collections made in 1957 included from five to six lots of sweet corn delivered to the Washington, D. C., market at different times from each of two farms, one in central [illegible] and one in eastern Pennsylvania. For lots from both farms, significant differences between the mean squares were found for boron, manganese, and copper.

	B	Mn	Cu
Delaware, Kent Co.	*	**	**
Pennsylvania, Luzerne Co.	*	**	**

The single and double asterisks denote significance at the 5 and 1% levels, respectively.

One aspect of the large differences among lots is that the significance of regional differences for certain elements becomes somewhat vague. The same conclusion was reached by others from a study of nutrients in field corn (*6*) and vegetables (*11*).

Single values for the element content of various vegetables were obtained by computing averages weighted according to delivery volume. Weighted averages for the element content in all vegetables collected in 1957 are shown in Table II. The assumption is made that the volume of vegetables shipped into a market approximates the level of human consumption of these vegetables within the market "reservoir." Information on delivery of carlot volumes of commercial fresh vegetables is published monthly by the Agricultural Marketing Service (*16, 17*).

A relatively large influx of Central California and New Jersey carrots with a low total manganese content and a decline in shipments of carrots from Texas and Southern California with a high manganese content apparently explains the marked depression in the seasonal curve for manganese [illegible]

Table I. Mean Values and Upper and Lower 95% Confidence Limits for Means of Element Content in Fresh Vegetables from Different Major Producing Areas

Vegetable and Year	Producing Area and Month	Total Solids, %	No. of Lots Collected		Element Content, Mg./100 G. B	P	Mg	Mn	Fe	Al	Ca	Cu	Na	r
Asparagus, 1957	Central Calif., April–May	7.9	6	Mean	0.12	57	19	0.30	0.90	0.53	20	0.16	2.3	[illegible]
				Upper	0.15	68	24	0.45	1.30	0.99	26	0.20	3.2	[illegible]
				Lower	0.10	49	15	0.20	0.59	0.29	16	0.13	1.6	[illegible]
	South N. J., May–June	7.6	6	Mean	0.12	62	22	0.25	0.84	0.33	24	0.16	1.2	[illegible]
				Upper	0.16	92	30	0.33	1.10	0.64	33	0.20	1.6	[illegible]
				Lower	0.09	41	16	0.18	0.63	0.17	18	0.13	0.9	[illegible]
Beans, snap, green, 1956	Md., August–September	8.0	3	Mean	[a]	35	25	0.49	0.75	<0.01	50	0.08	0.32	[illegible]
				Upper	...	51	39	0.80	1.20	...	68	0.10	0.46	[illegible]
				Lower	...	24	19	0.30	0.47	...	37	0.06	0.23	[illegible]
	N. J., September	9.1	3	Mean	[a]	34	29	0.32	0.65	<0.01	57	0.07	0.32	[illegible]
				Upper	...	46	37	0.52	1.00	...	78	0.12	0.42	[illegible]
				Lower	...	26	22	0.20	0.41	...	42	0.05	0.24	[illegible]
	N. Y., September	9.1	3	Mean	[a]	44	30	0.55	0.62	<0.01	49	0.07	0.37	[illegible]
				Upper	...	65	39	0.90	1.00	...	66	0.17	0.62	[illegible]
				Lower	...	31	22	0.34	0.40	...	36	0.05	0.21	[illegible]
Cabbage, domestic, round, 1956	Central Md., August–September	7.7	3	Mean	[a]	19	13	0.28	0.34	<0.01	35	0.02	6.0	[illegible]
				Upper	...	27	15	0.51	0.57	...	53	0.05	18.0	[illegible]
				Lower	...	14	6	0.14	0.20	...	22	0.02	2.0	[illegible]
	N. J., July–September	7.9	3	Mean	[a]	19	11	0.24	0.26	<0.01	38	0.02	8.2	[illegible]
				Upper	...	26	15	0.47	0.44	...	60	0.03	25.0	[illegible]
				Lower	...	13	8	0.12	0.16	...	23	0.01	2.7	[illegible]
	N. Y., September	8.8	3	Mean	[a]	17	11	0.33	0.34	<0.01	35	0.02	6.0	[illegible]
				Upper	...	23	15	0.48	0.42	...	42	0.03	18.0	[illegible]
				Lower	...	12	8	0.33	0.22	...	28	0.02	2.0	[illegible]
Carrots, 1957	Central Ariz., April–July	13.8	6	Mean	0.13	25	20	0.30	0.45	0.08	47	0.06	50	[illegible]
				Upper	0.20	34	30	0.48	0.65	0.15	62	0.10	99[b]	[illegible]
				Lower	0.09	19	15	0.19	0.31	0.05	36	0.04	16	[illegible]
	South Calif., May–June	15.4	4	Mean	0.16	39	30	0.31	0.45	0.09	52	0.16	59	[illegible]
				Upper	0.20	50	43	0.43	0.62	0.11	65	0.23	[illegible]	[illegible]
				Lower	0.13	30	21	0.20	0.33	0.06	41	0.12	[illegible]	[illegible]
	Central Calif., June–October	13.0	5	Mean	0.17	30	25	0.12	0.60	0.07	44	0.08	41	[illegible]
				Upper	0.22	38	31	0.21	1.20	0.11	62	0.12	52	[illegible]
				Lower	0.13	24	20	0.07	0.31	0.05	32	0.05	32	[illegible]
	North N. J., July–October	11.7	6	Mean	0.15	34	25	0.10	0.31	<0.04	57	0.03	18	[illegible]
				Upper	0.22	46	33	0.18	0.42	...	47	0.06	20[b]	[illegible]
				Lower	0.11	26	20	0.06	0.23	...	29	0.02	13[b]	[illegible]
	Northwest N. M., September–October	12.4	4	Mean	0.15	24	21	0.12	0.27	<0.04	37	0.05	33	[illegible]
				Upper	0.25	34	29	0.24	0.40	...	48	0.10	75[b]	[illegible]
				Lower	0.10	17	15	0.06	0.18	...	29	0.02	1[b]	[illegible]
	South Tex., April–October	13.3	4	Mean	0.14	37	22	0.52	0.52	0.07	46	0.12	72	[illegible]
				Upper	0.17	46	26	0.63	0.88	0.13	60	0.20	133[b]	[illegible]
				Lower	0.12	30	17	0.32	0.31	0.04	36	0.07	12[b]	[illegible]
Celery, pascal, 1956	Calif., July–September	5.2	3	Mean	[a]	17	9.4	0.05	0.10	<0.01	27	0.03	101	[illegible]
				Upper	...	35	17	0.07	0.15	...	56	0.04	123[b]	[illegible]
				Lower	...	9	5.1	0.03	0.07	...	13	0.02	7[b]	[illegible]
	Mich., September–October	5.0	3	Mean	[a]	18	12	0.06	0.10	<0.01	55	0.02	55	[illegible]
				Upper	...	31	24	0.18	0.14	...	74	0.03	61[b]	[illegible]
				Lower	...	10	6.1	0.02	0.07	...	41	0.01	47[b]	[illegible]
	N. Y., August–September	4.4	3	Mean	[a]	20	6.5	0.32	0.08	<0.01	24	0.02	81	[illegible]
				Upper	...	30	10	0.93	0.11	...	31	0.02	135[b]	[illegible]
				Lower	...	13	4.4	0.11	0.05	...	18	0.02	2[b]	[illegible]
Corn, sweet, yellow, 1957	Central Del., July–August	24.6	6	Mean	0.04	110	51	0.25	0.71	<0.05	<2.0	0.05	0.14	[illegible]
				Upper	0.06	132	63	0.35	0.94	...	...	0.09	0.22[b]	[illegible]
				Lower	0.03	91	41	0.18	0.54	...	...	0.05	0.08[b]	[illegible]
	South Fla., April–June	21.6	6	Mean	0.10	91	40	0.20	0.51	0.16	4.5	0.05	0.24	[illegible]
				Upper	0.14	102	57	0.30	0.76	0.31	9.7	0.07	0.45[b]	[illegible]
				Lower	0.07	82	29	0.13	0.35	0.08	2.1	0.03	0.01[b]	[illegible]
	Central Fla., May–July	22.1	4	Mean	0.07	90	43	0.25	0.63	0.13	5.9	0.06	0.20	[illegible]
				Upper	0.12	155	58	0.40	1.30	0.21	8.5	0.15	0.39[b]	[illegible]
				Lower	0.05	51	33	0.16	0.50	0.07	1.8	0.02	0.02[b]	[illegible]
	Eastern Shore, Md., July	25.7	4	Mean	0.05	115	47	0.20	0.42	<0.05	<2.0	0.05	0.13	[illegible]
				Upper	0.07	151	82	0.39	0.62	...	...	0.10	0.36[b]	[illegible]
				Lower	0.03	88	27	0.10	0.28	...	...	0.03	0.07[b]	[illegible]
	Central Md., July–August	23.2	5	Mean	0.04	90	43	0.16	0.34	<0.05	<2.0	0.05	0.14	[illegible]
				Upper	0.06	117	67	0.23	0.54	...	...	0.09	0.16[b]	[illegible]
				Lower	0.03	69	27	0.11	0.22	...	...	0.04	0.12[b]	[illegible]
	West N. Y., August–September	26.2	5	Mean	0.05	117	56	0.15	0.64	<0.05	<2.0	0.05	0.15	[illegible]
				Upper	0.07	141	67	0.21	0.94	...	...	0.07	0.19[b]	[illegible]
				Lower	0.03	97	48	0.11	0.44	...	...	0.04	0.12[b]	[illegible]
	Northeast N. C., June–July	32.1	3	Mean	0.06	159	54	0.25	0.72	0.14	<2.0	0.03	0.19	[illegible]
				Upper	0.08	231	87	0.42	1.29	0.20	...	0.13	0.28[b]	[illegible]
				Lower	0.04	73	30	0.14	0.40	0.10	...	0.03	0.09[b]	[illegible]
	Southwest N. C., July	29.0	3	Mean	0.06	129	52	0.18	0.53	0.14	<2.0	0.03	0.22	[illegible]
				Upper	0.11	215	99	0.58	1.19	0.22	...	0.08	0.35[b]	[illegible]
				Lower	0.03	77	30	0.06	0.26	0.09	...	0.05	0.07[b]	[illegible]
	East Pa., August–September	28.1	5	Mean	0.05	101	50	0.22	0.70	<0.05	<2.0	0.05	0.17	[illegible]
				Upper	0.07	126	63	0.29	1.00	...	...	0.08	0.30[b]	[illegible]
				Lower	0.04	80	39	0.17	0.49	...	...	0.05	0.04[b]	[illegible]

Table I. Mean Values and Upper and Lower 95% Confidence Limits for Means of Element Content in Nine Fresh Vegetables from Different Major Producing Areas (*Continued*)

Vegetable and Year	Producing Area and Month	Total Solids, %	No. of Lots Collected		Element Content, Mg./100 G. B	P	Mg	Mn	Fe	Al	Ca	Cu	Na	K
Lettuce, iceberg, 1956	Central Calif., August–September	4.7	3	Mean	[a]	21	10	0.14	0.31	0.02	13	0.03	13	159
				Upper	...	33	13	0.25	0.31	0.03	22	0.05	26	232[b]
				Lower	...	14	7.1	0.08	0.24	0.01	8	0.01	6	50[b]
	North N. J., July–September	3.7	3	Mean	[a]	19	7.4	0.16	0.28	0.02	16	0.01	1.4	157
				Upper	...	24	11	0.28	0.48	0.05	31	0.02	2.5	243[b]
				Lower	...	17	5.0	0.09	0.16	0.01	8	0.01	0.65	[illegible]
	Central N. Y., October	4.0	3	Mean	[a]	21	7.2	0.20	0.24	0.01	15	0.01	0.90	175
				Upper	...	27	11	0.36	0.30	0.02	19	0.03	1.9	166[b]
				Lower	...	16	5.0	0.11	0.19	0.01	12	0.01	0.43	156[b]
Lettuce, iceberg, 1957	Central Ariz., April–May	5.0	6	Mean	0.05	25	11	0.23	0.27	0.03	18	0.07	7.5	135
				Upper	0.09	31	15	0.41	0.35	0.05	24	0.09	10	202[b]
				Lower	0.03	20	8	0.13	0.21	0.02	13	0.05	5.2	1[illegible]
	Central Calif., May–August	4.6	6	Mean	0.05	22	13	0.21	0.37	0.03	19	0.03	8.2	1[illegible]
				Upper	0.08	31	15	0.33	0.49	0.06	22	0.03	9.8	[illegible]
				Lower	0.03	22	11	0.13	0.28	0.01	16	0.02	6.6	11[illegible]
	Central N. J., June–September	3.9	6	Mean	0.04	21	10	0.22	0.34	0.04	17	0.03	1.0	142
				Upper	0.05	25	13	0.36	0.44	0.07	22	0.04	1.8	185[b]
				Lower	0.03	17	8	0.13	0.26	0.02	14	0.02	0.58	99
	Central N. Y., August–September	3.9	6	Mean	0.04	21	9	0.15	0.31	0.03	18	0.02	0.7[illegible]	13[illegible]
				Upper	0.05	25	11	0.23	0.45	0.04	21	0.03	0.88	195[b]
				Lower	0.04	17	7	0.09	0.21	0.02	16	0.01	0.65	[illegible]
Onions, dry, yellow, 1956	Calif., July–September	8.8	3	Mean	[a]	23	11	0.29	0.21	<0.01	38	0.05	8.4	126
				Upper	...	31	17	0.70	0.32	...	95	0.11	14	159[b]
				Lower	...	17	7	0.06	0.14	...	15	0.02	5.1	114[b]
	Mich., September–October	10.4	3	Mean	[a]	26	11	0.09	0.16	<0.01	19	0.03	1.1	187
				Upper	...	41	15	0.15	0.23	...	28	0.06	1.5	2[illegible]
				Lower	...	17	7	0.05	0.11	...	13	[illegible]	[illegible]	[illegible]
	N. Y., August–September	8.7	3	Mean	[a]	25	9	0.19	0.21	<0.01	16	0.03	1.1	162
				Upper	...	41	10	0.31	0.16	...	22	0.06	1.5	188[b]
				Lower	...	17	6	0.05	0.09	...	12	0.01	0.74	134[b]
Tomatoes, 1957	Central Calif., August–October	5.6	6	Mean	0.06	15	13	0.10	0.23	0.03	5.3	0.06	3.0	180
				Upper	0.07	19	18	0.14	0.26	0.05	7.1	0.07	5.4	248[b]
				Lower	0.05	13	10	0.07	0.20	0.02	4.0	0.05	1.7	126[b]
	South Fla., April–May	6.2	5	Mean	0.07	28	12	0.12	0.22	0.04	6.5	0.05	1.9	253
				Upper	0.10	38	18	0.25	0.50	0.09	9.8	0.09	2.9	286[b]
				Lower	0.05	20	8	0.06	0.09	0.02	4.3	0.02	1.3	180[b]
	Central Fla., May–June	6.4	3	Mean	0.06	24	13	0.09	0.31	0.05	6.5	0.04	2.5	258
				Upper	0.09	32	22	0.17	0.43	0.11	11.0	0.16	4.4	351[b]
				Lower	0.05	18	7	0.05	0.23	0.02	3.7	0.01	1.4	165[b]
	Central Md., September	6.1	4	Mean	0.05	27	13	0.14	0.27	0.04	7.5	0.08	1.4	295
				Upper	0.07	41	25	0.29	0.56	0.07	16.0	0.11	1.9	367[b]
				Lower	0.04	18	7	0.06	0.13	0.03	5.5	0.06	1.1	223[b]
	East Pa., August–September	7.5	6	Mean	0.07	18	13	0.21	0.23	<0.02	6.7	0.09	1.1	295
				Upper	0.09	26	18	0.30	0.40	...	10.0	0.12	1.3	418[b]
				Lower	0.05	13	10	0.15	0.14	...	4.4	0.07	0.87	189[b]
	South Tex., May–June	6.2	4	Mean	0.07	22	15	0.15	0.27	0.04	7.6	0.07	1.7	327
				Upper	0.10	29	21	0.29	0.55	0.09	16.0	0.13	3.6	492[b]
				Lower	0.05	16	11	0.08	0.14	0.02	3.9	0.03	0.84	162[b]

[a] Not determined.
[b] Flame photometer assay.
[c] Including seeds and skins.

Table II. Average Content of Elements in Edible Portion of Fresh Vegetables, Weighted According to Delivery Volume by Areas for All Lots Collected in Washington, D. C., Market, April–October 1957

Vegetable	Total Lots Collected	Total Solids, %	Element Content, Mg./100 G. B	P	Mg	Mn	Fe	Al	Ca	Cu	Na	K
Asparagus	12	7.8	0.12	59	21	0.28	0.87	0.44	22	0.16	1.8	280
Carrots	29	13.6	0.16	34	25	0.29	0.48	<0.06	46	0.11	53.0	349
Corn, sweet, yellow	41	25.2	0.07	104	48	0.20	0.61	<0.06	<2	0.06	0.19	297
Lettuce, iceberg	24	4.5	0.05	22	12	0.22	0.34	0.03	18	0.04	6.3	152
Tomatoes[a]	28	6.3	0.06	22	13	0.13	0.25	<0.03	6.5	0.06	2.1	255

[a] Including seeds and skins.

The same pattern was exhibited by the sodium content of lettuce (Table I), where a relatively large influx of New Jersey lettuce with one tenth the sodium content of California lettuce entered the market during the May–June 1957 period. The manganese curve for carrots [illegible] considerably above the weighted average of 0.29 mg. per 100 grams, fresh weight. On the other hand, the curve for phosphorus in carrots (Figure 2) varies but slightly about the weighted average of 34 mg. per 100 grams fresh weight. This is characteristic of the majority of the elements in [illegible] lots collected in 1957.

Results of this study thus clearly indicate the existence of a series of average values for manganese content of carrots and sodium content of lettuce at different seasons. While the seasonal patterns for such highly variable elements as manganese in carrots [illegible] sodium in lettuce are exceptional [illegible]

as the present study is concerned, they illustrate the importance of knowing ahead of time something about the various market parameters, in order that collections of vegetable samples may be made at the proper seasons.

Information obtained from this study indicates the feasibility of estimating the inorganic element content of various vegetables in an urban market. Because many consumer groups obtain their food from such well-defined urban markets, the application of this approach to additional [illegible] would seem desirable. This suggests that a market analysis could be made by sampling lots from different producing areas according to delivery vehicle at different seasons. The number of lots to be collected and the number of spectrographic plates for each lot, would depend on the vegetable and the producing area sampled.

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Adrenocorticosteroid hormones and manganese metabolism[1,2]

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HUGHES, EDWIN R., AND GEORGE C. COTZIAS. *Adrenocorticosteroid hormones and manganese metabolism.* Am. J. Physiol. 201(6): 1061-1064. 1961.—An effect of two glucocorticosteroids on the transport of the essential trace element, manganese, is described. Mice receiving high doses of prednisolone or cortisol accumulated more radiomanganese in their carcasses and retained less in their livers than did untreated controls. In some respects this effect was similar to that seen when manganese salts were administered as significant metabolic loads. It was brought on regardless of whether the injected isotope was in the divalent ionic form or in the plasma-bound form. Treatment with mineralocorticosteroids alone did not affect manganese metabolism as tested here. Furthermore, Zn^{65} did not respond to the glucocorticoid effects exhibited by Mn^{54}.

It was reported earlier that the pathway of manganese through the body leads largely into the mitochondria (1). This pathway is highly specific for that element (2). It was postulated that observation of the disappearance of radiomanganese from the blood might lead to an assay for the functioning mitochondrial mass of the intact body (3). The visible liver mitochondria were found by Lowe, MacKinney, and Sarkaria (4) to become scarce following administration of the glucocorticosteroid, cortisone, to rats. Additional work has shown that the glucocorticosteroids affect the function of isolated mitochondria (5).

The sum of these observations appeared to provide the means of altering mitochondrial activity and correlating this alteration with changes in the kinetic behavior of radiomanganese. In the present experiments sufficient hormone was used so that the changes, if any, in the metabolism of radiomanganese would be indisputable. This was deemed necessary in defining whether only one class of metabolic steroids is effective and, if so, whether the effect might be specific for manganese or common to other members of the transition group of elements.

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MATERIALS AND METHODS

Animals. 100 Swiss albino male mice (BNL strain) were housed in groups of three. These mice were 7 weeks old at the beginning of the experiments and weighed 18-20 g.

Diet. Purina fox chow (0.02% $MnSO_4$ added by manufacturer) and water were offered ad libitum.

Isotopes. Carrier-free $Mn^{54}Cl_2$ and $Zn^{65}Cl_2$ in 20% HCl were supplied by Nuclear Science and Engineering Corporation in solutions containing 200-500 μc/cc. These solutions were diluted 20-50-fold with saline prior to intraperitoneal injection of 0.1-cc aliquots (0.5-1.0 μc). Total-body radioactivity and the radioactivity of groups of organs were assayed with an assembly previously described (2). The radioactivity in the intracellular organelles was assayed in a sodium iodide crystal scintillation counter. The counting error was kept within ±1%.

Hormones. The preparations used were aqueous suspensions of prednisolone acetate (Schering), deoxycorticosterone-trimethyl-acetate (DOC-TMA, Ciba) and cortisol acetate (Upjohn), and deoxycorticosterone acetate (DOCA, Bel-Mar Laboratories) in oil. One milligram per day (= 50 mg/kg) was injected subcutaneously as a daily dose (prednisolone and DOCA) or as aggregated doses every 5 days (DOC-TMA and cortisol acetate).

Liver fractionation. The method of Schneider and Hogeboom (6) was employed for the centrifugal separation of intracellular inclusions, by means of a Spinco model L centrifuge (no. 30 rotor) and a nomogram for the calculation of centrifugal force (7). After radioactivity was measured, these fractions were dialyzed against water (0 C) and assayed for macromolecular nitrogen by the digestion and titration procedures of Hiller, Plazin, and Van Slyke (8) using the distillation apparatus of Kirk (9).

EXPERIMENTAL PROCEDURE

Protocols. The control and experimental groups were allowed to become adjusted to their quarters for several days. On the 1st day of the experiments the mice were

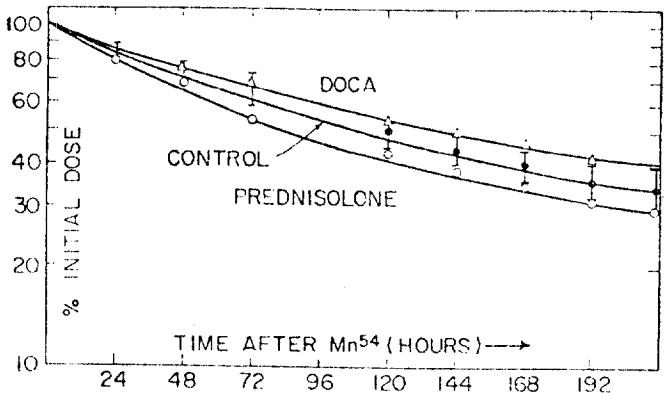

FIG. 1. Adrenal hormone effect on body turnover of radioactive manganese in mice on Purina chow diet. *Curves* depict percentage of original amount of radioactivity remaining in animals at various times after intraperitoneal injection of isotope (semilogarithmic plot). *Bars* represent range of data for 9 animals at each point in control group.

weighed, marked, and injected subcutaneously with either saline or hormone solution. The isotope was injected intraperitoneally in 0.1 ml of saline in all of the experiments except one; in this experiment, the $Mn^{54}Cl_2$ was incubated with homologous mouse plasma for 18 hr under 95% O_2 and 5% CO_2. The tracer then became plasma bound (10). This plasma-bound tracer was injected intraperitoneally.

When partition of the isotope among the organs was to be determined, the animals were sacrificed by a blow on the head. The G.I. tract, including spleen and pancreas, was removed en bloc, care being taken to prevent contamination of the carcass with isotope-rich feces. The remainder of the animal (carcass = skin, muscle, skeleton, heart, lungs, brain) was separated from the liver and the kidneys. These parts were then assayed for radioactivity. The sum of the radioactivities from Mn^{54} or Zn^{65} recovered in the small organs (other than the liver, kidney, and G.I. tract) was less than 10% of the total body burden and was not altered by any of the experimental maneuvers used. Therefore, they were combined in the above manner for the sake of simplicity.

In the experiments designed to study intracellular distributions, the animals were bled from the portal vein, and the livers were removed, weighed, placed on ice and taken to a cold room (3 C) where the liver fractionation described above was carried out.

OBSERVATIONS

Whole-Body Turnover

Curves depicting the rate of disappearance of Mn^{54} from control mice and mice treated with prednisolone or deoxycorticosterone can be seen in Fig. 1. Although small differences between the curves of the control and treated groups are apparent, when the curves are separated into their two exponential components and the lines of best fit and their standard error of estimate are determined, the slopes and intercepts for all three curves are the same. (Neither treated group is statistically different from control by Student's *t*-test; $P > 0.9$ for both the slopes and

TABLE 1. *Body distribution of radioactive Mn^{54} and Zn^{65}; % of total radioactivity at time of sacrifice*

	Time After Injection		
	6 hr	5 days	10 days
	Manganese 54		
Control			
Liver	29 (27–30)	27 (23–30)	17 (16–1
Carcass	22 (20–23)	34 (32–35)	44 (40–4
G.I. tract	44 (42–45)	34 (28–41)	31 (23–3
Kidneys	5 (4–5)	6 (6–7)	8 (5–9)
Prednisolone			
Liver	26 (21–29)	19 (14–23)	6 (5–7)
Carcass	22 (16–25)	40 (35–45)	59 (51–6
G.I. tract	48 (45–50)	35 (31–36)	25 (15–3
Kidneys	4 (4–5)	6 (6–7)	10 (9–11
	Zinc 65		
Control			
Liver	13 (11–14)	14 (11–15)	12 (10–1
Carcass	42 (40–44)	60 (57–62)	68 (62–7
G.I. tract	42 (41–43)	24 (23–25)	18 (14–2
Kidneys	3 (2–3)	2 (1–2)	2 (1–2)
Prednisolone			
Liver	16 (14–17)	15 (14–16)	13 (10–1
Carcass	38 (33–42)	56 (54–56)	66 (62–6
G.I. tract	43 (38–48)	27 (25–27)	19 (17–2
Kidneys	3 (2–3)	2 (1–2)	2 (1–2)

Percentile means (and range of percentage) in organs mice are shown in control and treated groups at 3 time inter after administration of Mn^{54} and Zn^{65}.

intercepts of the components between 5 and 10 da The prednisolone-treated animals failed to gain wei and some deaths occurred after 15 days of treatme probably due to infection. The average weight of con and of prednisolone-treated animals at the end of 30 d was 30 and 20 g, respectively. In spite of this sev catabolic effect of the hormone, no comparable loss Mn^{54} was noted. This indicated a possible reutilizat of the isotope and led to the study of its body distribut under the above experimental conditions.

Body Distribution

The distribution of the isotope was studied 2, 6, a 24 hr, and 5, 10, 15, 20, and 30 days following taggi Table 1 summarizes such data at three representat time intervals.

Manganese 54

The alteration of the Mn^{54} distribution with time c be gauged from the upper half of Table 1. The numb represent the percentage of radioactivity associated w each organ on the basis of the total-body Mn^{54} pres at the time of sacrifice. It should be recalled that there a fall in total-body radioactivity (Fig. 1) due to fe excretion of the tag. As a consequence, the absol amount of tracer in all the organs decreases with ti Obviously, the percentile distribution among the orga can rise or fall due to the characteristic rates of loss or accumulation of the tracer for each organ.

The control animals showed a gradual decline of t percentile concentration of tracer in the liver and G

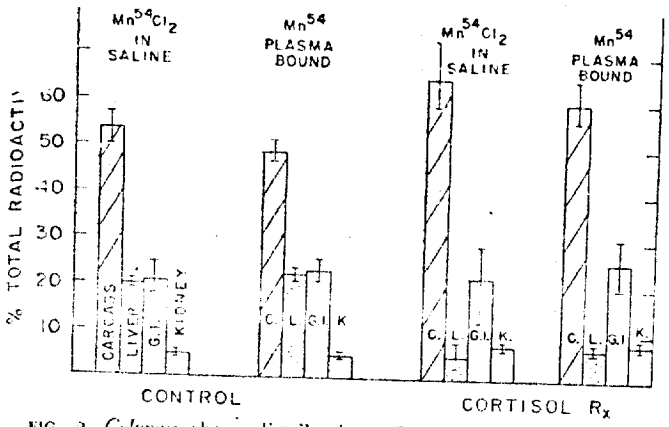

FIG. 2. *Columns* show distribution of total-body radioactivity among organs and carcass of groups of 3 animals receiving isotope intraperitoneally either in free or plasma-bound form. *Bars* represent range of all data.

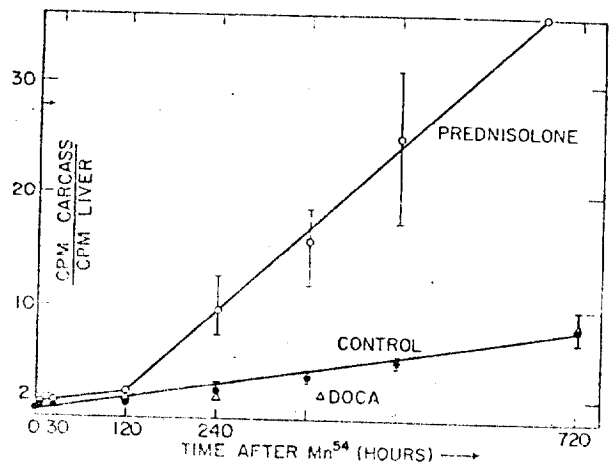

FIG. 3. Adrenal hormone effect on carcass/liver distribution of radioactive manganese in mice on Purina chow diet. Data from groups in Mn^{54} study are presented as ratio of radioactivity found at time of sacrifice in carcass and liver. Ratio found in DOCA-treated groups is superimposed on control curve at 5, 10, and 30 days for comparison. Last *point* on prednisolone curve represents only 1 animal, and *bars* enclose range of ratios from 3 animals at other points.

tract with a concomitant percentile increase in the carcass and kidneys. This process was markedly accentuated in the prednisolone-treated animals. In these treated animals there was a consistent increment in the absolute amount of radioactivity associated with the carcass which was by the end of 10 days 10–20% higher than the values for the control mice. These observations were confirmed with cortisol in animals receiving their Mn^{54} after it was incubated aerobically for 18 hr at 20 C in homologous plasma. This was done to control the possi- eration of the metabolism of Mn^{54} consequent to ig bound to plasma (10) as well as to test another ocorticoid (Fig. 2).

In animals observed for as long as 30 days, deoxycorticosterone did not alter the distribution of Mn^{54}. However, in one experiment in which the animals received both DOCA and prednisolone, the decrease in liver Mn^{54} was less pronounced than when prednisolone was given alone. These results are illustrated in Fig. 3, where they are plotted as the ratio of the radioactivity in the carcass to that in the liver. Hence, the magnitude of the change brought on by the glucocorticosteroid is emphasized.

Zinc 65. It was thought that the experiments with Mn^{54} might reflect a nonspecific cellular effect of prednisolone which would affect the distribution of any transition-group metal in a like manner. To test this, Zn^{65} was used in experiments identical to that described above. This isotope is kinetically similar to Mn^{54} in that it concentrates intracellularly but it differs from Mn^{54} in one pertinent particular: it does not concentrate preferentially in the mitochondria but rather in the other subcellular inclusions (Cotzias, Borg, and Selleck, submitted for publication).

The lower half of Table 1 summarizes the partition of this isotope among the segments of the body already described. It is evident that prednisolone brought on no change of the partition of Zn^{65}. Also, the total-body turnover of Zn^{65} as observed for 15 days was identical in prednisolone-treated and untreated animals.

Cellular Fractionation

Groups of three animals each were tagged with $Mn^{54}Cl$ and were either treated with prednisolone for 15 days or were observed as controls. The livers of these animals were removed and cellular fractionation was performed as described. Table 2 summarizes these results and indicates that in spite of causing a marked loss of Mn^{54} from the liver, the hormone had no appreciable effect on the

TABLE 2. *Intracellular distribution of radioactive manganese and nitrogen*

Fraction	Control			Prednisolone		
	Mn^{54}, % total	N, mg/g	Mn^{54} partition based on N	Mn^{54}, % total	N, mg/g	Mn^{54} partition based on N
Nuclear	15 (11–19)	4.7 (3.7–5.6)	15 (14–16)	16 (11–18)	4.9 (3.4–6.2)	15 (13–18)
Mitochondrial	54 (51–56)	4.0 (3.5–4.5)	63 (63–67)	52 (49–56)	4.1 (3.2–5.2)	62 (59–66)
Microsomal	16 (14–18)	5.0 (3.4–6.0)	15 (13–18)	16 (14–19)	5.1 (3.9–6.8)	16 (13–19)
Supernatant	15 (14–16)	14.1 (12.3–15.2)	5 (4–6)	16 (14–18)	12.0 (10.0–14.3)	7 (5–10)
	100%	27.8 (26.7–29.8)	100%	100%	26.1 (22.7–28.3)	100%

Percentage Mn^{54} found in each fraction for 3 control and 3 prednisolone-treated animals are shown in 1st column. Total nitrogen determinations on liver fractions are listed in 2nd column (avg. of 2 aliquots). In 3rd column, distribution is recalculated based on radioactivity per milligram of nitrogen present in fractions. Numbers in parentheses are ranges for 3 animals.

partition of the remaining isotope among the intracellular inclusions.

The intracellular partition of the isotope given in the plasma-bound form was almost identical to the distribution of the ionic isotope. Furthermore the intracellular partition remained unaffected by cortisol, when tested on two groups of three animals each. An experiment where animals were observed for 5 days after tagging with Zn^{65} likewise showed no change in intracellular partition of this isotope as a consequence of treatment with the glucocorticoid.

DISCUSSION

It had been expected at the outset of this work that intensive treatment of animals with a glucocorticosteroid would bring on alterations in their metabolism of manganese. This expectation was fulfilled by these experiments, which showed removal of radiomanganese from its location in the liver, accompanied by a percentile increase in the carcass as a consequence of this treatment. The initial expectation was based on two assumptions: *a*) the report from this laboratory that in the rat, mitochondria are a primary site of accumulation of radiomanganese (1); and *b*) on the work of Lowe et al. (4) which revealed that treatment with a glucocorticoid changed the morphology of the mitochondria drastically.

The first of these two assumptions was confirmed experimentally in the mouse with two different techniques, namely, by either delivering the manganese tracer as the aquo-ion or as the plasma bound form. With regard to the second assumption, the washed mitochondria contained identical nitrogen and isotope concentrations, irrespective of their having been obtained from steroid-treated or untreated animals. It should be stressed, however, that these bodies tend to concentrate 65% of the intrahepatic Mn^{54} per unit of nitrogen and only one-tenth as much of the intrahepatic Zn^{65} (Cotzias, Borg, and Selleck, submitted for publication). Therefore, the apparent metal specificity of the steroid effect might be a consequence of the markedly different mitochondrial enrichment by these two isotopes. Hence, our failure to demonstrate a diminution of macromolecular nitrogen content of mitochondria following this treatment does not prove that these bodies had not been altered by the glucocorticosteroids.

Alteration of manganese metabolism was exhibited by the glucocorticosteroids and not by the mineralocorticosteroid investigated. This also implies possible specificity with regard to the type of steroid hormone.

Another observation was that the action of this gl corticosteroid on the distribution of radiomanganese similar to the change brought on by metabolic l with manganese salts (2). Following such metal loads, the tracer tended also to be shifted from the l and to enrich the carcass. Nonetheless, there were s significant differences between the effect of the adm tration of metal and that of steroid: the former brou on an immediate mobilization of the tag (from entire animal as well as the liver) whereas the l ter hibited a period of induction (Fig. 3). This indic possible differences in the observed effect of these age In the use of metal loads immediate metal-isotope changes were highly probable while the induction pe found with the steroid effect argues for an indirect ac on manganese metabolism. Hence these experim should not be interpreted as bearing on the homeost control of this metal even though similar but less nounced results were obtained with one-tenth of dose of hormone tested here (Hughes and Cot manuscript in preparation).

An explanation for the changes in body distributio the Mn^{54} as a function of time is necessary both for normal as well as for the hormone-treated animals the present, it might be assumed that this change is sequence of gradual establishment of constant spe activity throughout the body. If this is the case, glucocorticoids might be accelerating this process but actual depletion of liver manganese could serve as alternative explanation. The recent development of accurate procedure for quantitation of the trace quan ties of manganese in tissue employing neutron act tion analysis (11) may provide the methodology for solving this issue.

It should be noted that the distribution of radiom ganese among both the organs and the liver organ of the mouse was identical regardless of whether element was administered in plasma-bound or in i form. It is also noteworthy that the steroid effect identical in the case of both methods of tagging the mals. These results add more general meaning to work previously reported from this laboratory in w manganese metabolism was studied only with the i form of this element.

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STUDIES ON ARGINASE

by

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Arginase determination was to date mainly implemented by determining the urea originating from the arginase effect with the aid of the urease method. While said method as seen is reliable, it nevertheless involves some inconveniences. Before mixing the test solution with the urease, it is necessary to deactivate the arginase, to adjust the pH to 7 pH; the distillation of the ammonia must be additionally performed. When investigating the effect of various substances on the arginase, it is likewise necessary to consider the possible susceptibility of the urease to said substances; therefore the ammonia content obtained through distillation does not always parallel the quantitative modifications of the arginase effect. Accordingly, I attempted to determine the residual non-precipitated arginase after hydrolysis through arginase. Some authors followed this line of thought when weighing the arginine precipitated as arginine flavianate for the purpose of arginase determination. Said gravimetric proceduer, however, is highly impractical during the performance of various tests. I therefore attempted a colorimetric determination of the arginine flavianate, in view of the fact that during alkaline reaction, a deep red discoloration occurs, caused by the reduction of the flavianic acid. Stannous chloride was used as reducing agent, and potassium sodium tartrate (Seignette-salt) was added for dissolving the stannous hydroxyde which forms in the course of the alkaline reaction.

Reagents;

1) Flavianic acid (1-naphtolic-2,4-dinitro-7-sulfonic acid + 3 molecules water of crystallization). From the business firm Takeda, Osaka. This flavianic acid was used for the preparation of the standard solution and for the precipitation of the arginine. I first recrystallized the preparation from hot 10% hydrochloric acid; the acid, desiccated in vacuum beside potash lye, was then converted into potash salt. The salt contains 1½ molecules water of crystallization. No difference resulted during colorimetric comparisons of said salt with the commercially available preparation. Therefore TAKEDA readily used the flavianic acid.

2) 5% potash lye solution.

3) 20% potassium sodium tartrate solution.

4) 5% stannous chloride-hydrochloric acid solution:
5 g stannous chloride for analysis was dissolved in 100 ccm of 1% hydrochloric acid, and stored in a dark flask. This solution showed a whitish turbidity, but was readily usable. It remains usable for approximately 10 to 15 days, but not for longer periods because its reducing capacity deteriorates.

The following reagent volume qualified best for obtaining a definite reddish discoloration:

Flavianic acid solution	1.0 ccm
5% potash lye	1.0 "
20% Seignette-salt	4.0 "
5% stannous chloride-hydrochloric acid solution	1.0 "
with water to	10.0 "

Following filling up to the mark, the mixture must be shaken vigorously. Maximal color development is obtainable within approx. 5 minutes in summer, and within approx. 10 minutes in winter, and the intensity of the coloring remains unchanged for at least 1 hour. The Seignette salt must not necessarily measure 4.0 ccm; it can vary to ± 20%; but 1 ccm potassium lye should be measured as precisely as possible; otherwise, turbidity develops in the solution which of course makes colorimetry impracticable.

First, The concentration range of the flavianic acid was determined within which the flavianic acid content parallels the chroma. It resulted that in case of colorimetry carried out according to the above-mentioned rule by using 1.0 ccm flavianic acid,(solution between m/50 and m/3000), the chroma is exactly proportionate to the flavianic acid concentration.

Furthermore, arginine flavianate was prepared from arginine nitrate, and a specific quantity of the desiccated recrystallized salt was weighed; 2 equivalents of alkali were added and dissolved, so as to prepare a solution of M/200. When said solution was colorimetrically compared with M/200 flavianic acid as specified above, the coloring grade of the arginine flavianate coincided--as evident from Table 1--with the coloring degree of the flavianic acid, within admissible error limits. The arginine content can be calculated from the determined quantity of the flavianic acid.

For the purpose of colorimetric determination, the arginine is first precipitated from the test solution in the form of flavianate; it must be a monoflavianate, which is obtainable only when the acidity of the solution is rather strong. Taking the elimination of the albumen into consideration which has to be carried out during the fermentative test, I used trichloracetic acid for acidification. The flavianate precipitation is performed in a 10 ccm centrifugal glass, graded like a measuring cylinder and equipped with a glass plug.

The following was added to 1.0 ccm of a M/200 arginine-monohydrochloride solution: 1 ccm of 8% trichloracetic acid, and 1 ccm 3% flavianic acid; the glass wall was vigorously rubbed.(100 to 200 times), so that the flavianate is deposited in fine crystallized form.

After approximately 30 minutes, the precipitate was removed by means of centrifugation and rinsed twice with pure alcohol for the purpose of eliminating surplus flavianic acid. 1.0 ccm potash lye and 4.0 ccm Seignette salt was subsequently added. The precipitation was dissolved in a boiling water bath; after cooling, 1.0 ccm of a stannous chloride-hydrochloric acid solution was added and thinned with water to 10 ccm. Thorough mixing followed. The above solution was colorimetrically compared with 1.0 ccm of flavianic acid which had been colored with stannous chloride; a coincidence within the experimental error limits was thereby obtained (Table II). I wish to add that complete flavianate precipitation occurs without cooling at room temperature, and that the arginine flavianate, provided that it is fully precipitated, rapidly sinks to the bottom spontaneously, producing a clear supernatant liquid. Instead of 1.0 ccm 5% potash lye, 1.0 ccm 2.5% lithium hydroxide can be used; the arginine flavianate will dissolve smoothly without heating in the latter. Since a small quantity of pure alcohol does not affect the chroma, the residual alcohol left at the bottom after rinsing did not require vaporization.

I then used said colorimetry for arginase determination, whereby it was initially found that the arginine flavianate is incompletely precipitated from the filtrate, obtained albumen-free with trichloracetic acid. This obstacle, however, was easily overcome by treating the filtrate with Jodlin. I determined the arginase effect at 37° as follows:

M/10 arginine-monohydrochloride	2.0 ccm	
M/10 soda lye	1.0 "	
Water	3.0 "	PH 9.2
Buffer (glycocoll-soda)	2.0 "	
Ferment solution	2.0 "	

The final arginine concentration is M/50. A small quantity of toluol is added to the above mixture. At a specified time 3.0 ccm are removed from the mixture and an identical amount of 8% trichloracetic acid is added. After approximately 15 minutes, filtering takes place; approx. 0.3 g of kaolin is added to the filtrate which is then agitated and filtered approx 10 minutes later. 1 ccm of 3% flavianic acid is added to 1.0 ccm of the filtrate in question, vigorously rubbed at the wall with a glass stirring rod, so that the flavianate is precipitated. The colorimetric examination is subsequently performed with the above-mentioned method, whebery a control solution has to be prepared from 1.0 ccm M/200 flavianic acid.

Using said method, I first determined the optimal acidity of the arginase. The composition of the control solution is specified in the experimental sector. Dialyzed autolysate of hog liver was used as arginase: it is preferable to the glycerin extract used by some authors because its action is vigorous and its albumen content is minor. The test proved to be most effective with a pH of 9.3 (at 37°, for 1 hour), as shown on Table III; this coincides well with the data of EDLBACHER and BONEM (1925), HUNTER and MORRELL (1933) and KAMIYA (1935).

Tests were subsequently performed with pH 9.2, using $CoCl_2$, $MgSO_4$, $CuSO_4$, $FeSO_4$, NaF, KCN, $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$, to determine whether these substances could have a certain effect on the arginase. I was unable to detect either activation or inhibition (Table V) during the test which lasted for 1 hour at 37°, within the limits of the added 1.0 ccm M/100 to M/5500 solution. A strong activation was, however, found under identical conditions with $MnSO_4$. When 1.0 ccm M/100, M/500 or M/5000 of $MnSO_4$ was added, the activity increased to 30, 26, and 21% respectively. KLEIN and ZIESE (1937) reported that the arginase can be activated by manganese salt, while according to KIRIAN and SOLOWJEW (1935) the Mn-ion is unable to activate the arginase. ADLBACHER and ZELLER (1936) agreed with the opinion of KLEIN and ZIESE. Our tests likewise indicate that the manganese ion can be regarded as an arginase activator.

As for the distribution of the arginase: a preliminary test must be performed to determine whether the arginase content in fresh organs is subject to certain quantitative modifications after the autolysis, or whether the organ fluid or the autolysate contains any substance precipitable with flavianic acid after albumen elimination and under the above-mentioned conditions. Accordingly, I first ground the liver, kidneys and testicles of rabbits, then added the quintuple volume of a 0.85% NaCl-solution. 2.0 ccm of the same was immediately used for the arginine precipitation test and the decomposition grade was simultaneously determined with the aid of the urease method, whereby the results of both determination procedures coincided completely (Table VI). The fact that the added arginine volume during the immediate test could be recovered unchanged and that the urease method and the colorimtry yielded a quite identical value indicates that no substance precipitable with flavianic acid can be detected in the organ fluid after albumen removal under the above-mentioned conditions.

Subsequently the liver, kidneys, testicles and muscles of rabbit, dog and fowl were ground and their arginase content was examined immediately or after autolysis. As Table VII shows, the presence of arginase could be determined in each organ, the muscles excepted (Table VII)during the test in the fresh organ suspension which was preserved by adding the quintuple volume of a physiological saline solution to the ground organs. The ferment was especially abundant in the liver of dogs and rabbits, and in the kidneys of fowls. The ratio differed somewhat when a test was performed with the ferment solution obtained from the organs by means of a three-day autolysis at 37° with the addition of toluol-chloroform; said solution was then preserved against water for 3 days after the dialysis. The arginase was detectable in the rabbit and dog liver only; but the activity was considerably reduced as compared to the activity of sediments from fresh organs.

No arginase effect was found in the other examined autolysates, including the kidneys of fowl (Table VIII). Fresh organs had to be tested during research aimed at the occurence of arginases.

As shown by the above experiments, arginase was in no case detectable in the muscle, a fact reported earlier by EDLBACHER and RÖHLER (1925). KLEIN and ZIESE (1935) reported recently that the muscle, specifically the muscle of rats, is unable to precipitate arginine; however, when Mn is added, an arginase effect can become apparent. In conjunction with the above, EDLBACHER and ZELLER (1936) reported that a very intensive arginase-reaction is triggered in the muscular system of pigeons when manganese salts are present. As stated above, arginase activation through manganese salts has been determined. But my test showed that--regardless of the presence of Mn--the fresh muscle of rat and pigeon is unable to precipitate the arginine even after 24 hours.

HELLERMAN and PERKINS (1936) furthermore reported that urease is capable of hydrolysing the arginine when Co, Mn or Ni is present. However, my test indicated that the use of the commercially available urease as well as the use of a preparation made here from soy beans failed to produce any arginase effect when such salts were added. As reported above, I was therefore able to examine the arginase effect from a variety of viewpoints with the aid of colorimetry.

EXPERIMENTAL SECTOR

Arginine monohydrochloride was prepared from gelatine, Kokkô trademark of Nitta, Tokyo, according to BERGMANN and ZERVAS (1927). The precise prescription is available in Organic Syntheses XII (1932).

The arginase solution was obtained as follows: 2500 ccm water and approx. 3 ccm of 36% acetic acid was added to 500 g ground fresh hog liver.

After adding chloroform and toluol the mixture was autolyzed for 72 hours at 37°; the filtrate was then dialyzed against water for 3 days. The ferment solution can be preserved for months when stored in the freezer.

The buffer solution consisted of M/2 acetic acid-acetate, or of M/10 glycocoll-soda; pH was determined in the test solution with the aid of diagram paper. Unless otherwise indicated, the test temperature was 37°.

The following ferment test tables show the arginine volume found in 1 ccm of the albumen-free filtrate. In cases when no arginine precipitation occurs, the detected arginine volume is 1,742 mg.

1. Colorimetry of the pure arginine flavianate.

TABLE I.

TABELLE I.

1	2		3		4
M/200 Arginin-flavianat	als Flaviansäure		als Arginin		% Fehler %
	5 ber. mg	6 gef. mg	5 ber. mg	6 gef. mg	
0.5 ccm	0.785	0.789	0.435	0.437	101	1
1.0 „	1.570	1.586	0.870	0.878	101	1
2.0 „	3.141	3.207	1.742	1.769	102	2

1) M/200 arginine flavianate
2) as flavianic acid
3) as arginine
4) error
5) reported, mg
6) found, mg

II. Colorimetric determination of the arginine in its pure solution.

TABLE II.

1 als Flaviansäure		2 als Arginin		%	3 Fehler %
4 ber. mg	5 gef. mg	4 ber. mg	5 gef. mg		
1.571	1.547	0.870	0.859	99	1

1) as flavianic acid
2) as arginine
3) error
4) reported, mg
5) found, mg.

III. pH-optimum of the arginase (1-hour test).

M/10 arginine monohydrochloride	2.0 ccm
M/10 soda lye	a "
water	4-a "
buffer	2.0 "
ferment solution	2.0 "

M/10 NaOH ccm	0	0	0.1	0.2	1.0	2.0	2.0
1 Puffer	Essigsäure-Acetat 2			3 Glykokoll-Natron			
Ph	6.0	6.2	7.7	8.3	9.3	10.2	11.2
4 Arginin mg	1.41	1.37	1.26	1.25	1.15	1.38	1.61
5 Arginin-spaltung %	17	21	28	28	34	21	7.5

1) Buffer
2) acetic acid-acetate
3) glycocoll-soda
4) arginine, mg
5) arginine precipitation %

IV. Arginase activation of the manganese salt (1-hour test with Ph 9.2).

M/10 arginine monohydrochloride	2.0 ccm
M/10 soda lye	1.0 "
$MnSO_4$-solution	1.0 "
water	2.0 "
M/10 glycocoll soda buffer	2.0 "
ferment solution	2.0 "

TABLE IV.

$MnSO_4$	1 ohne Mn			2 mit Mn			3
	4 sofort mg	5 1 Std. mg	6 Spaltung %	7 sofort mg	8 1 Std. mg	9 Spaltung %	Aktivierung %
M/100 1.0ccm	1.71	1.20	31	1.74	1.04	40	30
M/500 1.0 „	1.71	1.21	31	1.71	1.06	39	26
M/5000 1.0 „	1.72	1.23	29	1.71	1.13	35	21
M/50000 1.0 „	1.70	1.24	28	1.72	1.23	29	0

1) without Mn 2) with Mn 3) activation %
4) immediately, mg
5) per hour, mg
6) precipitation %
7) immediately, mg
8) per hour, mg
9) precipitation %

V. Effect of the salts on the arginase activity (Ph 9.2).

M/10 arginine monohydrochloride	2.0 ccm
M/10 soda lye	1.0 "
salt solution	1.0 "
water	2.0 "
M/10 glycocoll soda buffer	2.0 "
ferment solution	2.0 "

TABLE V.

1 Salze		2 Arginin mg sofort 3	2 Arginin mg 1 Std. 4	5 Hydrolyse %
6 ohne $CaCl_2$		1.71	1.23	29
7 mit $CaCl_2$	M/100 1.0 ccm	1.69	1.22	30
	M/500 1.0 „	1.69	1.27	27
6 ohne $COCl_2$		1.74	1.21	30
7 mit $COCl_2$	M/30 1.0 „	1.71	1.26	28
	M/100 1.0 „	1.71	1.24	29
	M/500 1.0 „	1.70	1.21	30
6 ohne $NiSO_4$		1.72	1.25	28
7 mit $NiSO_4$	M/100 1.0 „	1.71	1.21	31
	M/500 1.0 „	1.71	1.25	28
6 ohne $MgSO_4$		1.72	1.26	28
7 mit $MgSO_4$	M/100 1.0 „	1.74	1.26	28
	M/500 1.0 „	1.72	1.20	31
	M/5000 1.0 „	1.69	1.24	29
6 ohne $CuSO_4$		1.71	1.28	27
7 mit $CuSO_4$	M/100 1.0 „	1.71	1.24	29
	M/500 1.0 „	1.68	1.20	31
	M/5000 1.0 „	1.74	1.22	30
6 ohne $FeSO_4$		1.70	1.23	29
7 mit $FeSO_4$	M/100 1.0 „	1.67	1.21	31
	M/500 1.0 „	1.70	1.20	31
	M/5000 1.0 „	1.72	1.25	28
6 ohne NaF		1.74	1.22	30
7 mit NaF	M/100 1.0 „	1.69	1.24	29
	M/500 1.0 „	1.69	1.26	28
6 ohne KCN		1.69	1.23	29
7 mit KCN	M/100 1.0 „	1.70	1.23	29
	M/500 1.0 „	1.70	1.27	27
6 ohne $K_4Fe(CN)_6$		1.74	1.26	28
7 mit $K_4Fe(CN)_6$	M/100 1.0 „	1.70	1.25	28
6 ohne $K_3Fe(CN)_6$		1.70	1.21	31
7 mit $K_3Fe(CN)_6$	M/100 1.0 „	1.69	1.25	28

1) Salts 2) arginine, mg 3) immediately 4) per hour
5) hyrolysis %
6) without . . .
7) with . . .

VI. Comparison of the colorimetric and urease methods for arginase determination, with pH 9.2, in fresh rabbit organs.

M/10 argininemonohydrochloride	6.0 ccm
M/10 soda lye	3.0 "
water	9.0 "
glycoroll-soda buffer	6.0 "
organ suspension	6.0 "

Colorimetric arginine determination was performed as specified above. Using the urease method, albumen was eliminated from 10.0 ccm of the test solution with the same volume of 5% trichloracetic acid, and the filtrate was neutralized by adding 1.5 ccm of N/10 soda lye; it was then mixed with 4 ccm of M/10 phosphate buffer (Ph 7) and 4 ccm of 2% urease; the filtrate was subsequently allowed to stand for 1 hour at 40°.

NH_3-determination with the distillation method.
9.0 ccm water was used for the control test instead of the argininehydrochloride and the soda lye; the NH_2 volume determined thereby with the urease method was substracted from the principal test result. The colorimetric arginine evidence of the control test was always negative.

TABLE VI.

a) *Leber.*

	1 Sofort	2 1 Stunde		3 24 Stunden	
	4 Arginin mg	5 Arginin mg	6 Spaltung %	4 Arginin mg	6 Spaltung %
7 Kolorimetrie	1.71	0	100	0	100
8 Ureasemethode		0.01*	99	0.03*	98

b) *Niere.*

	1 Sofort	2 1 Stunde		3 24 Stunden	
	4 Arginin mg	5 Arginin mg	6 Spaltung %	4 Arginin mg	6 Spaltung %
7 Kolorimetrie	1.74	1.44	17	0	100
8 Ureasemethode		1.46*	16	0*	100

c) *Hoden.*

	1 Sofort	2 1 Stunde		3 24 Stunden	
	4 Arginin mg	5 Arginin mg	6 Spaltung %	4 Arginin mg	6 Spaltung %
7 Kolorimetrie	1.74	1.70	2	1.50	14
8 Ureasemethode		1.66*	4	1.46*	16

* Aus dem bemessenen NH_3 berechnete ungespaltene Arginiumenge in 1.0 ccm eiweissfreien Filtrats.

a) Liver. b) Kidney. c) Testicles.

1) immediately
2) 1 hour
3) 24 hours
4) arginine, mg.
5) " " "
6) precipitation %
7) colorimetry
8) urease method

* Non-precipitated arginine volume calculated from the measured NH_3 in 1.0 ccm of albumen-free filtrate.

VII. Test with pH 9.2 concerning the occurence of the arginase in various organs.

TABLE VII.

TABELLE VII.

	1 Kaninchen		2 Hund		3 Huhn	
	4 Argininspaltung %		4 Argininspaltung %		4 Argininspaltung %	
	5 1 Std.	6 24 Stdn.	5 1 Std.	6 24 Stdn.	5 1 Std.	6 24 Stdn.
7 Leber	100.0	100.0	100.0	100.0	2.0	10.5
8 Niere	22.1	100.0	5.5	29.8	29.7	100.0
9 Hoden	3.0	15.0	10.2	47.7	8.2	37.2
10 Muskel	2.0	1.0	1.0	1.0	0	2.0

1) Rabbit. 2) Dog. 3) Fowl.

4) arginine precipitation %
5) 1 hour
6) 24 hours
7) liver
8) kidney
9) testicles
10) muscle

VIII. Arginase effect of the dialyzed organ autolysate with pH 9.2.

TABLE VII.

TABELLE VIII.

	1 Kaninchen		2 Hund		3 Huhn	
	4 Argininspaltung %		4 Argininspaltung %		4 Argininspaltung %	
	5 1 Std.	6 24 Stdn.	5 1 Std.	6 24 Stdn.	5 1 Std.	6 24 Stdn.
7 Leber	38.2	100.0	49.9	100.0	0	1.0
8 Niere	2.0	3.0	0	1.0	1.0	2.0
9 Hoden	2.0	2.0	1.0	2.0	1.0	0
10 Muskel	1.0	2.0	0	1.0	0	1.0

1) Rabbit. 2) Dog. 3) Fowl.

4) arginine precipitation %
5) 1 hour
6) 24 hours
7) liver
8) kidney
9) testicles
10) muscle.

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STUDIEN ÜBER DIE ARGINASE.

Von

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Arginasebestimmung wurde bisher meistenteils derart durchgeführt, dass man den durch Arginasewirkung entstandenen Harnstoff mit der Ureasemethode bestimmte. Diese Methode ist zwar an sich zuverlässig, doch ist sie auch mit einigen Lästigkeiten verbunden: Vor der Mischung der Versuchslösung mit der Urease muss die Arginase inaktiviert, P_H der Lösung auf 7 eingestellt, und dazu noch Destillieren des Ammoniaks ausgeführt werden. Bei der Untersuchung über Einflüsse verschiedener Substanzen auf die Arginase muss auch eine mögliche Beeinflussbarkeit der Urease durch diese Substanzen in Betracht gezogen werden, sodass der durch Destillation erhaltene Ammoniakgehalt nicht immer der quantitativen Veränderung der Arginasewirkung parallel geht. So habe ich versucht, das nach der Hydrolyse durch Arginase ungespalten zurückgebliebene Arginin zu bestimmen. Unter diesem Gedankengange wurde schon von einigen Autoren die Abwägung des als Argininflavianat zur Fällung gebrachten Arginins für die Arginasebestimmung angewandt. Aber dieses gravimetrische Verfahren ist bei der Ausführung zahlreicher Experimente sehr unpraktisch. So habe ich gemäss der Tatsache, dass durch Reduktion der Flaviansäure in alkalischer Reaktion tief rote Verfärbung entsteht, das Argininflavianat kolorimetrisch zu bestimmen versucht. Als Reduktionsmittel wurde Stannochlorid verwendet und zur Auflösung des in alkalischer Reaktion entstehenden Stannohydroxydes wurde Kaliumnatriumtartrat (Seignette-Salz) hinzugesetzt.

Reagentien:

1) Flaviansäure (1-Naphthol-2,4-dinitro-7-Sulfonsäure+3 Molekül Kristallwasser): Von Firma Takeda, Osaka. Diese Flaviansäure wurde zur Herstellung der Standardlösung und zur Fällung des Arginins gebraucht. Zuerst habe ich das Präparat aus heisser 10% Salzsäure umkristallisiert und die in Vakuum neben Kalilauge getrocknete Säure in Kaliumsalz übergeführt. Das Salz enthält $1\frac{1}{2}$ Molekül Kristallwasser. Kolorimetrische Vergleichung dieses Salzes mit dem Handelspräparat ergab keine Differenz. Daher wurde die Flaviansäure von Takeda ohne weiteres verwendet.

2) 5% Kalilaugelösung.

3) 20% Kaliumnatriumtartratlösung.

4) 5% Zinnchlorür-Salzsäurelösung: 5 g Zinnchlorür zur Analyse wurde in 100 ccm 5%iger Salzsäure aufgelöst und in einer dunklen Flasche aufbewahrt. Diese Lösung zeigte eine weissliche Trübung, konnte aber ohne weiteres verwendet werden. Sie ist ungefähr 10-15 Tage brauchbar, aber darüber hinaus nicht wegen der Abschwächung des Reduktionsvermögens.

Zur ganz klaren rötlichen Verfärbung war die folgende Menge der Reagentien am geeignetesten.

Flaviansäurelösung	1.0 ccm
5% Kalilauge	1.0 „
20% Seignettesalz	4.0 „
5% Zinnchlorür-Salzsäurelösung	1.0 „
mit Wasser auf	10.0 „

Nach dem Auffüllen mit Wasser bis zur Marke muss das Gemisch stark geschüttelt werden. Die maximale Farbentwicklung ist im Sommer in ca. 5 Minuten und im Winter in ca. 10 Minuten zu erreichen und die Farbintensität bleibt wenigstens 1 Stunde unverändert. Seignettesalz braucht man nicht genau 4.0 ccm zu messen, es kann ±20% variiert werden, aber 1 ccm Kalilauge soll möglichst genau abgemessen werden, sonst entsteht in der Lösung eine Trübung, die selbstverständlich die Kolorimetrie unmöglich macht.

Zuerst wurde der Konzentrationsbereich der Flaviansäure fest-

gestellt, in dem der Flaviansäuregehalt mit dem Farbgrad parallel geht. Es ergab sich, dass bei Kolorimetrie, die nach oben erwähnter Vorschrift unter Verwendung von 1.0 ccm Flaviansäure zwischen $M/50$ und $M/3000$ Lösung ausgeführt wird, der Farbgrad genau der Flaviansäurekonzentration proportional ist.

Weiter wurde Argininmonoflavianat aus Argininnitrat hergestellt und eine bestimmte Menge des getrockneten umkristallisierten Salzes abgewogen, 2 Äquivalente Alkali zugesetzt und in Lösung gebracht, um eine $M/200$ Lösung zu bereiten. Als diese Lösung mit $M/200$ Flaviansäure, wie oben beschrieben, kolorimetrisch verglichen wurde, stimmte der Färbungsgrad des Argininflavianats, wie aus der Tabelle I ersichtlich, innerhalb den zu erlaubenden Fehlergrenzen mit dem der Flaviansäure überein. Man kann den Arginingehalt aus der ermittelten Flaviansäuremenge errechnen.

Zur kolorimetrischen Bestimmung wird das Arginin zuerst aus der Versuchslösung als Flavianat niedergeschlagen und es muss, was bei ziemlich starker Azidität der Lösung zu verwirklichen ist, Monoflavianat sein. Mit Rücksicht auf die beim fermentativen Versuch auszuführende Eiweissung brauchte ich zur Ansäuerung Trichloressigsäure. Die Flavianatfällung wird in 10 ccm Zentrifugenglas ausgeführt, das wie Messzylinder graduiert und mit Glasstopfen versehen ist.

Zu 1.0 ccm $M/200$ Argininmonohydrochloridlösung wurde 1 ccm 8%iger Trichloressigsäure, ferner 1 ccm 3% Flaviansäure hinzugefügt und die Glaswand stark (100-200 Mal) gerieben, so dass sich Flavianat schön kristallisiert abscheidet. Der Niederschlag wird nach etwa 30 Minuten abzentrifugiert und 2 Mal mit absolutem Alkohol gewaschen, um den Überschuss von Flaviansäure zu entfernen. Dann wurden 1.0 ccm Kalilauge und 4.0 ccm Seignettesalz hinzugefügt. Der Niederschlag wurde am siedenden Wasserbad aufgelöst, nach Abkühlen 1.0 ccm Zinnchlorür-Salzsäure-Lösung hinzugesetzt und mit Wasser auf 10 ccm verdünnt und gut gemischt. Diese Lösung wurde mit 1.0 ccm $M/200$ Flaviansäure, die mit Zinnchlorür verfärbt worden war, kolorimetrisch verglichen, wobei eine Übereinstimmung innerhalb der experimentellen Fehlergrenze erzielt wurde (Tabelle II). Ich möchte noch hinzufügen, dass die

Flavianatfällung ohne Abkühlen schon bei Zimmertemperatur vollständig vonstatten geht, und dass das Argininflavianat, falls es vollständig abgeschieden wird, schnell von selbst zu Boden sinkt und eine klare überstehende Flüssigkeit liefert. Anstatt 1.0 ccm 5% Kalilauge kann man 1.0 ccm 2.5% Lithiumhydroxyd verwenden, worin sich das Argininflavianat ohne Erhitzen glatt auflöst. Da eine geringe Menge absoluten Alkohols keinen Einfluss auf den Farbgrad ausübt, brauchte im Versuch der nach dem Auswaschen am Boden zurückgebliebene Alkohol nicht verdunstet werden.

Nun wandte ich diese Kolorimetrie zur Arginasebestimmung an, wobei zuerst festgestellt wurde, dass aus dem durch Trichloressigsäure enteiweisst erhaltenen Filtrat das Argininflavianat unvollständig ausgeschieden wird. Dieses Hindernis liess sich aber durch Kaolinbehandeln des Filtrats leicht beseitigen. Ich bestimmte bei 37° die Arginasewirkung folgendermassen:

M/10 Argininmonohydrochlorid	2.0 ccm	
M/10 Natronlauge	1.0 „	
Wasser	3.0 „	PH 9.2
Puffer (Glykokoll-Natron)	2.0 „	
Fermentlösung	2.0 „	

Die endliche Argininkonzentration ist $M/50$. Diesem Gemisch wird eine geringe Menge Toluol zugesetzt. Davon werden zur bestimmten Zeit 5.0 ccm genommen und die gleiche Menge 8%iger Trichloressigsäure hinzugesetzt, nach ca. 15 Minuten filtriert, dem Filtrat ca. 0.3 g Kaolin zugesetzt, geschüttelt und nach etwa 10 Minuten filtriert. 1.0 ccm von diesem Filtrat wird 1 ccm 3%iger Flaviansäure hinzugefügt, mit einem Glasstäbchen an der Wand kräftig gerieben, sodass sich Flavianat ausscheidet. Dann wird auf obenbeschriebene Weise die kolorimetrische Untersuchung ausgeführt, wobei die Vergleichslösung aus 1.0 ccm $M/200$ Flaviansäure herzustellen ist.

Mit dieser Methode habe ich zuerst die optimale Azidität der Arginase bestimmt. Zusammensetzung der Versuchslösung wird im experimentellen Teil angegeben. Als Arginase wurde dialysiertes Autolysat der Schweineleber verwendet, welches wegen starker Aktivität und geringen Eiweissgehalts den von manchen Autoren

gebrauchten Glyzerinextrakt übertrifft. Der Versuch fiel bei PH 9.2 (bei 37° eine Stunde) am stärksten aus, wie Tabelle III zeigt, was mit den Angaben von Edlbacher und Bonem (1925), Hunter und Morrell (1933) und Kamiya (1935) gut übereinstimmt.

Dann wurde es bei PH 9.2 mit $CaCl_2$, $CoCl_2$, $NiSO_4$, $MgSO_4$, $CuSO_4$, $FeSO_4$, NaF, KCN, $K_4Fe(CN)_6$ und $K_3Fe(CN)_6$ daraufhin untersucht, ob diese Substanzen auf die Arginase gewisse Einflüsse ausüben können. So konnte ich bei 1 stündigem Versuch und 37° in den Grenzen des Zusatzes von 1.0 ccm $M/100$—$M/5500$ Lösung weder Aktivierung noch Hemmung konstatieren (Tabelle V). Aber unter derselben Bedingung wurde mit $MnSO_4$ eine starke Aktivierung festgestellt. Die Aktivitätssteigerung betrug beim Zusatz von 1.0 ccm $M/100$, $M/500$ oder $M/5000$ $MnSO_4$ 30, 26 resp. 21% (Tabelle IV). Klein und Ziese (1935) gaben an, dass die Arginase durch Mangansalz aktiviert werden kann, während nach Kirian und Solowjew (1935) das Mn-ion die Arginase nicht zu aktivieren imstande sei. Edlbacher und Zeller (1936) schlossen sich der Ansicht von Klein und Ziese an. Aus meinen Experimenten geht ebenfalls hervor, dass man im Mangananion einen Aktivator der Arginase ersehen kann.

Was die Verteilung der Arginase anbetrifft, muss ein Vorversuch angestellt werden, um zu entscheiden, ob der Arginasegehalt in frischen Organen nach der Autolyse gewisse quantitative Veränderungen erfährt und ob der enteiweisste Organsaft oder das Autolysat unter den obenangeführten Bedingungen irgendeine durch Flaviansäure fällbare Substanz enthält. So habe ich zuerst Kaninchen-Leber, Niere und Hoden zermahlen und 5 faches Volumen 0.85%iger NaCl-Lösung hinzugesetzt. 2.0 ccm davon wurden sofort zum Versuch der Spaltung des Arginins verwendet und zugleich wurde der Spaltungsgrad mittels der Ureasemethode bestimmt, wobei das Resultat der beiden Bestimmungsverfahren völlig übereinstimmend war (Tabelle VI). Dass beim sofortigen Versuch die zugesetzte Argininmenge unverändert wiederzufinden war und die Ureasemethode und die Kolorimtrie einen völlig übereinstimmenden Wert ergaben, weist darauf hin, dass im ent-

eiweissten Organsaft unter den obengenannten Bedingungen keine durch Flavianäure fällbare Substanz nachweisbar ist.

Dann wurden Leber, Niere, Hoden und Muskel von Kaninchen, Hund und Huhn zermahlen und sobald oder nach der Autolyse auf die Arginasemenge hin untersucht. Wie die Tabelle VII zeigt, liess der Versuch mit der frischen Organsuspension, welche durch Zusatz von 5 fach grossem Volumen physiologischer Kochsalzlösung zu den zermahlenen Organen erhalten wurde, die Existenz der Arginase in jedem Organe, abgesehen vom Muskel, nachweisen (Tabelle VII). Das Ferment war besonders reichlich bei Hunden und Kaninchen in Leber und beim Huhn in Niere vorhanden. Das Verhältnis war etwas anders, als der Versuch mit der Ferment-Lösung ausgeführt wurde, welche aus den Organen durch 3 tägige Autolyse bei 37° unter Zusatz von Toluol-Chloroform und dann nach der Dialyse 3 Tage lang gegen Wasser erhalten wurde. Die Arginase war nur in Leber des Kaninchens und Hunden nachzuweisen, aber ihre Aktivität war stark abgeschwächt in Vergleich mit der von den frischen Organaufschwemmungen. In den übrigen untersuchten Autolysaten, einschliesslich der Hühnerniere, wurde keine Arginasewirkung beobachtet (Tabelle VIII). Bei der Untersuchung auf das Vorkommen der Arginasen mussten frische Organe zum Experiment genommen werden.

Wie aus den obigen Experimenten hervorgeht, wurde im Muskel keinesfalls Arginase nachgewiesen, wie es schon früher von Edlbacher und Röthler (1925) berichtet wurde. Klein und Ziese (1935) haben kürzlich angegeben, dass der Muskel, besonders der von Ratten, Arginin nicht zu spalten vermag, aber beim Zusatz von Mn eine Arginasewirkung in Erscheinung treten kann. Dem anschliessend, berichteten Edlbacher und Zeller (1936), dass in der Tauben-Muskulatur eine sehr intensive Arginase-Reaktion bei Gegenwart von Mangansalzen ausgelöst wird. Die Arginase-aktivierung durch Manganosalz wurde, wie schon oben angegeben, festgestellt. Mein Experiment ergab aber, dass der frische Muskel von Ratte und Taube unabhängig von Anwesenheit des Mn selbst nach 24 Stunden das Arginin nicht zu spalten vermag.

Weiter haben Hellerman und Perkins (1936) berichtet, dass Urease in Anwesenheit von Co, Mn oder Ni das Arginin zu hydrolysieren imstande sei. Mein Experiment ergab aber, dass bei Verwendung sowohl der käuflichen Urease als auch des bei uns aus Sojabohne hergestellten Präparats keine Arginasewirkung durch Zusatz von solchen Salzen hervorgerufen wurde. Somit konnte ich, wie vorstehend berichtet, an Hand der Kolorimetrie des Arginins Arginasewirkung von den verschiedensten Richtungen betrachten.

Experimenteller Teil.

Argininmonohydrochlorid wurde aus Gelatine, Kokkō Marke von Nitta, Tokyo nach Bergmann und Zervas (1927) hergestellt. Genaue Vorschrift ist in Organic Syntheses XII (1932) vorhanden.

Die Gewinnung der Arginaselösung wurde wie folgt ausgeführt. 500 g zerkleinerter frischer Schweineleber wurden 2500 ccm Wasser und ca. 3 ccm 36%iger Essigsäure zugesetzt. Unter Zusatz von Chloroform und Toluol wurde das Gemisch bei 37° 72 Stunden autolysiert und dann das Filtrat 3 Tage gegen Wasser dialysiert. In Eiskammer aufbewahrt ist die Fermentlösung monatelang haltbar.

Pufferlösung war $M/2$ Essigsäure-Acetat oder $M/10$ Glykokoll-Natron. PH wurde an der Versuchslösung mittels Indicatorpapier bestimmt. Versuchstemperatur war, sonst nichts angegeben, 37°.

In den nachstehenden Tabellen der Fermentversuche wird die in 1 ccm des eiweissten Filtrats gefundene Argininmenge angegeben. Falls keine Argininspaltung entsteht, ist die nachgewiesene Argininmenge 1.742 mg.

I. Kolorimetrie des reinen Argininflavianats.

Tabelle I.

M/200 Arginin-flavianat	als Flavianäure		als Arginin		%	Fehler %
	ber. mg	gef. mg	ber. mg	gef. mg		
0.5 ccm	0.785	0.789	0.435	0.437	101	1
1.0 „	1.570	1.586	0.870	0.878	101	1
2.0 „	3.141	3.207	1.742	1.769	102	2

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II. Kolorimetrische Bestimmung des Arginins an seiner reinen Lösung.

Tabelle II.

als Flaviansäure		als Arginin		%	Fehler %
ber. mg	gef. mg	ber. mg	gef. mg		
1.571	1.547	0.870	0.859	99	1

III. Ph-Optimum der Arginase (1 stündiger Versuch).

M/10 Argininmonohydrochlorid	2.0 ccm
M/10 Natronlauge	a „
Wasser	4−a „
Puffer	2.0 „
Fermentlösung	2.0 „

Tabelle III.

M/10 NaOH ccm	0	0	0.1	0.2	1.0	2.0	2.0
Puffer	Essigsäure-Acetat		Glykokoll-Natron				
Ph	6.0	6.2	7.7	8.3	9.3	10.2	11.2
Arginin mg	1.44	1.37	1.26	1.25	1.15	1.38	1.61
Arginin-spaltung %	17	21	28	28	34	21	7.5

IV. Arginaseaktivierung des Manganosalzes (1 stündiger Versuch bei Ph 9.2).

M/10 Argininmonohydrochlorid	2.0 ccm
M/10 Natronlauge	1.0 „
$MnSO_4$ Lösung	1.0 „
Wasser	2.0 „
M/10 Glykokollnatronpuffer	2.0 „
Fermentlösung	2.0 „

Tabelle IV.

$MnSO_4$	ohne Mn			mit Mn			Aktivierung %
	sofort mg	1 Std. mg	Spaltung %	sofort mg	1 Std. mg	Spaltung %	
M/100 1.0 ccm	1.71	1.20	31	1.74	1.04	40	30
M/500 1.0 „	1.71	1.21	31	1.71	1.06	39	26
M/5000 1.0 „	1.72	1.23	29	1.71	1.13	35	21
M/50000 1.0 „	1.70	1.24	28	1.72	1.23	29	0

V. Einfluss der Salze auf die Arginasewirkung (Ph 9.2).

M/10 Argininmonohydrochlorid	2.0 ccm
M/10 Natronlauge	1.0 „
Salzlösung	1.0 „
Wasser	2.0 „
M/10 Glykokollnatronpuffer	2.0 „
Fermentlösung	2.0 „

Tabelle V.

Salze		Arginin mg		Hydrolyse %
		sofort	1 Std.	
ohne $CaCl_2$		1.71	1.23	29
mit $CaCl_2$	M/100 1.0 ccm	1.69	1.22	30
	M/500 1.0 „	1.69	1.27	27
ohne $COCl_2$		1.74	1.21	30
mit $COCl_2$	M/30 1.0 „	1.71	1.26	28
	M/100 1.0 „	1.71	1.24	29
	M/500 1.0 „	1.70	1.21	30
ohne $NiSO_4$		1.72	1.25	28
mit $NiSO_4$	M/100 1.0 „	1.71	1.21	31
	M/500 1.0 „	1.71	1.25	28
ohne $MgSO_4$		1.72	1.26	28
mit $MgSO_4$	M/100 1.0 „	1.74	1.26	28
	M/500 1.0 „	1.72	1.20	31
	M/5000 1.0 „	1.69	1.24	29

ohne $CuSO_4$		1.71	1.28	27
mit $CuSO_4$	M/100 1.0 „	1.71	1.24	29
	M/500 1.0 „	1.68	1.20	31
	M/5000 1.0 „	1.74	1.22	30
ohne $FeSO_4$		1.70	1.23	29
mit $FeSO_4$	M/100 1.0 „	1.67	1.21	31
	M/500 1.0 „	1.70	1.20	31
	M/5000 1.0 „	1.72	1.25	28
ohne NaF		1.74	1.22	30
mit NaF	M/100 1.0 „	1.69	1.24	29
	M/500 1.0 „	1.69	1.26	28
ohne KCN		1.69	1.23	29
mit KCN	M/100 1.0 „	1.70	1.23	29
	M/500 1.0 „	1.70	1.27	27
ohne $K_4Fe(CN)_6$		1.74	1.26	28
mit $K_4Fe(CN)_6$	M/100 1.0 „	1.70	1.25	28
ohne $K_3Fe(CN)_6$		1.70	1.21	31
mit $K_3Fe(CN)_6$	M/100 1.0 „	1.69	1.25	28

VI. *Vergleich der kolorimetrischen und Ureasemethode bei dem Arginasenachweis bei Ph 9.2 in den frischen Organen des Kaninchens.*

M/10 Argininmonohydrochlorid	6.0 ccm
M/10 Natronlauge	3.0 „
Wasser	9.0 „
Glykokoll-Natronpuffer	6.0 „
Organsuspension	6.0 „

Kolorimetrische Argininbestimmung wurde wie oben erwähnt ausgeführt. Zur Ureasemethode wurde 10.0 ccm der Versuchslösung mit gleichem Volumen 5% Trichloressigsäure enteiweisst und 10.0 ccm Filtrat wurde durch Zusatz von 1.5 ccm N/10 Natronlauge neutralisiert, mit 4 ccm M/10 Phosphatpuffer (Ph 7) und 4 ccm 2% Urease versetzt und bei 40° 1 Stunde stehen gelassen.

NH_3-Bestimmung durch Destillierungsmethode. Im Kontrollversuche wurde anstatt Argininhydrochlorid und Natronlauge 9.0 ccm Wasser gebraucht und die dabei durch Ureasemethode ermittelte NH_3-Menge wurde vom Hauptversuchsresultate abgezogen. Kolorimetrischer Argininnachweis verlief bei dem Kontrollversuch immer negativ.

Tabelle VI.

a) *Leber.*

	Sofort	1 Stunde		24 Stunden	
	Arginin mg	Arginin mg	Spaltung %	Arginin mg	Spaltung %
Kolorimetrie	1.71	0	100	0	100
Ureasemethode		0.01*	99	0.03*	98

b) *Niere.*

	Sofort	1 Stunde		24 Stunden	
	Arginin mg	Arginin mg	Spaltung %	Arginin mg	Spaltung %
Kolorimetrie	1.74	1.44	17	0	100
Ureasemethode		1.46*	16	0*	100

c) *Hoden.*

	Sofort	1 Stunde		24 Stunden	
	Arginin mg	Arginin mg	Spaltung %	Arginin mg	Spaltung %
Kolorimetrie	1.74	1.70	2	1.50	14
Ureasemethode		1.66*	4	1.46*	16

* Aus dem bemessenen NH_3 berechnete ungespaltene Argininmenge in 1.0 ccm enteiweissten Filtrats.

VII. Versuch bei Ph 9.2 über das Vorkommen der Arginase in verschieden Organen.

TABELLE VII.

	Kaninchen		Hund		Huhn	
	Argininspaltung %		Argininspaltung %		Argininspaltung %	
	1 Std.	24 Stdn.	1 Std.	24 Stdn.	1 Std.	24 Stdn.
Leber	100.0	100.0	100.0	100.0	2.0	10.5
Niere	22.1	100.0	5.5	29.8	29.7	100.0
Hoden	3.0	15.0	10.2	47.7	8.2	37.2
Muskel	2.0	1.0	1.0	1.0	0	2.0

VIII. Argininwirkung der dialysierten Organautolysate bei Ph 9.2.

TABELLE VIII.

	Kaninchen		Hund		Huhn	
	Argininspaltung %		Argininspaltung %		Argininspaltung %	
	1 Std.	24 Stdn.	1 Std.	24 Stdn.	1 Std.	24 Stdn.
Leber	38.2	100.0	49.0	100.0	0	1.0
Niere	2.0	3.0	0	1.0	1.0	2.0
Hoden	2.0	2.0	1.0	2.0	1.0	0
Muskel	1.0	2.0	0	1.0	0	1.0

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THE ACCELERATING EFFECT OF MANGANOUS IONS ON PHAGE ACTION*

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In previous papers dealing with the properties of an antistaphylococcus phage and its mode of action on a strain of *Staphylococcus aureus* (1–9) it has been shown that:

1. In a mixture of phage and growing bacteria, phage in or on the cells is in equilibrium with the phage free in the medium.

2. Phage formation is intimately related to bacterial growth and as far as can be determined does not occur in its absence. The rate of phage production is considerably greater than the rate of bacterial reproduction; as a consequence the ratio of phage to bacteria is constantly increasing. Upon accumulation of a certain high ratio of phage to bacteria (lytic threshold) lytic destruction of bacteria begins and proceeds rapidly to completion.

3. Phage can be completely inactivated by high concentrations of $HgCl_2$ and subsequently can be reactivated by removal of the Hg^{++} ions. Similarly inactivation with KCN can be reversed by conversion of the CN^- into $Ag(CN)_2^-$.

None of the experimental evidence so far elicited indicates clearly the nature of phage; it may be animate or inanimate—either concept will fit the few known facts. Additional data about phage and its mode of action upon bacteria constitute an essential requirement before any serious attempt to define its nature can be made.

To continue the study of one particular phage and bacterium, the antistaphylococcus phage and *Staphylococcus aureus* above mentioned, systematic experiments on the effect of electrolytes upon the phage-bacterium reaction were undertaken. It was found, as would be

* Supported by Grant-in-Aid from Eli Lilly and Company.

anticipated, that a variety of electrolytes in appropriate concentrations served to inhibit the reaction. Of greater interest, however, was the acceleration noted in some instances, particularly in the case of Mn^{++} salts. The Mn^{++} ion in extremely low concentrations lessens the time required for lysis to begin in a given mixture of phage and bacteria, heightens the final yield of phage obtained, and shifts the equilibrium between intracellular and extracellular phage in favor of the latter fraction.

The Acceleration Effect

Cultures of *Staphylococcus aureus* were grown in infusion broth containing 0.00016 molar $MnCl_2$ at 36°C. When $[B]^1 = 1 \times 10^8$ 1.0 ml. of the suspension was added to 4.0 ml. amounts of various concentrations of phage containing 0.00016 molar $MnCl_2$. These tubes were shaken in the 36°C. water bath together with identical controls containing no manganese. The results are recorded in Table I. For any given phage concentration the manganese-containing cultures lysed 0.5 hour ahead of the controls. To determine whether this result was due to the anion or cation, experiments were run with equivalent solutions of NaCl and $MnSO_4$. There was no accelerating action due to NaCl in the concentration used while the $MnSO_4$ produced the same effect as the $MnCl_2$. The action was therefore attributed to the Mn^{++} ion.

Analysis of the Mn^{++} Effect

In considering the mechanism underlying the accelerating effect of Mn^{++} ions three general possibilities suggested themselves; first, that the ion stimulates bacterial growth, increasing the rate of phage production and hastening attainment of the lytic threshold; second, that phage formation is somehow enhanced independently of bacterial growth; third, that the Mn^{++} ion reduces the lytic threshold and renders the cells more susceptible to phage action.

In order to test out the first of these hypotheses, that bacterial growth stimulation primarily was involved, a series of experiments was run following in each case the bacterial growth curve in phage-bacteria mixtures and in similar mixtures to which had been added manganese.

[1] $[P]$ = phage concentration in activity units/ml. $[B]$ = concentration of bacteria or staphylococci/ml. P.U. = phage activity unit.

TABLE I

Acceleration of Phage Action by Mn^{++}

Initial phage concentration	1×10^3 P.U./ml.	1×10^4 P.U./ml.	1×10^4 P.U./ml.	1×10^3 P.U./ml.
Initial bacterial concentration	2.5×10^7 *B*/ml.	2.5×10^7 *B*/ml.	2.5×10^7 *B*/ml.	2.5×10^7 *B*/ml.
Time of onset of lysis: with Mn^{++}	0.8	1.5	2.15	2.9
Time of onset of lysis: without Mn^{++}	1.3	2.0	2.6	3.4

Temperature = 36°C. pH = 7.2.
For the Mn^{++} series the bacteria were grown in broth containing 0.00016 M $MnCl_2$.
The phage dilutions used contained the same concentration of $MnCl_2$.

The growth curves were found to be identical as is indicated in Fig. 1, record of one experiment.

To test for the possible action of Mn^{++} on the phage-forming mechanism, mixtures of phage and growing bacteria with and without manganese were incubated at 36°C. and samples were taken at intervals for titration of phage. Employing the procedures outlined below for quantitative determination of phage and bacteria, it was found that the rate of phage formation was not increased by manganese.

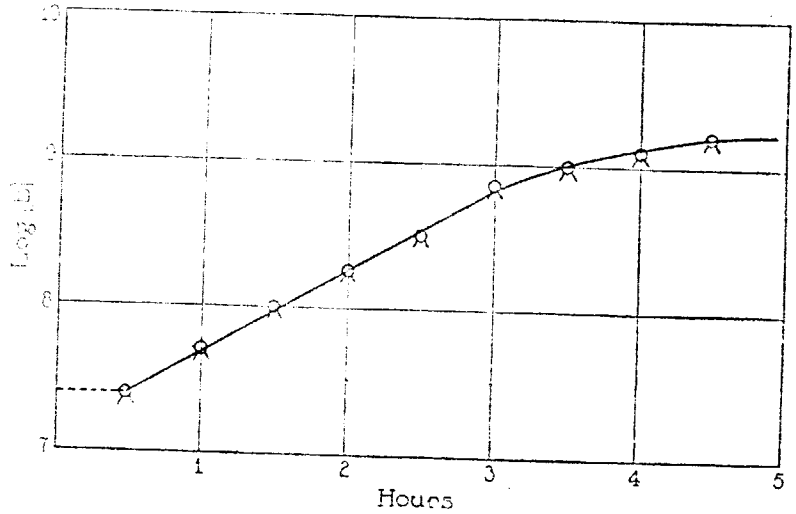

FIG. 1. Bacterial growth curves with and without $MnCl_2$ as measured by turbidity method (see text). 36°C. pH = 7.2. ○ = initial [phage] = 1×10^4 [bacteria] = 2.5×10^7, $MnCl_2$ 0.00016 M. ● = initial [phage] = 1×10^4 [bacteria] = 2.5×10^7, no manganese.

However, there was a decided difference in the distribution of phage between the medium and the cells. Manganese increases the extracellular phage fraction to about four times the ordinary free phage concentration found in the absence of manganese. This is shown in Fig. 2. Distribution of phage evidently is of the simple type previously described (4) both in the presence of and in the absence of manganese. The average value of the partition coefficient K calculated from the equation $K = \frac{Pb/B \cdot 25 \times 10^{10}}{P_E}$ (where Pb = phage in bac-

teria/ml., B = bacteria/ml., and P_E = phage free in medium/ml.) was 15×10^9 in the absence of manganese. With manganese present the value for a number of experiments was 4×10^9.

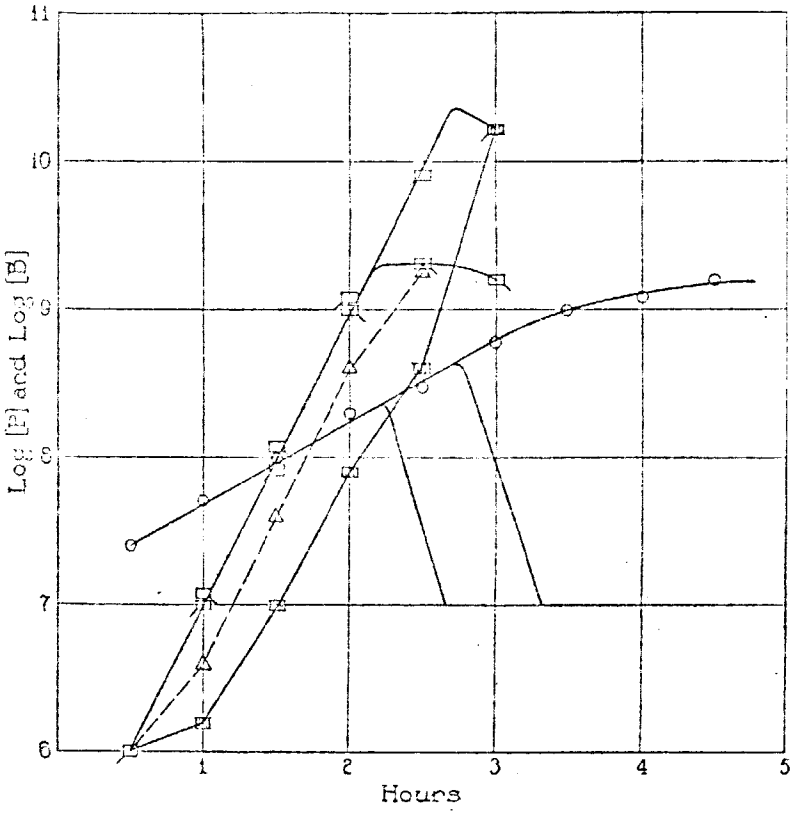

FIG. 2. The distribution of phage between bacteria and medium during growth and phage production in plain broth and in $MnCl_2$ broth. 36°C. pH = 7.2. ○ = bacterial growth curve; □ = total phage/ml. in absence of Mn^{++}; ▦ = extracellular phage/ml. in absence of Mn^{++}; □ = total phage/ml. in presence of Mn^{++}; △ = extracellular phage/ml. in presence of Mn^{++}.

It is clear from Fig. 2 that lysis requires a lower ratio of phage to bacteria in the case of the manganese mixtures. As determined in current experiments, the usual ratio of phage to bacteria at the time of

lysis in the absence of manganese is 54 phage units/cell. In the presence of manganese this is reduced to a figure of about 12 (average of several experiments).

TABLE II

The Effect of $MnCl_2$ on the Lytic Threshold

Standard phage 1×10^{10} P.U./ml.	8.0	7.0	6.0	5.0	4.0	3.0	2.0
Broth/ml.	1.0	2.0	3.0	4.0	5.0	6.0	7.0
Bacteria (8×10^8/ml.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Time	Lytic curves followed by means of turbidity measurements B = Cells $\times 10^8$/ml.													
	[B] With Mn	[B] Without Mn	[B] With Mn	[B] Without Mn	[B] With Mn	[B] Without Mn	[B] With Mn	[B] Without Mn	[B] With Mn	[B] Without Mn	[B] With Mn	[B] Without Mn	[B] With Mn	[B] Without Mn
hr.														
0.2	8	8	8	8	8	8	8	8	8	8	8	8	8	8
0.4	6	6	6	6	6	8	8	8	8	8	8	8	8	8
0.6	3	4	4	4	4	10	6	10	6	10	8	8	10	8
0.8	0	0	0	0	0	10	4	12	4	10	6	10	12	10
1.0						6	0	12	0	12	4	12	10	12
1.2						4		12		12	0	12	8	12
1.4						0		8		12		12	6	14
1.6								4		10		10	0	12
1.8								0		6		8		10
2.0										0		6		8
2.2												0		4
2.4														0

Determination of critical phage/bacteria ratio for lysis in absence of bacterial growth.

Staphylococcus-phage mixtures containing: (*a*) 0.00016 molal $MnCl_2$; (*b*) no $MnCl_2$.

[Bacteria] constant and [phage] varying. Young bacterial cultures used and growth inhibited by preliminary icing. Tubes transferred to 36°C. bath and turbidity measurements made every 0.2 hour. The critical phage/bacteria ratio for lysis with Mn^{++} present = 38 and with no Mn^{++} = 88.

If the lytic threshold in growing mixtures is lowered by manganese it would be expected that similar results could be obtained with static (non-growing) mixtures of phage and bacteria. To test this point

bacteria were grown in the presence of manganese and were then iced to inhibit growth. A unit volume of bacteria was added to varying concentrations of phage, the mixtures were iced to avoid bacterial growth and phage formation during the time required for establishment of equilibrium between intracellular and extracellular phage, and they were then shaken in the 36°C. water bath. Turbidity measurements were taken every 0.1 hour. Table II shows the results obtained in the presence and in the absence of manganese. Evidently the lytic threshold in static mixtures of phage and bacteria is lowered as in the case of the actively growing mixtures although in the absence of growth higher phage/bacteria ratios are required for lysis.

That the accelerating effect of manganese is due to depression of the lytic threshold is evidenced also by the reduction in final titre of phage when Mn^{++} is present. Experimentally, for any given initial phage concentration the end titre is uniformly about 1/10th of that developed in the absence of manganese. This would be anticipated if a lower ratio of phage to bacteria is required to institute lysis. (See Fig. 2.)

Methods

A. Materials and Titration Technique.—The broth used in our work was beef infusion containing in each liter 10 gm. Fairchild's peptone and 5 gm. NaCl. The hydrogen ion concentration was adjusted to between pH 7.2 and pH 7.4.

The stock manganese solutions contained 0.08 molal $MnCl_2$ or $MnSO_4$ and were diluted in physiological saline solution to 0.0016 molal strength before each run. Final 1:10 dilutions were made in broth so that the latter contained 0.00016 molal $MnCl_2$ or $MnSO_4$.

18 hour cultures of *Staphylococcus aureus* grown in Blake flasks were suspended in saline solution for each day's experiments. In order to have a uniform cell suspension the bacteria were seeded into manganese broth and plain broth and were grown at 36°C. until the bacterial concentration was sufficient for use. Measurements of bacterial concentrations were done by a simple turbidity comparison method as described by Krueger (1).

The bacteriophage was the antistaphylococcus phage used in previous experimental work (1-9). The standard phage solution contained 1×10^{10} activity units/ml.

Quantitative determinations of phage were routinely done by the method previously described by Krueger (1). In this method varying dilutions of unknowns and of standard phage are mixed with a unit concentration of susceptible bacterial cells. The time of lysis, *i.e.* the length of time required to reduce the

growing suspension to a particular turbidity end point, is determined under controlled conditions. The smaller the amount of phage present the longer it takes for the suspension to lyse and the initial phage concentration of an unknown is calculated from a graph on which the time of lysis of the standard controls is plotted against the logarithms of phage units present. The phage unit is an arbitrary one and represents the minimum quantity producing complete clearing of 1.25×10^8 cells in 5.0 ml. pH 7.6 broth at 36°C.

For determining phage free in solution in mixtures of phage and bacteria, the bacterial cells were centrifuged down and the supernatants diluted and titrated as mentioned above. In the presence of Mn^{++} ions such dilutions do not yield true titration values because of the accelerating action of manganese on the titration mixture: the values are always too large. For this reason it was found necessary to add some substance which would inhibit the manganese effect on the titration set up; 0.00016 Na_2SiO_3 in broth was found satisfactory for this purpose. In practice manganese-containing phage unknowns were diluted for titration in the silicate broth. Manganese containing controls were diluted with plain broth and with silicate broth to make sure that the manganese effect was successfully blocked by the silicate.

To determine total phage/ml., *i.e.* intracellular phage + extracellular phage, various dilutions of the phage-bacteria mixtures were titrated just as in the case of the supernatants. It was found that Na_2SiO_3 would not inhibit the manganese effect in such mixtures and consequently they titrated too high. It was therefore necessary to include control sets with each titration consisting of a number of bacterial concentrations representing points on the logarithmic growth curve to which had been added varying amounts of phage. The mixtures were iced to allow the intracellular and extracellular phage fractions to come to equilibrium and were then diluted. The usual aliquot of bacterial suspension for phage titration was added to each tube and the time of lysis was determined in the routine way. By means of this additional series of controls it was possible to determine the amount of phage in the given manganese-containing mixture providing the concentration of bacteria originally present was known.

B. The Accelerating Effect of Mn^{++} on Phage Action.—Phage dilutions in plain broth and in manganese broth were prepared using 4.0 ml. amounts of 1×10^8, 1×10^7, 1×10^6, 1×10^5, and 1×10^4 phage units/ml. To the plain broth-phage dilutions were added 1.0 ml. of a growing staphylococcus suspension containing 12.5×10^7 bacteria/ml. The manganese-phage mixtures were similarly seeded employing a staphylococcus culture growing in manganese broth. All the tubes were shaken in the 36°C. water bath and turbidity measurements were made at intervals as described in a previous paper (1).

C. The Effect of Manganese on Phage Production and on Phage Distribution.—To 35 ml. of broth there was added 5.0 ml. of phage solution containing the proper concentration of phage units/ml. and 10.0 ml. of a broth culture of growing staphylococci containing 12.5×10^7 bacteria/ml. An identical set-up was prepared at the same time using manganese broth, phage diluted in manganese broth, and

bacteria growing in manganese broth. Samples for the determination of total phage/ml. and extracellular phage/ml. were taken at half-hour intervals. The bacterial growth curves and lytic curves were followed by the turbidity method mentioned above. All dilutions for phage titration were kept in ice water until completion of the experiment when all were titrated at once. An experiment of this sort is shown in Fig. 2. In this particular case 5.0 ml. of phage suspension containing 1×10^7 phage units/ml. was added to 35.0 ml. of broth together with 10.0 ml. of bacterial suspension. The final phage concentration therefore was 1×10^6 phage units/ml.

D. Determination of Lytic Threshold. The lytic threshold in growing mixtures was determined from the accumulated experimental data of the distribution experiments. For the threshold in the absence of bacterial growth, bacteria were grown in plain broth and manganese broth. When $[B] = 8 \times 10^8$ the suspension was iced for 15 minutes to stop growth. Mixtures with various dilutions of plain phage and similar dilutions of phage in manganese broth were then made and the tubes were iced 0.5 hour. The mixtures were transferred to the 36°C. water bath and turbidity measurements were made every 0.1 hour.

DISCUSSION

It has long been known that dilute solutions of certain electrolytes will stimulate the action of enzymes. Falk (10) showed that dilute solutions of $MnSO_4$, $MnCl_2$, $MgSO_4$, $CaCl_2$, and $BaCl_2$ increased the activity of castor bean lipase toward ethyl butyrate. Calcium and magnesium salts accelerate tryptic digestion and the neutral salts of strong monobasic acids enhance the activity of salivary amylase (11). Aluminum sulfate and monophosphates in dilute solution have been found to stimulate enzyme activity (12); potassium bromate in low concentrations stimulates the digestion of casein by trypsin (13). In some cases the mechanism of the stimulating action of electrolytes on enzymes has been worked out. For example, the chlorides of the alkaline earths stimulate the hydrolytic action of pancreatic lipase and Pekelharing (14) has shown that the action probably consists in saponification of the fatty acids thereby removing them from the system and allowing hydrolysis to proceed. Not only is it possible to stimulate the activity of enzymes with salts but their presence is also capable of increasing enzyme production by microorganisms (15).

The experiments performed in connection with the accelerating effect of manganous salts on phage action have shown that it is not due to a stimulation of bacterial growth nor to an enhancement of

phage formation. There is a clear-cut lowering of the lytic threshold and also a change in the distribution of phage between the bacterial cell and its environment. The Mn^{++} ion increases the extracellular fraction at the expense of the phage fraction associated with the cell. In previous work it was not possible to determine which of the two

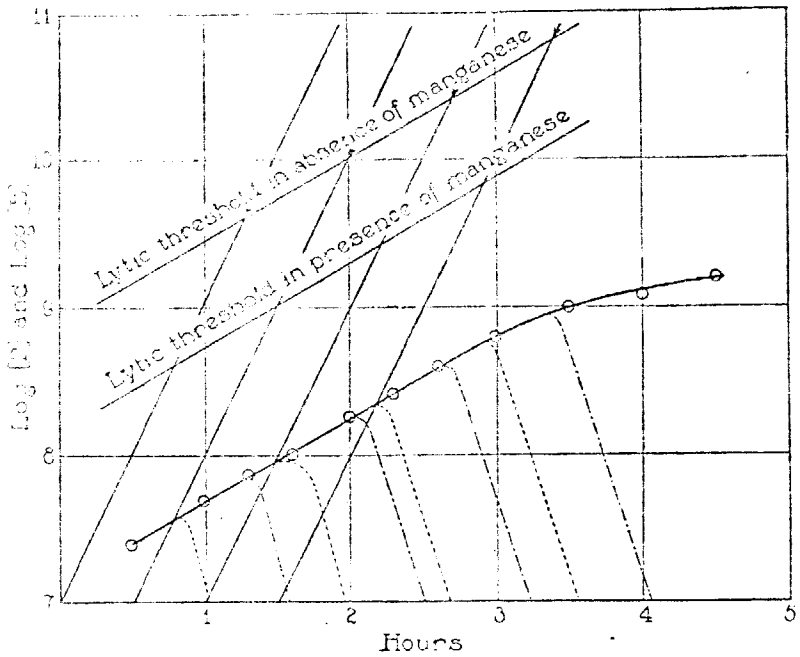

FIG. 3. Graphic representation of bacterial growth, phage production, and lytic ratios with and without $MnCl_2$. Originating in the bacterial growth curve (O) are the curves of bacterial lysis (- - - - - -) with $MnCl_2$ and adjacent broken lines (- - - - - -) the identical mixtures without $MnCl_2$. Above are two lines paralleling the logarithmic phase of the growth curve; both are crossed by a series of steep curves representing phage formation for various initial phage concentrations. The intercepts of these latter lines with the two parallel lines indicate attainment of the lytic phage/bacteria ratios requisite for lysis. At corresponding time intervals on the curve for bacterial growth the curves of lytic destruction of bacteria begin. With Mn^{++} present the total phage concentrations at lysis are constantly one-tenth of those prevailing in its absence, predicting that for any given initial [phage] in a growing mixture of phage and bacteria, the presence of Mn^{++} will reduce the end titre to one tenth that of a control mixture.

fractions, intracellular or extracellular phage, is requisite for lysis. The conclusion was reached that the significant condition for the occurrence of lysis is attainment of either a particular concentration of phage inside each bacterium or a certain concentration of phage in each ml. of surrounding solution; these two quantities are always in constant ratio to each other and it was not possible to invoke either fraction alone under equilibrium conditions.

In the manganese mixtures lysis occurs when total phage/bacterium = 12 as compared with a total phage to bacteria ratio of 54 in controls. In the present experiments the intracellular phage/bacteria ratios and extracellular phage/bacteria ratios at lysis for various mixtures of phage and bacteria were not constant. The essential ratio for initiation of lysis seems to be total phage/bacteria, that is, intracellular + extracellular phage per bacterium.

The essential features of manganese acceleration are graphically shown in Fig. 3. In this plot the bacterial growth rate is seen to be identical with and without manganese. Similarly the rate of phage production is not changed by Mn^{++} nor is the rate of bacterial destruction altered once lysis begins. The reduced threshold for lysis is represented by a line paralleling the logarithmic portion of the growth curve. Intercepts of the phage curves with this line indicate points at which the critical ratio of phage to bacteria requisite for lysis is attained and the bacterial lytic curves take their origin from the growth curve at these points. In the absence of manganese, bacterial growth and phage production continue until about ten times more phage has been produced, when lysis ensues. The lytic threshold in the absence of manganese is represented by a line paralleling the growth curve and connecting points on the phage production curves which represent the critical ratios of phage to bacteria essential for lysis. From this plot it is apparent that for any given initial concentration of phage the end titre in the manganese-containing mixture after lysis will be one-tenth that developed in the control.

SUMMARY AND CONCLUSIONS

Dilute solutions of $MnCl_2$ or $MnSO_4$ accelerate the lytic effect of phage upon susceptible staphylococci. Under the conditions of our

experiments the manganese containing mixtures lysed regularly 0.5 hour sooner than the controls.

The effect is shown to be due to a lowering of the lytic threshold, i.e. the quantity of phage/bacterium requisite for lysis; Mn^{++} reduces the ratio from 54 to about 12. In the presence of Mn^{++} phage distribution is altered and in growing phage bacteria mixtures the extracellular phage concentration is increased by manganese to approximately 3 times that occurring in the absence of manganese. There appears to be no enhancement of phage formation nor any affect on the rate of bacterial growth. As would be anticipated, for any given initial phage concentration the end titre after completion of lysis is less in the presence of manganese than in its absence. This is due to the reduced lytic threshold produced by Mn^{++}, there consequently being less phage needed to bring about lytic destruction of the bacteria.

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MICRODETERMINATION OF MANGANESE IN BIOLOGICAL MATERIAL BY MEANS OF CATALYSIS*

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The standard methods of manganese determination in biological material (1, 2), based on the oxidation of Mn^{++} to permanganate, are not sufficiently sensitive. Since the Mn^{++} content of animal tissues is very low, 20 to 100 gm. of material are required for analysis. The color reaction of manganese [illegible] and benzidine was successfully applied for the quantitative detection of Mn^{++} in tissues by Wiese and Johnson (3). This method made it possible to determine manganese from 5 gm. tissue samples. However, [illegible] experiments it is desirable to use even less material.

It will be shown in this paper that the catalytic properties of Mn^{++} [illegible] a possible means of microanalysis of manganese, by which mere [illegible] of Mn^{++} can be determined from small tissue samples.

EXPERIMENTAL

The method is based on Szebelledy's and coworkers' experimental results (4, 6). It was shown by them that the oxidation of certain phenol derivatives can be catalyzed by traces of Mn^{++} ion. The most sensitive reaction for the measurement of Mn^{++} catalysis proved to be the oxidation of an aqueous solution of diethylaniline by an aqueous solution of potassium periodate. At the present time the kinetics of this reaction as well as the structure of the oxidation product are unknown. When this reaction is carried out in unbuffered solutions, no strict correlation between the rate of the reaction and the concentration of the catalyst was found. The use of a phosphate-citrate-borate buffer at pH 7.0 (7), however, increased the reproducibility of the measurements by maintaining a constant environment for the catalysis (Table I).

Markus (6) found that Fe^{++} and Mg^{++} do not alter this Mn^{++} catalysis. Fe^{++} ion, which interferes with the catalysis in an unbuffered medium, did not show any effect in the buffered mixture, even when its concentration was 10 times as high as that calculated to be present in biological material [illegible]. Other heavy metals did not interfere when the procedure was carried

* Part of this work was carried out with Geza Frendl in the biological laboratory of the chemical and pharmaceutical factory of Palik and Company, Ltd., Budapest. Aided by a grant (Contract N6ori-20, Task Order 11) from the United States Navy, Office of Naval Research, and by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

out at this pH and in the buffered media. The sensitivity of this catalytic reaction could be increased by using a 5 times more dilute solution of diethylaniline and potassium periodate, with the aid of the Beckman spectrophotometer.[1] The optimal wave-length as determined with this instrument was found to be 470 mμ.

Figs. 1 and 2 were constructed from the same data, obtained with the Beckman spectrophotometer. The optical density ($\log I_e/I_z$), measured at the 200th second, was plotted against the wave-length, expressed in mμ (Fig. 1). A sharp maximum at 470 mμ was found.

Fig. 2 shows the correlation between the amount of Mn^{++} and the optical density measured under the same conditions as those mentioned for Fig. 1. The optical density measured at the 200th second after the addition of the KIO_4 solution plotted against the amount of Mn^{++} on a semilogarithmic paper gave a straight line relationship for the concentration range used.

TABLE I

Catalytic Activity of Various Concentrations of Mn^{++} in Phosphate-Citrate-Borate Buffer, pH 7.0

The optical density of the yellow oxidation product of saturated diethylaniline by saturated KIO_4 was measured by means of a Lange-Roth photoelectric colorimeter with a blue filter at the 120th second after the addition of the KIO_4 solution.

	Mn^{++} concentration, micrograms per ml.				
	Mn^{++}-free	[illegible]	0.010	0.1	0.5
	Optical density				
Mean (10 measurements)	0.116	0.124	0.134	0.169	0.198
S.D.	±0.001	±0.0011	±0.003	±0.0013	±0.0013

This correlation made it possible to work out a method for the determination of Mn^{++}. If one measures the catalytic activity of an unknown amount of Mn^{++}, that is the optical density of the dye, reached at a given time (200 seconds), then the amount of Mn^{++} can be easily read from a standard curve.

Reagents—1. Saturated diethylaniline solution. 4 drops of diethylaniline base are diluted with redistilled water to 100 ml. This is thoroughly shaken and is left to stand for 24 hours at room temperature in a dark place. Immediately before use the solution is filtered.

2. Saturated potassium periodate. This solution is prepared by shaking 2 gm. of crystalline KIO_4 in 100 ml. of redistilled water and treating it in

[1] The spectrophotometric measurements were made in the laboratory of Dr. L. S. G. Barron, who kindly provided us with the use of his Beckman spectrophotometer.

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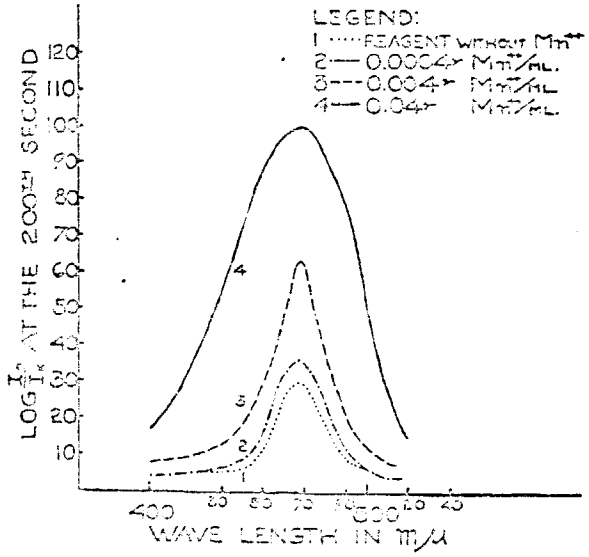

FIG. 1. Correlation between the wave-length used in the spectrophotometer and the optical density of the reaction mixture, containing varying amounts of Mn^{++} from 0.0004 γ per ml. to 0.04 γ per ml. Readings were made at the 200th second after the addition of the KIO_4 reagent. There is a maximum at 470 mμ.

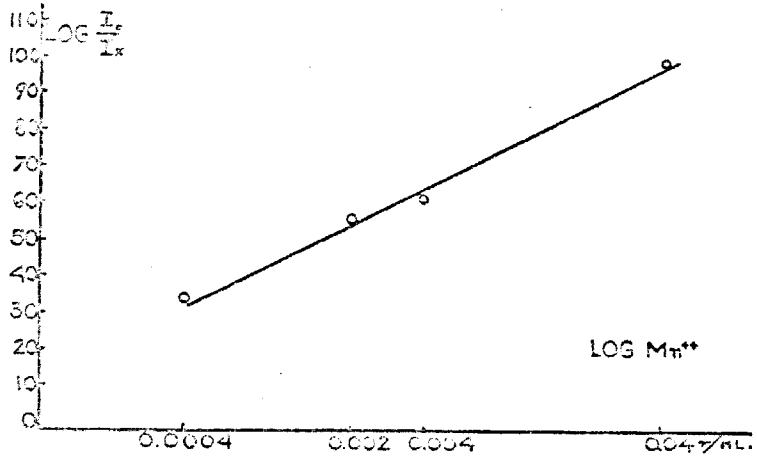

FIG. 2. Relationship between the amount of Mn^{++} and the optical density of the reaction mixture measured at the 200th second after the addition of KIO_4 shown on semilogarithmic paper. The points are obtained from the same data as in Fig. 1.

the same manner as Reagent 1. When a Beckman spectrophotometer is used, it is necessary to dilute Reagents 1 and 2 five times with redistilled water.

3. Manganese standard solution. Crystalline $MnSO_4$ is dried for 3 hours at 120°. This substance is used for standard Mn^{++} solutions.

4. 0.1 M oxalic acid.

5. 0.1 N HCl.

6. Phosphate-citrate-borate buffer (pH 7.0).

Procedure

The vessels used must be carefully washed with a mixture of HCl and HNO_3 (1:1), and then with redistilled water five times. If the material is blood or any other fluid, it can be used as such. For each analysis (50 parallel measurements) 0.1 to 0.5 gm. of tissue or 0.5 ml. of fluid is required. The sample is measured into a platinum crucible with 10 drops of 0.1 N HCl and ashed at 870° in an electric oven for 4 hours. When the crucible is cooled, 2 ml. of 0.1 M oxalic acid and 5 drops of 0.1 N HCl are added and the mixture kept at 80–90° for 20 minutes. This reduction is necessary because Mn^{++} is oxidized at 870° to Mn^{+++}, which is inactive as a catalyst in this case. The contents of the crucible are quantitatively washed into a 100 ml. flask and diluted to the mark with redistilled water. For each determination 2 ml. of this extract are required.

The measurements are made as follows: 2 ml. of extract plus 4 ml. of buffer (pH 7.0) are placed in a test-tube. The mixture and the reagents are kept with the standard Mn^{++} solutions at room temperature for ½ hour. Samples containing known amounts of Mn^{++} are prepared in the same way as tissue extracts. Immediately before the measurements are made, 2 ml. of diethylaniline solution plus 2 ml. of KIO_4 reagent are added. From the moment this latter reagent is added the time must be measured exactly by a stop-watch for 200 seconds. During this time the test-tube is shaken for 30 seconds; then the mixture is poured into the photometer cup. The extinction is read at the 200th second after the addition of KIO_4. From the straight line obtained by plotting the known amounts of Mn^{++} against the E values read at the 200th second on semilogarithmic paper, the Mn^{++} concentration of the extract can be easily calculated. To insure accuracy oxalic acid and HCl are added to the Mn^{++} standard solution in the same concentration as contained in the tissue extract.

To check the described method, its results were compared with those of a known micromethod. The Mn^{++} concentration of dried brewers' yeast was first determined by the catalytic method on yeast samples of 0.1 gm. Then five parallel measurements were made with a colorimetric method (9) whereby Mn^{++} was determined as $KMnO_4$ on 50 gm. yeast samples. With

he microprocedure 1.8 to 2.0 mg. of Mn^{++} per 100 gm. of yeast (Mn^{++}) were obtained, while the macroprocedure gave 2.0 to 2.1 mg. per 100 gm. In ten samples 0.01 γ of Mn^{++} was added in the form of $MnSO_4$ to 1 gm. of yeast. This amount could be recovered with a standard deviation of ±0.0018. Analyses of human tissues carried out by means of this method are shown in Table II, in which human muscle (quadriceps femoris), liver, and kidney were analyzed with both micro- and macromethods.

TABLE II

Mn^{++} Content of Human Tissues

Organ	Catalytic method	Macromethod (γ/gm.)
	From 1.5 gm. material	From 5.0 gm. material
	γ/gm.	γ/gm.
Muscle	0.0082	0.0080
	0.0090	0.0071
	0.0083	0.0092
	0.0100	0.0084
	0.0096	0.0076
Mean	0.0090	0.0080
Liver	0.092	0.086
	0.072	0.096
	0.087	0.080
Mean	0.083	0.077
Kidney	0.030	0.025
	0.021	0.031
	0.027	0.037
Mean	0.026	0.031

SUMMARY

A colorimetric method, based on the catalytic properties of Mn^{++}, has been developed and used for the microdetermination of Mn^{++} in biological material. Mn^{++} catalyzes the oxidation of diethylaniline by potassium periodate to a yellow dye. The speed of this color development can be measured by means of a spectrophotometer. The speed of the reaction is a function of the amount of Mn^{++} present in the reaction mixture. With this tool, 0.0001 γ to 0.01 γ of Mn^{++} per ml. has been determined with a precision of ±15 per cent. 0.5 gm. of tissue or 0.1 to 0.5 ml. of biological material is sufficient for 50 parallel measurements.

The author wishes to take this opportunity to thank Dr. E. M. K. Geiling for making possible the publication of this paper.

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THE MANGANESE CONTENT OF PLANT AND ANIMAL MATERIALS.*

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Although the fundamental importance of the mineral elements in plant and animal nutrition has long been recognized, there is a marked lack of analytical data on the mineral content of various food materials. In a previous publication (1) attention was called to this deficiency and a report was given of the calcium, phosphorus, and iron content of cabbage grown in various parts of the United States. Within the last 2 months Paul E. Howe (2), Chairman, Subcommittee on Animal Nutrition, National Research Council, has requested research workers to assist in supplying analyses not only for "those inorganic elements ordinarily considered as nutrients—namely, sodium, potassium, calcium, magnesium, sulphur, phosphorus, chlorine, iodine and iron, but also those others the function or effects of which are less perfectly known—namely, manganese, fluorine, bromine, silicon, boron, aluminum, copper, arsenic and zinc."

In the present paper data will be given on the manganese content of 84 samples covering the principal classes of human foods. A number of papers relating to trees and leaves (3–6), to plant food materials (7–23), and to animal food materials (24–28) has already been published on this subject, but in most cases a rather limited number of food materials was analyzed. There is also great lack of uniformity in regard to the methods of analysis employed and the basis on which the manganese is calculated.

The authors fully appreciate that the figures herewith presented do not necessarily represent the manganese content of these

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materials generally. Only when an extensive series of samples, grown on different soil types and under different climatic conditions, has been analyzed, will it be possible to state the probable manganese content of any plant foodstuff.

EXPERIMENTAL.

The samples used in this study constituted a representative group of food materials, such as fruits, vegetables, cereals, nuts, and meats bought in the local market. The materials were carefully washed, spread out in thin layers, and left until the water had evaporated. They were then cut in small pieces, dried to constant weight at 100° C., ground in a glass mortar, and bottled. Before taking a sample for analysis the material was dried several hours to remove any absorbed moisture.

The manganese determinations were made according to the periodate method of Willard and Greathouse (23) as described in Methods of Analysis of the Association of Official Agricultural Chemists (50).

Reagents of the highest grade obtainable were used, and all were tested for manganese before being used in the analysis of the samples. Porcelain dishes were used for the ignition of the samples, and these were especially tested. Since the glaze is destroyed when the dish is used several times, more or less of the porcelain may be included with the sample. A test for manganese in the porcelain of the dish was therefore made by placing potassium carbonate in the dish and heating to the fusion point. This treatment removed many times more porcelain than could be taken up by a sample in the regular determination. Three dishes were fused with potassium carbonate and the hydrochloric acid extracts were found to be free from manganese.

The material to be analyzed was thoroughly ashed in a muffle furnace at a temperature of dull redness until the carbon was completely destroyed.

Because of the wide variations in manganese content of the materials used, a single preliminary analysis of each material was made in order to determine the size of sample necessary to produce a depth of color which is to be read against the standard. Samples varying in weight from 1 to 15 gm. were subsequently used.

After ashing, the residue was taken up with 25 to 30 cc. of HCl (1:4) and evaporated to dryness in order to render the silica insoluble. The residue was moistened with 2.5 cc. of concentrated HCl and 30 cc. of water. After heating on the sand bath for 1 hour, the sample was filtered and the insoluble residue washed thoroughly. To the filtrate, which amounted to about 150 cc., 4 cc. of concentrated H_2SO_4 were added, and the whole evaporated on a hot plate to 8 cc. After cooling, 2½ cc. of concentrated HNO_3 were added, and the evaporation continued until white fumes began to come off. After the addition of 30 cc. of water, the solution was heated to dissolve the salts of iron. Then 0.3 gm. of KIO_4 was added, and the solution gently boiled to oxidize the manganese and bring out the full permanganate color. The solution was carefully concentrated to slightly less than 25 cc., transferred to a 25 cc. volumetric flask, and made up to volume. The color was compared with that of a standard permanganate solution in a Duboscq colorimeter.

Duplicate determinations were made in all cases, and a third "recovery" determination was made in some cases.

Preparation of the Standard.—A $KMnO_4$ solution was prepared so as to contain 1 mg. of manganese in 1 cc. of solution. This solution is slightly weaker than a 0.1 N solution. The exact strength of the solution was determined by standardizing against analyzed $Na_2C_2O_4$. This was the stock solution and from it more dilute solutions were made at the time they were to be used.

Iron salts in solution affect the permanganate color to a slight extent, and because of this fact $Fe(NO_3)_3$ solution containing 1.0 mg. of iron in 1 cc. was added to the standard to give it an iron content about equal to that of the material analyzed. The standard was treated with H_2SO_4 and KIO_4 in exactly the same manner as the sample solution and was also made up to 25 cc. Two or more standards were usually prepared, and the one was chosen which had approximately the same intensity of color as the sample. Usually the standard prism was set at 20 mm. In some cases, where dilute standards had to be used, the prism was set at 30 mm. and in three or four determinations it had to be placed as high as 60 mm. At this extreme limit it is probable that the accuracy of the determination is much reduced.

Recovery of Manganese.—As a check upon the method, a known.

quantity of $KMnO_4$ solution was added to the third sample of every different material before ashing. This check for recovery of manganese was used in a sufficient number of cases to show that the method was giving satisfactory results.

In Table I will be found detailed data illustrative of the entire procedure. The data for three different materials are given. As will be noted, the size of the samples and their manganese content varied widely, but the recovery of added manganese, ranging from 92.9 to 106.0 per cent, was good in each case.

Manganese Content of Foods.—In Table II are given the moisture and manganese content of 84 food materials. The figures

TABLE I.

Details of Four Illustrating Procedure for Determination of Manganese in a Few Representative Food Materials.

Sample.	Weight of sample.	Manganese added.	Standard. Manganese.	Standard. Reading.	Unknown reading.	Manganese.	Recovery of manganese added.	Manganese in sample, dry basis.
	gm.	mg.	mg.	mm.	mm.	mg.	per cent	per cent
Barley (average)	2.5		0.20	20.0	20.0	0.25000		0.00800
"	2.5	0.04	0.20	20.0	13.5	0.21520	106.0	
Beans, Lima, (average)	5.0		0.05	20.0	24.5	0.05125		0.00122
Beans, Lima	5.0	0.04	0.05	20.0	15.2	0.09038	93.8	
Potatoes (average)	10.0		0.05	20.0	22.0	0.04515		0.00045
Potatoes	10.0	0.01	0.05	20.0	12.1	0.05634	92.9	

for one foodstuff, cabbage, are the averages of twenty-two different samples grown on different types of soil and in different localities.

Manganese varies widely—from none in several species of fish to 0.02162 per cent in northern grown lettuce. Tree fruits grown in temperate regions are low in manganese (0.00039 per cent, average), whereas some tropical tree fruits are high (0.00333 per cent, average) and others are low (trace, grapefruit). The average percentage of manganese, arranged in ascending order, for some of the principal classes of foods are: 7 animal tissue, 0.00062; 12 berry and vine foods, 0.00178; 10 roots and tubers, 0.00184;

TABLE II.

Manganese Content of Food and Animal Materials.

Sample.	Moisture.	Manganese in sample, dry basis.	Sample.	Moisture.	Manganese in sample, dry basis.
	per cent	mg. per kg.		per cent	mg. per kg.
Apples, greening	82.5	3.0	Grapes, Malaga	79.6	Trace.
" sweet	83.9	1.6	Grapefruit	92.8	"
Apricots	[illegible]	4.8	Halibut	76.6	None.
Asparagus	[illegible]	12.8	Hog, liver	68.7	12.2
Banana	75.4	33.3	Kohlrabi	91.5	13.0
Barley	6.8	16.2	Kraut, No. 329	92.0	7.7
Beans, kidney	12.5	13.7	" " 332	91.4	4.0
" lima	12.3	12.[illegible]	Kumquat	85.0	3.3
" navy	[illegible]	29.[illegible]	Lettuce, northern	95.0	216.2
Beef, kidney	79.6	0.5	" southern	95.0	100.0
" liver	7[illegible].6	8.7	Oats	8.2	42.1
" spleen	77.1	None.	Onion	93.7	7.9
" round steak	75.1	"	Orange peel	76.2	5.2
Beet greens (roots)	87.2	22.[illegible]	" pulp and juice	87.0	Trace.
" " (tops)	90.3	12[illegible].8	Parsley	88.2	80.0
Beets	82.3	76.2	Peach	83.4	19.9
"	84.7	35.1	Peanuts	1.9	16.0
Blackberries	84.1	37.5	Pears, Bartlett	83.9	4.0
Blueberries	81.3	122.4	Peas, green	78.6	19.4
Bluefish	74.0	None.	Parsnips, scraped	82.7	2.0
Brussels sprouts	87.5	21.5	" unscraped	75.6	13.9
Cabbage (average of 22 samples)	92.8	10.6	Peppers, red	91.7	18.2
			Pepsin	5.4	10.1
Cabbage, red	91.4	13.7	Pineapple	92.0	133.9
Calf, liver		12.0	Pistachio nuts	4.0	6.6
Carrots	90.1	6.0	Pomegranate, seeds	79.6	8.6
Celery	94.0	27.1	" skins	73.5	16.6
Chard	91.4	93.5	Potatoes	78.2	4.5
Cherries	87.5	2.5	" sweet	72.1	5.4
Codfish	81.7	None.	" "	73.3	19.6
Corn, yellow	8.6	5.3	Prunes	41.2	3.1
Currants	28.8	4.4	Quince	82.5	Trace.
Dates	22.3	1.0	Raisins, seeded	23.6	4.2
Figs	37.0	5.5	" seedless	27.4	4.7
Gooseberries	91.1	4.8	Rye	6.4	33.3
Grapes, raisin, California	81.2	4.5	Salmon	75.7	None.
Grapes, table, Concord	77.3	3.0	Shrimp	69.6	Trace.
" " Tokay	72.5	3.0	Spinach	91.3	86.5

TABLE II—*Concluded.*

Sample.	Moisture.	Manganese, calculated to dry basis.	Sample.	Moisture.	Manganese, calculated to dry basis.
	per cent	mg. per kg.		per cent	mg. per kg.
Squash, Hubbard	90.4	16.7	Walnuts, English	3.3	18.6
Sweet peas	10.0	6.5	Watermelon	92.7	2.5
Tomatoes	[illegible]	2.6	Wheat	7.6	37.0
Turnip, white	90.0	None.	" bran	9.8	101.0
Walnuts, black	5.0	19.1			

4 nuts, 0.00166; 4 leguminous seeds, 0.00200; 5 cereals, 0.00268; 12 leafy vegetables, 0.00970. Leafy vegetables, which are generally high in all mineral elements, likewise contain the largest amount of manganese. This is about 3 times that contained in the next two groups, cereals and legumes.

Variations are subject among the tree fruits, berries, roots, tubers, and leafy material. For example, leafy materials range from [illegible] per cent for southern to 0.02162 per cent for northern grown lettuce. Two different samples of the same material have widely differing manganese percentages, varying presumably with soil conditions. Northern grown lettuce contains twice as much manganese as southern grown lettuce. The maximum for the twenty-two samples of cabbage analyzed was 0.00159 per cent, and the minimum was 0.00052 per cent. Beets and sweet potatoes show similar variations.

The pineapple is produced in a part of the world where the soils are known to be rich in manganese. On the other hand, many common food materials produced within the state of Wisconsin are still higher in manganese than the pineapple. Lettuce, broccoli (tops), blueberries, and wheat bran are all unusually high in manganese.

SUMMARY.

The manganese content of 34 samples covering the principal classes of food materials is given.

The figures (dry basis) range from none in fish to 0.02162 per cent in northern grown lettuce. The classes of foods in decreasing order of manganese content are as follows: leafy vegetables, cereals, legumes, nuts, roots and tubers, bush and vine fruits, animal tissues, and tree fruits.

Different samples of the same foodstuff vary in manganese content. For example a sample of lettuce grown in the north contained more than twice as much manganese as a sample grown in the south. In twenty-two samples of cabbage the manganese ranged from 0.00052 to 0.00159 per cent.

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SIMPLIFIED NEUTRON ACTIVATION ANALYSIS METHOD FOR COPPER AND MANGANESE

KEY WORDS: copper, manganese, activation analysis, serum, ion exchange

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ABSTRACT

An activation analysis procedure has been developed to determine manganese and copper serum values in one milliliter of serum or less rapidly and with good precision. The sample is irradiated in a neutron flux of $10^{12} cm^{-2} sec^{-1}$ for two hours followed by nitric acid digestion. The activity plus carrier is absorbed from saturated lithium chloride solution on Dowex1-8 contained in a syringe. After washing the resin with more lithium chloride, the syringe is counted in a five inch well crystal multichannel spectrometer. The carrier is washed off the syringe by dilute acid and analyzed. The sample concentration is determined by comparison to irradiated standards correcting the unknown for decay and chemical yield.

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INTRODUCTION

The role of trace metals is of extreme importance in studying and understanding the actions and reactions of biological system. Enzymatic reactions and biological control systems frequently are dependent upon trace metal concentrations in the parts per million to parts per billion ranges. The accurate determination of these elements is vital to the comprehension of their roles and the biological consequences of the metal deficiency or excess. Of the trace metals involved, both copper and manganese are among the most important.

The copper concentration in serum is in the parts per million range, while manganese is in the parts per billion range. The relatively high concentration of copper permits analysis by conventional techniques, such as atomic absorption and standard colorimetric procedures. Manganese, because of its extremely low concentration, often less than one part per billion, is very difficult to measure. Chemical and spectroscopic methods in these low concentrations are very difficult and require large amounts of serum. However, neutron activation has been used with considerable success but the reported methods are technically difficult and time consuming[1,2,3]. In the proposed procedure, designed to determine manganese, the determination of copper is an added bonus.

Any activation analytical procedure involving trace metals in a biological matrix has a basic problem. Biological systems have large amounts of sodium and chlorine. For example, one milliliter of serum irradiated for one hour at 10^{12} n/cm^{-2} sec^{-1} may have up to 10^6 cpm

of Na^{24} and only a few hundred cpm of Mn^{56}. Therefore, a separation procedure to isolate the element of interest from the overwhelming interfering activity is essential.

PROCEDURE

One milliliter samples of serum collected by procedures known to be free of contamination are irradiated in copper and manganese-free polyethylene vials for 2 hours at a nominal neutron flux of 10^{12} $n/cm^2\ sec^{-1}$. Standards are simultaneously irradiated. The samples are transferred to 50 ml round bottom flasks and then 5 ml of concentrated nitric acid and 0.1 mg each of copper and manganese carrier added. After refluxing for one hour the solutions are taken to dryness. Three milliliters of 12M LiCl is then added. The LiCl solution is taken up on 1 cc of specially treated Bio-Rad Ag 1-8 resin[4] contained in a plastic syringe which is shown in Figure 1.

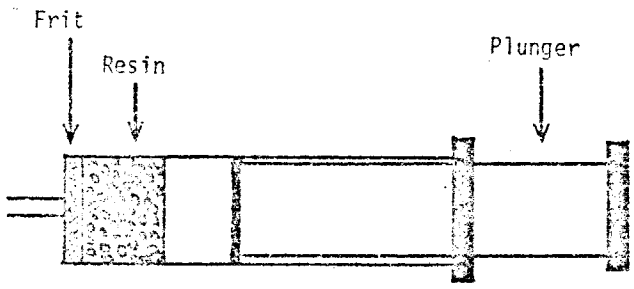

FIG. 1

Syringe with Resin

The solution is shaken with the resin and is allowed to equilibrate, then the solution is discarded by expressing the liquid from the syringe.

This procedure is repeated three times; the syringe is placed in a finger cot and counted in dual 5 inch well sodium iodide crystal counter equipped with a split 400 channel memory multichannel analyzer (200 channels being available for each crystal). The standards are merely transferred and counted. The output of the analyses is taken both on printed tape and punched tape. An example is shown in Figure 2.

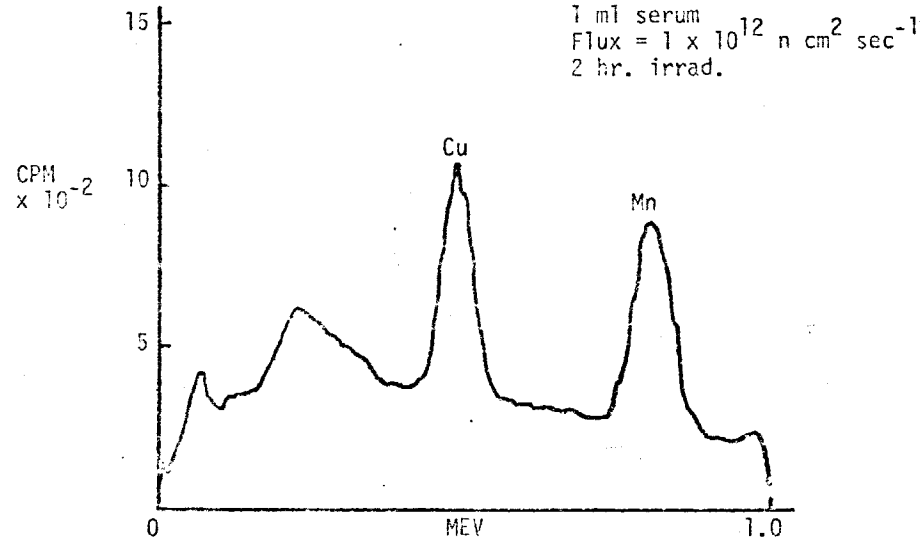

FIG. 2

Spectrum of Typical Syringe Sample

To conserve time, a computer is used to determine the areas under the surves. After decay, the copper and manganese are removed from the syringe with 0.01 M HCl and diluted to 25 ml in volumetric flasks. An atomic absorption spectrophotometer is used to determine the chemical yield, the recovery being about 70-80%. The concentration of the copper and manganese is calculated in the conventional fashion.

NEUTRON ACTIVATION ANALYSIS METHOD

All the glassware must be free of contamination, which can be accomplished by washing with pure nitric acid and de-ionized, glass distilled water whose purity is checked by activation analysis. The collection methods and storage procedures must be monitored continuously to prevent accidental contamination by the elements in question.

RESULTS AND DISCUSSION

Because of its inherent simplicity, ion exchange was adopted as the separation procedure. To determine the suitability of anion exchange separation, a synthetic radioisotope sample was separated on a pressurized Bio-Rad Ag 1-8 ion exchange column in a manner similar to the method suggested by Kraus and Moore[5], and Worwood[6] for the separation of the transition elements. The 2 ml fractions were counted in a five inch well crystal utilizing a 400 channel multichannel analyzer. A one milliliter serum sample was irradiated, ashed in nitric acid and also chromatographed. The copper and manganese were found in the same fractions in both samples. The separation of Na^{24} was not sufficient to permit the accurate determination of manganese in the serum sample. While manganese could be detected, the high dead time due to the Na^{24} overlap presented a great problem.

Using the increased (circa 4 to 550) Kd resulting from the lithium chloride effect[7], the analysis of irradiated serum was repeated with 25 milliliters of 12 M lithium chloride preceding the HCl eluants. The Na^{24} and other non anionic forms were effectively washed through the column with the 25 ml of LiCl. The copper and manganese were retained on the column. Elution with the HCl gradient suggested by Kraus then desorbed the copper and manganese in separate fractions with a dead time of less than 5% in the appropriate tubes. The Na^{24} was effectively removed and only copper and manganese were detected. Both the energy and fraction position proved the chemical identity and no other isotopes were detected by the system. The isotopic purity was such that the fractions could be counted in an auto-gamma without significant error. However, column chromatographic techniques are too slow for practical use.

When the syringe method was used, three washings were sufficient to reduce the Na^{24} count to an insignificant value. Two washings were sufficient to reduce the Na^{24} from an initial activity of 4.8×10^5 cpm in a sample to 483 cpm. The Na^{24} reduction is comparable to that achieved by the Sb_2O_5 procedure of Girardi[8].

The procedure has a high precision. Ten replicates of pooled serum, five each on different days, gave a value of $1.60 \pm 0.07 \times 10^{-8}$ gm/ml for manganese and 1.05×10^{-6} gm/ml for copper. The average of 18 beagle serum samples gave results similar to the average manganese results obtained for human serum using more complex and tedious procedures. One such procedure at the Walter Reed Army Institute of Research yields an average value of 1.1×10^{-3} ppm for manganese in human serum which is comparable to the 1.1×10^{-3} ppm found for beagles (average of 18 samples). The serum copper for beagles is 0.72 ppm (average of 18 samples) when determined by the syringe technique which is identical to the value of 0.72 ppm (average of 56 samples) as determined by atomic absorption techniques.

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EFFECT OF DIFFERENT CONCENTRATIONS OF MANGANESE SULPHATE ON THE GROWTH OF PLANTS IN ACID AND NEUTRAL SOILS AND THE NECESSITY OF MANGANESE AS A PLANT NUTRIENT[1]

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INTRODUCTION

In a study of the literature on the nutrition of plants, one is impressed with the lack of definite knowledge concerning the necessity of a number of elements frequently found in appreciable amounts in the ash of different species.

For many years it has been taught and accepted by agronomists and plant physiologists that only 10 of the elements are essential for the normal growth and maturation of plants. These essential elements are carbon, hydrogen, oxygen, nitrogen, calcium, magnesium, potassium, phosphorus, sulphur and iron. In addition to these, it is also well known that as many as 20 or 30 others are frequently found in small amounts in plants which have grown under natural conditions.

Within recent years many data have been obtained which indicate that a few of these so-called nonessential elements may have important functions in the plant's economy. During the past 25 years, perhaps, more attention has been given to the acquisition of knowledge concerning the occurrence, distribution, and probable functions of manganese in soils and plants than to any other one of the elements referred to.

The number of contributions to the literature on the relation of manganese to various phases of agriculture number no less than 100. As a matter of convenience these contributions may be divided into two general classes, depending upon the object to be attained. Investigators of one class, apparently, have been interested in determining whether or not manganese has any commercial value from the standpoint of a necessary fertilizer, while those of the other class have sought to determine whether or not manganese is an essential element in the vegetable economy and, if so, to ascertain its functions.

HISTORICAL

Manganese was discovered by Scheele (*9*)[2] in 1774. He found that the soil contained small amounts of this element and that it was assimilated by plants that grew in the soil; however, he made no effort to determine whether manganese was necessary for plant growth.

In 1864 Sachs (*8*) conducted experiments, the purpose of which was to determine whether or not manganese was necessary in plant economy. He proved that manganese could not replace iron in plant growth, but

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This investigation, begun at the Kentucky Agricultural Experiment Station in 1916, was continued, with the consent of the Director, at Cornell University where part of the results were offered in partial fulfillment of the requirements for the degree of doctor of philosophy. Since September, 1922, the work has been carried on at the Kentucky Station. The paper presents in part the results of the investigation at Cornell, the earlier results, and the recent findings at the Kentucky Station.

[2] Reference is made by number (italic) to "Literature cited," p. 793-794.

he did not prove that manganese was also necessary when iron was available.

In 1883 Yoshida (*12*) isolated from the sap of the lac tree a product possessing peculiar enzymic properties which he was unable to explain.

In 1894 Bertrand (*1*) undertook further work on the nature and composition of the product which had been isolated from the sap of the lac tree, and found that it contained considerable manganese, which gave to the product its peculiar enzymic properties. After several years of research on the relation of manganese to the growth of plants he concluded that this element is necessary for the normal growth of both autotrophic and heterotrophic plants and that its functions can not be performed by any other element.

In 1914 Brenchley (*2*) published the results of an investigation concerning the effect of manganese sulphate on the growth of barley seedlings. She found that with a concentration of 1/100,000 of $MnSO_4$ and less a decided stimulation in the growth was produced; with greater concentrations than this, however, toxic effect resulted. In her conclusion with respect to the function of manganese in plants she suggests the probability that manganese may prove to be an essential element in the economy of plant life, even though the quantity usually found in plants is very small.

Kelly (*5*) made a study of the effect produced on different species of plants when grown in a natural soil containing a large amount of manganese as compared with the effect produced on the same species when grown on a soil containing only a normal amount of manganese. From his results he concluded that the small amount of manganese occurring in soils probably performs a twofold function in plant growth: (1) It acts catalytically, increasing the oxidations in the soil and accelerating the auto-oxidations in plants; and (2) it tends to modify the absorption of calcium and magnesium, perhaps by partially replacing calcium from insoluble combinations, but especially through a direct effect by which the absorption of calcium is increased and that of magnesium is decreased.

McHargue (*6*) grew wheat plants to maturity in solution cultures free from and containing manganese and found that in the absence of manganese the plants became etiolated and did not develop in a normal way, thus showing that manganese is essential to the normal growth and maturation of this plant.

Previous to 1902 but little attention had been given to the occurrence and distribution of manganese in soils. During that year Ewell (*3*) made an interesting observation with respect to the occurrence of soluble salts of manganese in a certain small area of soil which failed to produce crops in a normal way. The results of his investigation showed that the small nonfertile area of soil contained relatively large amounts of soluble salts of manganese, while the adjacent fertile soil contained none. He assumed that the difference in the fertility of the two soils was due to the presence of the soluble compounds of manganese. The publication of his conclusions apparently awakened a wide interest regarding the rôle manganese plays in soils.

In 1907 the United States Bureau of Soils (*10*) inaugurated a series of experiments with manganese at the Arlington Experiment Farm. Manganese sulphate was applied at the rate of 50 pounds per acre to an acid silty clay loam soil, and crops were grown on the treated and untreated soil for six years. The application of manganese to the acid soil resulted

in decreased yields; however, upon neutralizing the acidity in the soil by the addition of calcium carbonate the plots receiving manganese produced very marked increases in yields, thus showing that the reaction of the soil is an important factor in determining whether or not an application of manganese sulphate will exert a beneficial effect on plant growth.

Funchess (4) has obtained data which tend to show that some acid soils contain soluble salts of manganese. He also offers the suggestion that the toxic effect associated with soil acidity may be due in part to soluble salts of this element. He describes experiments with acid soils that contained soluble salts of manganese and were toxic to the growth of plants. He found that the toxic effect of the soil was destroyed after neutralization with calcium hydroxid.

In 1920 Olaru (7) published results showing the effect of manganese on some of the more important microorganisms concerned with nitrogen transformations in soils and leguminous plants. With pure cultures of *Bacillus radiocicola*, *Azotobacter chroococcum*, *Clostridium pasteurianum*, and *Micrococcus ureae* and with concentrations of manganese sulphate varying from zero to 10 mgm. per liter he obtained a marked stimulation in the functions of each of these organisms. The maximum stimulation appears to have been obtained with concentrations of about 1 mgm. of manganese per liter. He therefore concludes that the small amount of manganese occurring in the soil serves a very useful function with respect to the organisms concerned in nitrogen transformation.

Robinson (11) has determined the manganese content of 26 different and representative soil types in this country. The maximum amount found in any soil was 0.51 per cent, the minimum 0.01 per cent, and the average 0.10 per cent of MnO, respectively. Various other reports concerning the amount of manganese contained in the soils of this and other countries will average approximately 0.10 per cent of this element, which is as much as the phosphorus and sulphur contained in soils that produce average yields of field crops.

In the foregoing review it has been the aim of the author to refer to those contributions on the subject which contain the more modern views concerning the probable function of manganese in its relation to agriculture.

The purpose of this investigation was, first, to show the effect of increased concentrations of manganese sulphate on the growth of plants in certain soils, and, secondly, to determine definitely if manganese is an essential element in the plant economy.

EXPERIMENTAL DATA

Citation (1) is a contribution whose data indicate that soluble salts of manganese are associated with and may in part be responsible for the toxicity of acid soils. To obtain further data on this subject, a series of experiments was planned in which plants were grown in acid and in neutralized portions of the same soil to which were added equal and graduated amounts of manganese sulphate. The soils selected for the experiments were not only acid but were lacking in capacity to produce good crops without the addition of certain plant nutrients.

EXPERIMENTS WITH VOLUSIA SILT LOAM SOIL

Volusia silt loam soil has a brownish-gray color, is low in organic matter, and contains considerable clay. It requires about 2,000 pounds of calcium carbonate per acre foot to neutralize its acidity.

About 500 pounds of this soil were obtained in a pasture field on Turkey Hill, Tompkins County, N. Y. The soil was well mixed and allowed to air-dry. Representative samples were taken for determination of the total and water-soluble manganese.

The total manganese content was determined by the potassium bisulphate fusion and the colorimetric periodate method. The amount of manganese found by this method was 0.08 per cent, or 800 parts per million of the air-dry soil. The amount of manganese dissolved by digestion with distilled water was 6.25 parts per million of the air-dry soil.

For pot experiments, 4,000 gm. portions of the air-dry soil were weighed into clean 1-gallon earthenware jars without drainage. Two series of 12 jars each were prepared, one with and the other without calcium carbonate, as shown in Table I, each treatment being made in duplicate. The mineral nutrients consisted of 10 gm. calcium nitrate, 10 gm. dipotassium phosphate, and 5 gm. magnesium sulphate per jar.

TABLE 1.—*Pot treatments in Series I and II*

Pot No.	Treatment, Series I.	Treatment, Series II.
1 and 1a...	No treatment.....	20 gm. $CaCO_3$.
2 and 2a...	Mineral nutrients only.....	Mineral nutrients and 20 gm. $CaCO_3$.
3 and 3a...	Mineral nutrients and 5 parts per million Mn.	Mineral nutrients, 20 gm. $CaCO_3$ and 5 parts per million Mn.
4 and 4a...	Mineral nutrients and 10 parts per million Mn.	Mineral nutrients, 20 gm. $CaCO_3$ and 10 parts per million Mn.
5 and 5a...	Mineral nutrients and 50 parts per million Mn.	Mineral nutrients, 20 gm. $CaCO_3$ and 50 parts per million Mn.
6 and 6a...	Mineral nutrients and 100 parts per million Mn.	Mineral nutrients, 20 gm. $CaCO_3$ and 100 parts per million Mn.

Purple-top radish seeds were sown in each pot and the moisture content of the soil in each pot was brought to one-half saturation, by weight, with distilled water. After the seedlings were up they were thinned to 10 plants in each pot and the moisture content kept at approximately one-half saturation with distilled water during the time the plants were making their growth.

Immediately after the crop of radishes was harvested soybeans were planted in each of the pots without further treatment of the soil. After the beans had reached the proper size they were thinned to 6 plants in each pot and allowed to grow until they were approaching maturity, the moisture content being meanwhile kept up to about one-half saturation with distilled water.

Plate 1, A, represents five pots from series No. 1. Pot No. 1 is one of the control pots and represents the productivity of the soil without treatment. Pot No. 2 shows the effect of the addition of appropriate amounts of phosphorus, potassium, calcium, magnesium, nitrogen and sulphur to the soil. This is one of the pots that received mineral nutrients

only and no manganese. Pot No. 3 received 5 parts per million of manganese in addition to the mineral nutrients. Pot No. 4 received 10 parts per million of manganese. Pot No. 5 received 50 parts per million of manganese. It is therefore readily apparent that 50 parts per million of manganese in the form of manganese sulphate are sufficient to produce toxicity in this soil. None of the seeds came up in the soil receiving this and greater concentrations of manganese sulphate. It is quite evident from Plate 1, A, that the maximum tolerance for manganese sulphate in this soil and with this particular plant lies between 10 and 50 parts per million.

Table II shows that the plants in the corresponding duplicate pots which are not shown made a similar growth.

There is no noticeable difference in the growth of the plants receiving the 5 and 10 parts per million of manganese, as compared with those that received no additional manganese. It is apparent that the small amount of manganese contained in the soil was sufficient for the requirements of the plants, under the conditions described.

The growth attained by the plants in the neutralized Volusia soil is shown in Plate 1, B, in which pot No. 1 is one of the control pots to which calcium carbonate was added. Comparison with the corresponding pot in Plate 1, A, shows that the plants made a slightly better growth in the neutralized soil than in the acid soil. Pot No. 2 received mineral nutrients and calcium carbonate and the plants did not make as good growth as did those in the corresponding pots with the acid soil. Pot No. 4 is one of two to which 10 parts per million of manganese was added. Neither of the two pots of soil that received 5 parts per million of manganese is shown in the photograph, but it will be seen in Table II that the plants that received 5 parts per million of manganese in series No. 2 made a slightly better growth than did the plants in the corresponding pots of soil in series No. 1. It will also be observed in Table II that the plants in the pots of soil that received 10 parts per million of manganese made a decidedly better growth in the neutralized soil than did the plants in the corresponding pots in the acid soil. Pot No. 5 shows the growth the plants attained in the neutralized soil to which 50 parts per million of manganese was added. It will be recalled that in the corresponding pots in the acid soil, the seeds did not germinate because of the toxic effect produced by an excess of manganese, while with the same concentration of manganese in the neutral soil the plants made a fair growth, though apparently retarded by an excess of this element. This experiment illustrates in a very striking way the effect of calcium carbonate in reducing the toxicity produced by an excess of manganese sulphate. In pot No. 6, to which was added 100 parts per million of manganese, no plants grew.

In Table II it will be observed that the applications of manganese to the acid soil reduced the yields, as shown by the green and dry weights of the plants, whereas with the same concentrations of manganese in the neutralized soil there was an appreciable gain.

TABLE II.—*Weights of radish and soybean plants grown in Volusia and Dunkirk soils, with and without nutrients, $MnSO_4$ and $CaCO_3$, duplicate pots*

Crop.	Soil.	Acid reaction of soil.				Neutral reaction of soil.				
		No treatment.	Minerals.	Minerals plus 5 parts per million Mn.	Minerals plus 10 parts per million Mn.	$CaCO_3$.	$CaCO_3$ plus minerals.	$CaCO_3$ plus minerals and 5 parts per million Mn.	$CaCO_3$ plus minerals and 10 parts per million Mn.	$CaCO_3$ plus minerals and 20 parts per million Mn.
		Gm.	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*	*Gm.*
Green weight of radish tops and roots.	Volusia	45.0	212.5	167.5	152.5	52.5	151.0	178.5	194.0	36.5
	Dunkirk	92.5	240.5	205.0	101.0	95.0	129.0	185.0	222.5	145.5
Dry weight of radish tops and roots.	Volusia	7.3	12.4	10.0	9.7	3.4	10.7	12.9	13.4	4.2
	Dunkirk	7.3	15.8	13.2	8.8	7.8	7.7	12.3	13.8	10.1
Green weight of soybeans.	Volusia	38.8	65.5	50.0	28.0	42.0	42.0	60.5	45.5	11.8
	Dunkirk	32.3	67.8	41.3	29.5	27.5	27.5	52.8	44.3	48.5
Dry weight of soybeans.	Volusia	4.4	12.5	9.6	6.0	9.3	9.9	12.5	10.1	2.6
	Dunkirk	6.9	11.9	8.2	3.6	6.2	7.1	9.5	8.8	10.0

EXPERIMENTS WITH DUNKIRK CLAY LOAM SOIL

Two series of experiments similar to those just described with the Volusia soil were conducted at the same time and under the same conditions with the Dunkirk clay loam soil. Apparently this soil is slightly more productive than the Volusia silt loam soil. It has an acid reaction and responds to applications of certain plant nutrients.

A manganese determination on the Dunkirk soil gave 0.093 per cent of this element. The amount of water-soluble manganese found was 9.30 parts per million of the soil.

The radish plants which were grown on the Dunkirk soil under the same conditions as those previously described on the Volusia soil were harvested on the same day and treated in like manner for the green and dry weights, which are given in Table II.

No photographs were made of the plants grown in the Dunkirk soil; however, it will be seen from the green and dry weights of the plants, shown in Table II, that results somewhat comparable to those obtained with the Volusia soil were obtained under similar treatments with this soil. In series No. 1 the maximum yields occurred with the applications of mineral nutrients. Addition of manganese gave a diminution in yields as compared with the pots receiving mineral nutrients alone.

In series No. 2, application of calcium carbonate to the soil of pots No. 1 and 2 affected the growth of the plants but little as compared with the corresponding pots in series No. 1, in which the plants grew in acid soil. It thus appears that the application of calcium carbonate under these conditions had no effect as far as the growth of the plants was concerned. There is an increase which is apparently due to the applications of manganese in this series, as compared with the pots receiving mineral nutrients alone. This is in accord with the results obtained with applications of manganese to the neutralized Volusia soil.

Equal quantities of the radish tops grown in duplicate pots were combined to make a composite sample for chemical analysis, the results of which are given in Table III.

TABLE III.—*Analyses of composite samples of radish tops grown in Volusia and Dunkirk soils, with and without the addition of nutrients, manganese sulphate, and calcium carbonate (results calculated on moisture-free basis)*

Element.	Soil.	Acid reaction of soil.					Neutral reaction of soil.				
		No treatment.	Minerals	Minerals plus 5 parts per million Mn.	Minerals plus 10 parts per million Mn.	$CaCO_3$	$CaCO_3$ plus minerals.	$CaCO_3$ plus minerals and 5 parts per million Mn.	$CaCO_3$ plus minerals and 10 parts per million Mn.	$CaCO_3$ plus minerals and 50 parts per million Mn.
		Per cent.	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
Ash	Volusia	16.87	27.60	22.51	24.45	22.06	24.96	25.49	26.00	21.72
	Dunkirk	20.15	24.83	24.18	26.70	17.55	19.06	25.08	25.56	25.45
Fe	Volusia	.077	.059	.050	.097	.047	.075	.096	.071	.08
	Dunkirk	.073	.073	.073	.073	.074	.084	.095	.041	.11
Mn	Volusia	.030	.035	.058	.94	.054	.026	.070	.087	1.15
	Dunkirk	.024	.024	.056	.67	.079	.021	.034	.059	.84
Ca	Volusia	3.89	3.59	3.12	3.25	3.01	4.39	4.44	4.54	2.81
	Dunkirk	3.19	3.32	3.23	3.95	2.72	2.85	2.91	2.96	2.98
Mg	Volusia	.36	.45	.47	.38	.43	.38	.40	.44	.38
	Dunkirk	.23	.40	.42	.43	.45	.42	.41	.42	.34
P	Volusia	.23	.25	.70	.72	.24	.70	.70	.75	.68
	Dunkirk	.27	.56	.55	.56	.25	.59	.56	.61	.62
K	Volusia	4.56	4.72	6.67	7.47	6.94	4.33	5.55	6.52	6.35
	Dunkirk	4.56	4.56	7.69	7.99	7.37	3.52	3.16	7.83	7.24
N	Volusia	6.04	6.79	6.58	6.71	6.15	6.38	6.52	6.45	7.81
	Dunkirk	5.56	6.77	7.22	6.81	3.19	3.58	5.27	5.04	5.56

An examination of the results shown in Table III reveals some points of interest. The plants which grew in the untreated soil contained less mineral matter than did those to which mineral nutrients were added. The addition of manganese to the soil in series No. 1 did not increase the mineral content of the plants receiving it, whereas it appears to have done so in the neutralized soil, except in pots 9 and 10, in which toxicity was produced. About as much mineral matter was taken up by the plants grown in the soil to which calcium carbonate was added as where it was omitted in the corresponding control pots of soil in series No. 1. A little more calcium was taken up by the plants under neutral conditions of the Volusia soil than under acid conditions; however, a little less calcium was found in the plants that grew in the neutralized Dunkirk soil than in those that grew in the acid soil. A larger percentage of potassium was found in the plants that grew in the acid Volusia and Dunkirk soils to which manganese was added than in those plants that grew in the soil to which no manganese was added. There was an increase in the percentage of nitrogen in the plants to which manganese was added in the neutral Dunkirk soil, while the percentage of nitrogen was nearly constant in the other experiments.

It appears, therefore, that in the soils dealt with in the foregoing series of experiments with radish plants, there has been a slight gain in each of the soils where manganese was added to the neutral soil in connection with mineral nutrients. Under acid conditions there has been a diminution in the yield of the plants receiving manganese when compared with the pots receiving mineral nutrients alone. All the soils have responded vigorously to an application of mineral nutrients when compared with no treatment.

An inspection of the green and dry weights of the soybean plants in Table II indicates that a more favorable growth was obtained in the neutralized soil plus manganese than in the acid soil to which manganese

was added. The protective action of calcium carbonate against the toxic properties of an excess of manganese sulphate is demonstrated with soybeans. The plants receiving 50 parts per million of manganese in the neutral soil made a moderate growth, while in the corresponding pots of acid soil no plants were produced.

After the dry weights were determined the leaves and stems of the soybean plants in duplicate pots were combined to make a composite sample for chemical analysis. The results of the analyses are given in Table VI.

TABLE IV.—*Analyses of composite samples of soybean plants grown after radish plants in Volusia and Dunkirk soils, with and without the addition of nutrients, $MnSO_4$ and $CaCO_3$ (results calculated on moisture-free basis)*

		Acid reaction of soil.				Neutral reaction of soil.				
Crop.	Soil.	No treatment.	Minerals.	Minerals plus 5 parts per million Mn.	Minerals plus 10 parts per million Mn.	$CaCO_3$.	$CaCO_3$ plus minerals	$CaCO_3$ plus minerals and 5 parts per million Mn.	$CaCO_3$ plus minerals and 10 parts per million Mn.	$CaCO_3$ plus minerals and 50 parts per million Mn.
		Per ct.	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*
Ash	Volusia	8.38	12.43	13.80	14.24	9.48	22.52	12.59	13.15	10.72
	Dunkirk	9.58	12.17	13.98	14.12	11.36	12.50	13.19	13.86	9.37
Fe	Volusia	.059	.078	.078	.069	.068	.059	.071	1.06	.114
	Dunkirk	.070	.070	.066	.051	.050	.032	.047	.052	.082
Mn	Volusia	.012	.012	.180	.373	.012	.014	.027	.042	.204
	Dunkirk	.013	.019	.161	.377	.009	.105	.346	.607	.150
Ca	Volusia	1.19	1.69	1.84	1.86	2.05	2.05	2.01	1.96	1.62
	Dunkirk	1.90	1.87	1.60	2.12	2.08	1.62	1.85	1.84	1.71
Mg	Volusia	.31	.29	.30	.28	.32	.32	.27	.28	.24
	Dunkirk	.33	.36	.31	.29	.31	.34	.28	.25	.36
P	Volusia	.13	.31	.35	.43	.18	.27	.24	.22	.36
	Dunkirk	.23	.45	.50	.55	.27	.48	.46	.40	.41
K	Volusia	1.43	3.40	3.62	3.92	1.40	3.12	3.05	3.05	3.03
	Dunkirk	1.34	2.80	3.39	2.20	3.51	3.71	3.51	3.34	3.21
N	Volusia	3.40	4.06	4.07	4.12	3.69	4.16	3.91	4.00	4.49
	Dunkirk	1.62	4.25	3.85	3.76	1.86	3.82	3.80	3.76	3.96

Table IV shows that the smallest percentage of ash was taken up by the plants grown in the untreated soils, while the largest percentage was found in the plants that had grown in the acid soil and to which 10 parts per million of manganese was added. Considerably more manganese than iron was taken up by the plants that grew in each of the acid soils to which manganese was added. Under neutral conditions more manganese than iron was absorbed by the plants that grew in the Dunkirk soil. The results for calcium vary to some extent between the two soils but apparently more calcium was taken up by the plants that grew in the neutralized soil. The plants that grew in the Dunkirk soil contained more phosphorus than those that grew in the Volusia soil. The amounts absorbed under acid and neutral conditions were nearly equal in the Dunkirk soil. Considerably more potassium was taken up by the plants receiving mineral nutrients and manganese than by either the untreated or the soils to which only calcium carbonate was added.

In the foregoing experiments with soils, the purpose was to determine the effect produced on the growth of plants in an acid and a neutral soil to which was added graduated amounts of manganese sulphate. It is readily apparent, therefore, that while such methods of experimentation are very important from an economic standpoint, they do not afford the desired proof to determine whether or not manganese is essential in the plant's economy.

The preparation of a medium that will, when the test is applied, show no manganese, requires certain procedures and precautions which, in so far as the writer has been able to discover, have not previously been taken into consideration in experiments planned to show the effect the absence of this element may have on the growth of plants. Tests for manganese in many different samples of chemical reagents which are used for plant nutrients show that manganese-free chemicals are rarely if ever found. So intimate is the association of manganese with iron that a manganese-free iron compound could not be purchased from dealers in chemicals and it was necessary to prepare it in the laboratory. Therefore in order to eliminate all possible sources of contamination with manganese it is necessary to test and purify all the reagents that enter the nutrient medium. Even though manganese has been eliminated from the nutrient medium it is necessary to grow plants until they approach maturity before any definite conclusions can be reached with respect to the effect of this element on their growth. The seeds of those plants with which the writer has carried on experiments apparently contained enough manganese to maintain a normal growth for the first six or eight weeks.

After having procured reagents that were proved by chemical tests to be free from manganese, a Knop's nutrient solution was made for water culture experiments.

Alaska garden pea seeds were germinated on moist cheesecloth in a porcelain-lined pan. When the seedlings were of the proper size they were transferred and held in place by means of plugs of cotton in holes made in squares cut from boards which were made to fit over the mouths of well-glazed one-half gallon earthenware jars. An attempt was made to waterproof the boards by keeping them submerged in paraffin kept near the boiling point for several hours. Although the squares of wood afford excellent means of support for the plants, they proved to be unsatisfactory in these and in previous experiments from the fact that the lower surface of the board, which is close to the top of the nutrient solution, affords favorable conditions for the growth of molds which, unless daily attention is given to washing them away, very soon attack the roots of the plants.

More recently supports have been made of well-glazed earthenware and no trouble from molds has been experienced.

The earthenware jars were filled nearly to the mouth with the Knop's solution and when the covers containing the seedlings were on, the roots were submerged in the solution. The pots containing the plants were kept at a suitable temperature for the growth of the peas in the greenhouse and the nutrient solutions were changed twice each week. The water lost by transpiration in the meantime was replaced with distilled water, thus keeping the concentration approximately constant.

Previous to starting the experiment new pots were obtained and tested for soluble manganese in the following manner: Three of the new pots were taken at random and filled to near the mouth with a mixture of equal parts of 1 : 1 nitric and hydrochloric acids and placed over holes on a water bath and kept at the temperature of boiling water for 48 hours. The mouths of the pots were covered with watchglasses during the digestion. The solutions were then transferred to porcelain dishes and brought to dryness. The residues were tested for the presence of manganese, and only a slight trace was found from any of the pots. The writer considers this a negligible source of manganese as it was thought

that even the trace found in the acid extraction would not be available to the plants growing in a Knop's solution.

Twelve Alaska pea seedlings were placed in each of four jars containing a Knop's solution. At the beginning of the experiment 1 part of manganese in the form of the sulphate to 1,000,000 parts of the solution was added to each of two of the pots. After the plants had been growing three or four weeks the concentration of manganese was increased to five parts per million of the Knop's solution. One cubic centimeter of a manganese-free ferric chlorid solution which contained 0.001 gm. iron was added to each of the pots at the time the solutions were changed.

During the first four or six weeks of growth there was little or no difference in the size or appearance of the plants. Therefore the plants which received no manganese except that contained in the seed began to show effects which can not be accounted for in any other way except for a lack of this element. The first effect to be observed was that the young buds as they unfolded were yellowish instead of a normal green color and later became flecked with small specks of brown, which was not observed at any time on the plants receiving manganese. The etiolated condition became more pronounced as the time progressed and finally resulted in the top branches dying back. The green and dry weights of the 12 plants that grew in each of the 4 pots were determined.

The average dry weights of the pea plants show an increase of 49.47 per cent in the total dry matter produced in the plants which were grown in the presence of manganese when compared with those plants that grew in a Knop's solution to which no manganese was added. There was also an increase of 24.63 per cent in the mineral nutrients assimilated, exclusive of nitrogen, in the plants that grew in the Knop's solution containing manganese. The plants which did not get any manganese contained a little more nitrogen than those to which manganese was added. The plants from which manganese was withheld contained only traces of this element, which was no more than could have been derived from the seeds from which the plants grew. It therefore appears that the trace of manganese derived from the seeds of the plants was not sufficient to maintain a normal metabolic process during the latter part of the time the plants were growing.

The duplicate sets of plants were combined to make a composite sample for chemical analysis and the results are given in Table V.

TABLE V.—*Analyses of pea, soybean, corn, and cowpea plants grown in media free from, and containing manganese (results expressed of the moisture-free material)*

	Ash (crude).	Iron (Fe).	Manganese (Mn).	Calcium (Ca).	Magnesium (Mg).	Potassium (K).	Phosphorus (P).	Nitrogen (N).
	Per ct.	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*	*Per ct.*
Alaska pea plants:								
Manganese	12.85	0.041	0.179	1.85	0.50	2.05	0.58	2.97
No manganese	15.41	.040	Trace.	2.41	.98	2.71	.68	4.71
Soybean leaves:								
Manganese	15.00	.051	.032	.80	.56	1.58	.62	3.42
No manganese	15.99	.094	Trace.	.87	.62	1.11	.48	3.85
Soybean stalks:								
Manganese	13.07	.036	.010	.57	.37	1.08	.45	1.69
No manganese	10.67	.033	None.	.65	.39	1.35	.46	2.21
Cowpea plants:								
Manganese	17.24	.046	.036	1.70	.54	5.56	.72	2.57
No manganese	20.14	.065	.003	1.32	.61	7.88	.51	4.51
Corn leaves:								
Manganese	8.71	.029	.040	.23	.31	3.80	.40	.22
No manganese	10.28	.034	.001	.24	.36	4.07	.35	1.47
Corn stalks:								
Manganese	6.37	.014	.023	.08	.13	3.02	.42	.77
No manganese	8.24	.011	Trace.	.10	.13	3.52	.37	.95

SAND CULTURE EXPERIMENTS

A quantity of quartz sand of medium sized grains was obtained for sand cultures. The sand, from external appearance, was good quality glass sand. Upon digesting 1,000 gm. in a mixture of 1:1 hydrochloric and nitric acids on a hot water bath for several hours, filtering out and washing the residue free of acids, it was found upon testing portions of the filtrate that the sand had contained small amounts of iron, manganese, zinc, arsenic, calcium, and magnesium. The residue of sand was snow white, except for a few black particles, which resisted further treatments of strong acids to dissolve them. A small amount of the black particles were separated, ground in an agate mortar, and fused with potassium bisulphate. The fused mass was extracted with dilute sulphuric acid and tested for manganese, but none was found.

Several hundred pounds of the sand were then washed with a mixture of nitric and hydrochloric acids and afterwards with distilled water until free of chlorids, as shown by a test with silver nitrate. The sand was then transferred to large, shallow, porcelain-lined agateware pans and dried. After the sand was dry 4,000 gm. portions were weighed into clean 1-gallon earthenware pots.

The sand prepared in this way possessed rather strong absorptive powers when wet with a Knop's nutrient solution. It, therefore, was necessary to saturate the absorptive capacity of the sand with respect to the plant nutrients before plants could be grown. This was accomplished by adding the following amounts of manganese-free mineral nutrients to each 1 gallon of dry sand (4,000 gm.): 25 gm. of $CaCO_3$, 10 gm. of K_2HPO_4, 10 gm. of $Ca(NO_3)_2$, 5 gm. of $MgSO_4$, 3 gm. of KCl and 3 gm. of Fe in the form of a suspension of $Fe(OH)_3$. These chemicals were all free from manganese and pots of sand receiving this treatment served as the controls. Equal numbers of other pots of the sand received 2 gm. each of manganese in the form of $MnCO_3$ and the above-mentioned nutrients. The mineral nutrients and the sand were thoroughly mixed by hand in a large porcelain-lined pan and returned to the respective pots. Equal numbers of plants of soybeans, cowpeas, and sweetcorn were grown in the purified sand, with and without manganese, until they approached maturity. They were then harvested and the dry weights determined and chemical analyses made of the plant material. The results are given in Tables V and VI.

TABLE VI.—*Dry weights of the plants*

Species of plant.	Manganese added.	No manganese added.	Increase due to manganese.
	Gm.	*Gm.*	*Per cent.*
Alaska peas (grown in water cultures)	28.10	18.80	49.5
Soybeans	19.80	12.25	61.6
Cowpeas	26.50	11.30	134.5
Corn	72.00	59.00	22.0

All the plants which grew in the pot cultures containing manganese made a very much better growth than did those from which manganese was withheld. The most striking result was obtained with cowpeas, in

which the increase in dry weight of the plants receiving manganese was 134.5 per cent. All the leguminous plants from which manganese was withheld made a normal growth for the first six or eight weeks; thereafter the young buds and leaves as they unfolded were etiolated and brown specks developed on the etiolated leaves later. After this condition had developed but very little growth was made, and the young and tender parts of the plants died back, while the plants to which manganese was added made a normal growth and no chlorosis developed during their growth. With corn, the only apparent effect produced on the growth of the plants was the failure in the production of dry organic matter. No chlorosis developed on the leaves of the corn, which was harvested after the plants had produced tassels. From these results it appears that leguminous plants are more sensitive towards the lack of manganese than are the nonlegumes.

In the cowpea and corn plants to which no manganese was added, enough of this element was present for a determination. In the other plants to which no manganese was added only a trace of manganese could be detected.

The stalk of the soybean plants which grew in the absence of manganese was the only material in which no manganese could be detected. The plants which grew in the pot cultures to which manganese was added contained a normal amount of this element.

More recent experiments with manganese-free sand cultures at the Kentucky Agricultural Experiment Station have shown the necessity of manganese in both nonleguminous and leguminous plants.

Plate 2, A, shows in a very striking way the beneficial influence of manganese on oats. The plants on the left had no manganese added to the sand in which they grew, while those on the right grew in the presence of manganese.

Plate 2, B, shows Canada field peas. The plants on the left received no manganese in the sand in which they grew, while to those on the right manganese was added, all other conditions being the same.

Sand cultures were carried out with the following species of plants: Wheat, oats, peas, cowpeas, beans, lettuce, tomatoes, onions, spinach, cabbage, carrots, radish, and clover, and similar effects were observed as those described and shown in Plate 2, A, B.

SUMMARY

(1) In the experiments with acid soil which contained approximately one-tenth of 1 per cent of manganese it was found that only small amounts of the total manganese were soluble in water. Applications of more manganese, in the form of the sulphate, to the acid soil caused a decrease in the yields of the crops, whereas like quantities of this compound, when applied to different portions of the same soil, after addition of calcium carbonate, caused an increase in the yields of other plants of the same species.

(2) The occurrence of soluble salts of manganese in an acid soil may be one of the causes of toxicity in such soils as exhibit toxic effects. An excess of manganese sulphate in a soil renders it sterile with respect to the growth of plants. Calcium carbonate in the soil causes a diminution in the toxic effects produced by an excess of manganese sulphate.

(3) In order to demonstrate whether or not manganese is essential for the normal growth of plants, it is necessary that great care should

be taken in the preparation of a manganese-free medium in which to grow plants.

(4) Manganese is intimately associated with compounds of iron, phosphorus, and calcium, and since very small amounts of this element are required for the growth of plants it is quite probable that the contamination of plant nutrients with manganese has hitherto been an unrecognized source of error in determining the necessity of this element in plant economy.

(5) Apparently leguminous plants are more sensitive to the lack of manganese than are the nonlegumes; however, further data obtained by growing nonleguminous plants for more than one generation in a manganese-free medium may be necessary to prove this point.

(6) A very small amount of manganese is required for the normal growth of plants.

(7) Seeds of the plants tested (radish, soybean, cowpea, field pea, and corn) do not contain enough manganese for the growth of the plant to maturity.

(8) The seeds of some plants contain enough manganese to maintain a normal development for the first four or six weeks of their growth; therefore experiments conducted for a shorter time in a manganese-free medium are not likely to give any indications as to the necessity of this element in the growth of plants.

(9) The lack of manganese affects the production of the dry matter in plants, thus indicating that it has some very important function in carbon assimilation.

(10) The etiolated condition of the young and tender leaves and buds obtained in well-controlled experiments indicates that manganese has a function in the photosynthetic process and the formation of chlorophyll.

(11) Manganese apparently is essential for the normal growth and development of plants.

(12) Further investigations on this subject are under way in which an attempt will be made to show the relation of manganese to other important phases of plant and animal life.

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Colorimetric Determination of Manganese with Periodate

A Spectrophotometric Study

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A NUMBER of early investigators (*2, 6, 10, 11*) studied the reaction between manganous and periodate ions with more or less conflicting results. But Willard and Greathouse (*16*) were the first to use it as the basis of a colorimetric method for the quantitative determination of manganese. Their method depends upon the oxidation in acid solution of manganous salts to permanganate by potassium periodate and they state that it is free from all the faults of the other methods for manganese and yields results of a high degree of accuracy. More recently it has been successfully applied to water (*1*), to animal and vegetable tissues (*4, 12, 13*), and to salt solutions (*9*).

The purpose of the work described in this paper was to make a study of this method by means of the photoelectric recording spectrophotometer (*9*), with particular attention to the effect of other ions upon the color system. Similar studies of other colorimetric methods have recently been made (*5, 7, 14, 16*). The recording spectrophotometer is an ideal instrument for making such an investigation, because if two solutions give identical spectral transmission curves they will have the same color under any condition of illumination and to any observer. By such curves very small differences in color intensity and in hue can be detected.

Apparatus and Solutions

All spectrophotometric measurements in the present work were made with the instrument built for the Department of Chemistry of Purdue University by the General Electric Co. (*8*).

A stock solution of potassium permanganate containing approximately 10 mg. of manganese per ml. was made by dissolving the proper amount of the salt in redistilled water and making the volume up to 1 liter. After 4 days this solution was filtered through asbestos and standardized against ferrous ammonium sulfate of known iron content. A 100-ml. volume of this solution was accurately measured into a beaker, 25 ml. of concentrated sulfuric acid and 6 grams of potassium metaperiodate were added, and the solution was boiled for 2 minutes. After 15 minutes on the hot plate, the solution was cooled to room temperature, accurately diluted to 1 liter in a volumetric flask, thoroughly mixed, and transferred to a brown, glass-stoppered bottle. Each milliliter contained 0.09865 mg. of manganese. Because of the presence of the periodate this solution was stable and could be used as a standard throughout the work.

Standard solutions of the metals were prepared from the nitrate or sulfate salts, while the sodium, potassium, or ammonium salts were used for the preparation of standard solutions of the anions. Redistilled water was used throughout. Each milliliter contained 10 mg. of the ion in question. For the stannous, stannic, and antimonous solutions it was necessary to evaporate a known volume of the chloride solution with concentrated sulfuric acid to remove all chloride and then make up to the original volume with dilute sulfuric acid. The resulting solutions gave negative chloride tests.

For producing the color system the procedure of Willard and Greathouse (*15*) was followed. To 5 ml. of the standard manganese solution, representing 0.4933 mg. of manganese, in a tall-form 250-ml. beaker about 25 ml. of water were added followed by 10 ml. of concentrated sulfuric acid, and the solution was diluted to 100 ml. After the addition and solution of 0.5 gram of potassium metaperiodate, the solution was carefully boiled for 1 minute, kept warm for 10 minutes, transferred to a 250-ml. volumetric flask, cooled to room temperature, made up to the mark, and thoroughly mixed.

The spectral transmission curves were determined for a solution thickness of 4.983 cm. The absorption of the glass cell was compensated for by placing in the rear beam of light a similar cell filled with redistilled water.

The transmittancy curves for varying amounts of manganese are shown in Figure 1. The peak of the absorption band is located at 522 mμ.

Conformity to Beer's Law

That the color system follows Beer's law, at least up to a concentration of 20 mg. of manganese per liter, is apparent from Table I, in which are shown the observed transmittancies at 522 mμ, the wave length of maximum absorption, for six

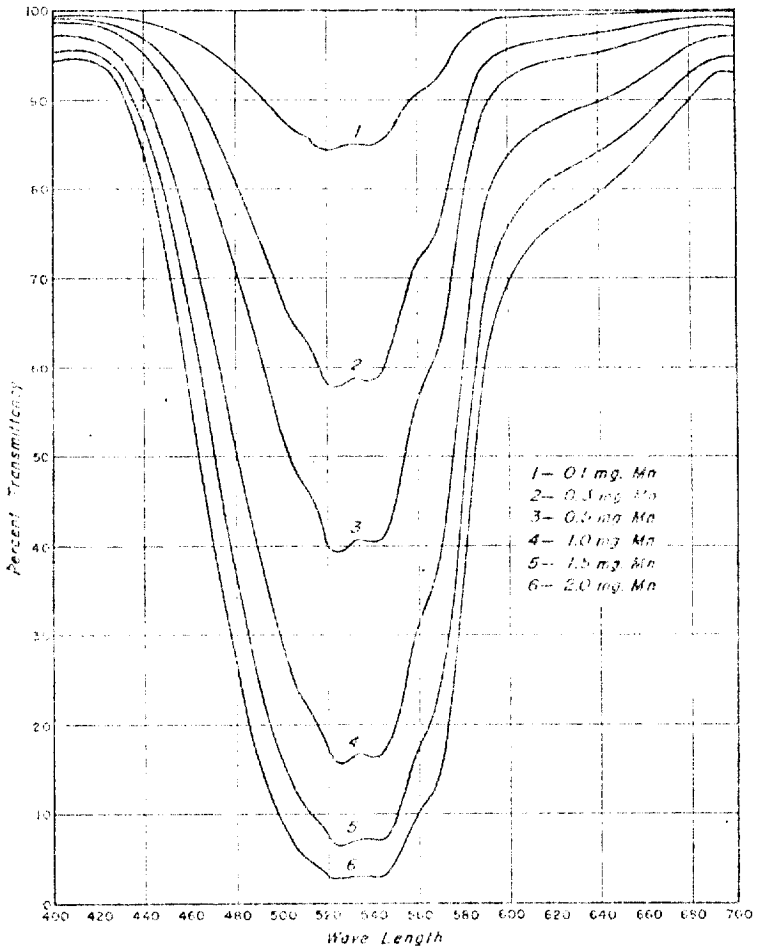

FIGURE 1. TRANSMITTANCY CURVES FOR MANGANESE

manganese solutions of different concentrations, together with the transmittancies, calculated by means of a special color slide rule from the transmittancy of the solution containing 5 mg. of manganese per liter. The calculations were made by use of the formula which expresses Beer's law

$$T_2 = T_1^{\frac{c_2}{c_1}}$$

where T_1 represents the observed transmittancy, expressed as a decimal, for the solution of concentration c_1, and T_2 the calculated transmittancy for the solution of concentration c_2. The observed and calculated values check satisfactorily.

TABLE I. VALIDITY OF BEER'S LAW

(1.961-cm. cell)

Manganese	Transmittancy at 522 mμ		Manganese	Transmittancy at 55[illegible] mμ	
	Observed	Calculated		Observed	Calculated
Mg./L.	%	%	*Mg./L.*	%	%
1	84.7	84.5	10	18.4	18.4
3	65.2	60.2	15	7.7	7.9
5	43.0	..	20	3.7	3.4

Additional evidence that Beer's law is followed is given by the fact that a straight line results when the logarithm of the observed transmittancy is plotted against the concentration. Concentrations above 20 mg. per liter give solutions too deeply colored for visual determination.

Effect of Reagents

Willard and Greathouse (*16*) note that the success of the reaction depends upon the presence of a sufficient concentration of acid to prevent the precipitation of manganic periodate or oxide and that the minimum concentration of acid required increases with the concentration of manganese. A number of metals, such as silver, lead, bismuth, and mercury, form iodates or periodates which are insoluble in dilute acids, but soluble in high concentration of acids. Because of the high concentration of acid needed, the pH values of the various solutions were so low—less than 1.0—that no attempt was made to determine them.

In studying the possible effect on the color of the amount and kind of acid present, the curve produced by the solution containing 5 ml. of the standard manganese solution (representing 0.4933 mg. of manganese), 10 ml. of concentrated sulfuric acid, and 0.3 gram of potassium periodate per 250 ml. was compared with the curves produced by a series of similar manganese solutions containing in place of the 10 ml. of sulfuric acid 15, 20, 25, and 30 ml. of concentrated sulfuric acid, 20, 30, and 40 ml. of concentrated nitric acid which had been decolorized by drawing air through it by suction, and 5, 10, 15, 30, and 50 ml. of concentrated phosphoric acid, respectively. From the transmittancies at 522 mμ of the standard solution and each of the other solutions and from the known manganese concentration of the standard solution, by use of the formula employed above in the Beer's law calculations, the apparent concentration of manganese in each of the other solutions was calculated by the aid of the special color slide rule. The difference between this value and the actual concentration multiplied by 100 and divided by the actual concentration gave the percentage error.

In accordance with the procedure of a former study (*7*) a 2 per cent error was arbitrarily set as the maximum allowable for negligible interference. Although visual methods of color comparison often have a precision of not less than 5 per cent, it was thought advisable to set a lower figure to provide for other possible factors. In all cases the curves were practically identical and the error was negligible except for the solutions containing 30 and 40 ml. of nitric acid, where it amounted to −4.0 and −5.0 per cent, respectively. Possibly these large amounts of nitric acid produced enough nitrogen oxides during the boiling and digestion to influence the color.

Varying the amount of potassium periodate added has no appreciable effect upon the color. Curves for a series of solutions containing in 250 ml. 0.4933 mg. of manganese and 10 ml. of concentrated sulfuric acid with 0.2, 0.4, 0.5, and 0.6 gram of potassium periodate, respectively, showed a negligible error when compared to the standard curve made from the solution containing 0.3 gram of potassium periodate. Therefore it is possible to add an excess of periodate when reducing agents are present and thus prevent reduction of the permanganate and consequent fading of the color of the solution.

Stability of the Color

Because of the presence of periodate in the solution there is practically no tendency of the color to fade on standing. Willard and Greathouse (*15*) found it stable for 3 months when measured by a visual colorimeter and Cha[illegible] (*4*) reported it stable for 7 months.

Curves made at intervals for six solutions containing, respectively, 0.25, 0.75, 1.25, 2.5, 3.75, and 5.0 mg. of manganese with 10 ml. of concentrated sulfuric acid and 0.3 gram of potassium periodate per 250 ml., after the solutions had stood in glass-stoppered Pyrex bottles in diffuse light, gave no evidence whatever of fading or other change in the color over a period of 2 months. Lack of time prevented tests over any longer period. Such marked stability makes possible the use of a series of permanent standards.

Effect of Anions

In the ion interference studies the curve produced by the standard manganese solution, containing 0.4933 mg. of manganese, 10 ml. of concentrated sulfuric acid, and 0.3 gram of periodate per 250 ml., was compared with the curve produced by a similar manganese solution containing a known amount of the added ion. Calculations of the percentage error were made as explained above.

A considerable number of the common anions, cyanide, thiocyanate, iodide, bromide, chloride, oxalate, tartrate, citrate, sulfite, nitrite, and arsenite, have the ability to reduce the permanganate and thus decrease the color intensity. However, by using larger amounts of periodate this reducing action may be prevented for moderate amounts of the ions and the permanganate may be kept in the oxidized state with the color unchanged. In the usual procedure for determining manganese these reducing ions will be removed during the boiling with nitric acid and the subsequent evaporation with sulfuric acid. In general it is preferable to remove them.

In Figure 2, curve 4 shows the effect of 10 mg. of oxalate per 250 ml., curve 5 the effect of 3 mg. of nitrite per 250 ml., and curve 6 the effect of 50 mg. of sulfite per 250 ml. In each case 0.3 gram of periodate was used. Certain colored ions, as dichromate, cause a change in hue. In Figure 2, curves 2 and 3 show the respective effects of 10 and 2.5 mg. of dichromate per 250 ml. Thiosulfate in addition to its reducing action also causes the precipitation of sulfur in the acid solution. Tungstate causes turbidity because of the precipitation of tungstic acid in the acid solution.

The effects of the common anions and their approximate limiting concentrations are listed in Table II.

Effect of Cations

In the tests involving barium, calcium, lead, and strontium ions it was necessary to substitute concentrated phosphoric acid for the sulfuric acid, and a standard permanganate solution to which no sulfuric acid had been added was used. In each case except for lead, where 10 ml. of phosphoric acid were necessary to prevent precipitation, 5 ml. sufficed.

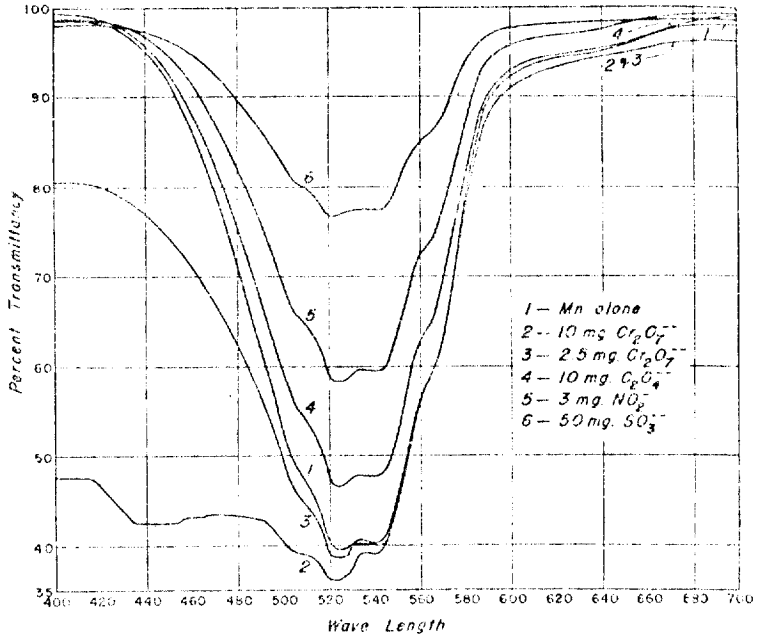

FIGURE 2. EFFECT OF ANIONS

0.4933 mg. of manganese, diverse ion, 10 ml. of concentrated sulfuric acid, and 0.3 gram of potassium iodate in 250 ml. of solution. 1.983-cm. cell

TABLE II. EFFECT OF COMMON ANIONS ON THE COLOR DEVELOPED BY MANGANESE

(0.4933 mg. of manganese in 250 ml. of solution)

Ion	Concentration Mg./250 ml.	Apparent Change in Manganese Concentration %	Approximate Limiting Concentration Mg./250 ml.
Acetate	60	Negligible	..
Arsenate	100 (As)	Negligible	..
Arsenite	50 (As)	Change in hue	..
	20 (As)	+ 63.7	
	10 (As)	+ 36.1	0.0
Borate	150 (B_2O_3)	Negligible	..
Bromide	50	−7.0	10
	50 (0.6 gram KIO_3)	Negligible	50
Carbonate	200	Negligible	..
Chlorate	100	Negligible	..
Chloride	100	−35.0	..
	50	− 3.0	30
Citrate	50	Decolorized	..
	50 (0.6 gram KIO_3)	Decolorized	..
	10	− 2.6	7
Cyanide	100	+ 10.0	
	50	− 3.5	25
Dichromate	10	Change in hue	
	2.5	Change in hue	0.0
Fluoride	50	Negligible	
Iodide	50 (0.6 gram KIO_3)	Negligible	50
	10	− 3.0	3
Molybdate	10 (Mo)	− 3.4	5
Nitrate	200	Negligible	..
Nitrite	50	Decolorized	..
	50 (0.6 gram KIO_3)	− 5.9	..
	10	Decolorized	..
	10 (0.6 gram KIO_3)	− 4.4	
	3	−43.6	0.0
Orthophosphate	200 (P_2O_5)	Negligible	..
Oxalate	50	Decolorized	..
	50 (0.6 gram KIO_3)	Decolorized	..
	10	−18.0	0.0
	10 (0.6 gram KIO_3)	Negligible	10
Perchlorate	100	Negligible	..
Pyrophosphate	200 (P_2O_5)	Negligible	..
Silicate	100 (SiO_2)	Negligible	..
Sulfate	300	Negligible	..
Sulfite	50	+71.6	..
	10	+12.5	0.0
	10 (0.6 gram KIO_3)	− 3.3	3
Tartrate	50	Decolorized	
	50 (0.6 gram KIO_3)	− 3.1	25
	10	− 2.0	10
Thiocyanate	100	Change in hue	
	100 (0.6 gram KIO_3)	− 6.2	10
	70	− 5.0	25
Thiosulfate	5	Turbidity	0.0
Tungstate	50	Precipitates	..
	10	Precipitates	0.0

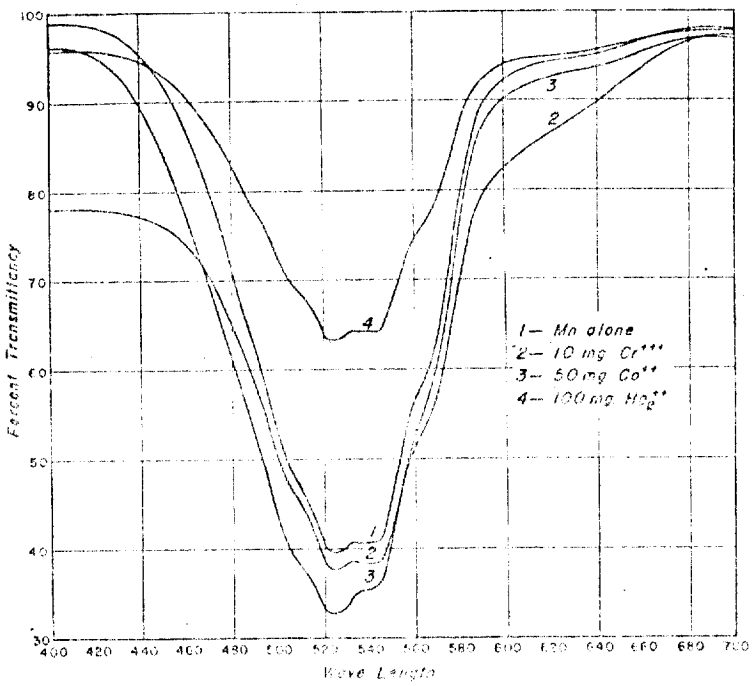

FIGURE 3. EFFECT OF CATIONS

0.4933 mg. of manganese, diverse ion, 10 ml. of concentrated sulfuric acid, and 0.3 gram of potassium periodate in 250 ml. of solution. 4.983-cm. cell

The most common interference caused by cations is due to those which impart a color of their own to the solution and thereby cause a change in hue. Examples are cupric, nickelous, cobaltous, chromic, uranyl, and ferric ions (Figure 3, curves 2 and 3). The color caused by ferric ion may be removed by the addition of sufficient phosphoric acid to form the colorless complex ion. The curve then coincides with the standard curve. Willard and Greathouse (*15*) suggest correcting for the color imparted by these colored cations by adding the same amounts to the color standard as are present in the sample. Silver, lead, and mercuric ions form iodates or periodates which are insoluble in dilute acids, but dissolve in high concentrations of acids. Bismuth and stannous ions give turbidity even in strongly acid solution and should be removed. Although antimonous ion readily reduces permanganate, the amount of the former left in solution after evaporation with sulfuric acid is so small that the periodate will easily oxidize it and prevent any reduction of the permanganate. Because of its reducing action mercurous ion causes a decrease in the color intensity (Figure 3, curve 4). Ferrous ion reduces the periodate to free iodine which colors the solution and causes a change in hue. It should, therefore, be oxidized by boiling with nitric acid.

In Table III are listed the effects of the common cations and their approximate limiting concentrations.

TABLE III. EFFECT OF COMMON CATIONS ON THE COLOR DEVELOPED BY MANGANESE

(0.4933 mg. of manganese in 250 ml. of solution)

Ion	Concentration *Mg./250 ml.*	Apparent Change in Manganese Concentration %	Approximate Limiting Concentration *Mg./250 ml.*
Aluminum	100	Negligible	..
Ammonium	100	Negligible	..
Antimonous	100	Negligible	..
Barium	100	Negligible	..
Beryllium	50	Negligible	..
Bismuth	50 (20 ml. H_2SO_4)	Turbidity	0.0
Cadmium	100	Negligible	..
Calcium	100	Negligible	..
Chromic	50	Change in hue	..
	10	Change in hue	0.0
Cobaltous	50	+18.0	..
	10	+ 3.0	5
Cupric	50	Change in hue	..
	10	Change in hue	5
Ferric	100 (10 ml. H_3PO_4)	Negligible	..
Ferrous	5	Change in hue	0.0
Lead	100 (10 ml. H_3PO_4)	Negligible	..
Lithium	100	Negligible	..
Magnesium	100	Negligible	..
Mercuric	100 (20 ml. H_2SO_4)	Negligible	..
Mercurous	100	−50.7	..
	50	− 1.1	50
Nickelous	50	Change in hue	..
	10	Negligible	10
Potassium	125	Negligible	..
Silver	100 (20 ml. H_2SO_4)	Negligible	..
Sodium	125	Negligible	..
Stannic	50	Negligible	..
Stannous	50 (20 ml. H_2SO_4)	Turbidity	0.0
Strontium	100	Negligible	..
Thorium	100	Negligible	..
Uranyl	50 (V)	− 2.6	50
Zinc	100	Negligible	..
Zirconium	100	Negligible	..

Summary

A spectrophotometric study shows that the periodate method for the determination of manganese colorimetrically is a most satisfactory one with very few limitations.

Sulfuric, nitric, or phosphoric acid may be present in widely varying amounts. A large excess of periodate may be used without interfering with the color.

The color system follows Beer's law. The color is stable in diffuse light for at least 2 months.

A study of the effect of fifty-six of the common ions was made. A few seriously interfere with the color, but in the course of the determination most of these would be removed.

Acknowledgments

The writer wishes to express his sincere appreciation to M. G. Mellon of Purdue University, in whose laboratory this investigation was conducted, and to thank him for the privilege of using the Purdue spectrophotometer. Thanks are also given to J. T. Woods and D. H. Byers for their aid in adjusting the spectrophotometer.

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Trace-Element Sensitivity: Comparison of Activation Analysis with Other Methods

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ACTIVATION analysis is a method for determining the constituents of a sample by utilizing certain nuclear properties of the isotopes of the elements in the sample. Nuclear particles are used to produce radioactive isotopes by activation of the [illegible] of the sample [illegible]. These radioisotopes can be detected and measured by their nuclear radiations. Thus with an exact knowledge of nuclear characteristics, a determination of the amount of element present can be made. This method is applicable to determination of trace elements in many types of materials used by the biologist, chemist, metallurgist, physicist, and others. Solid, as well as liquid, samples can be used for the analysis, a few milligrams or less of material is sufficient for precise work. When chemical separations are required in activation analysis to remove interfering ions, no errors are introduced by trace contaminants in the reagents.

Analysis by radioactivation was first suggested by Hevesy and Levi (*1*) in 1936 when they used neutrons from a 300-mc radium-emanation beryllium source to activate certain rare earth elements. It was not until the advent of the nuclear reactor, however, that sources of sufficiently high neutron flux became available to give high sensitivity to this method.

In the past decade activation analysis has been viewed by many as representing the ultimate in sensitivity. The completion of new reactors with increasingly high fluxes brought a steady improvement in this sensitivity. Most analysts, however, had to forego the reportedly high sensitivities and had to use the more conventional methods of analysis because there were few reactor facilities available and access to them was limited.

In 1952 Oak Ridge National Laboratory made available to the public an activation analysis service (*2, 3*) that has since enjoyed a favorable response. At the present time several non-AEC laboratories are building research-type reactors (*4*) and the prospects for many more reactor facilities being built in the near future are good.

This increase in access and availability of sources of high neutron flux will proportionately increase the ease of obtaining analysis by activation. Not only will it be possible to send samples out to any of several laboratories for this type of analysis, but in areas near a reactor an investigator should be able to make his own analysis at the reactor facility.

Thus some critical evaluation of activation analysis as a method for determining trace quantities of elements is necessary to ascertain its advantages and limitations relative to other existing methods of trace analysis. Limits of sensitivity for various methods can be found in the literature. Although these limits vary with investigators, types of equipment, experimental procedure, and so forth, rough comparisons of these limits for each element can provide invaluable insight into the value of activation analysis relative to other methods of trace analysis.

Activation analysis. A number of general papers (*5-15*) on activation analysis should be consulted for background information on the method. The general equation governing analysis by activation is

$$\text{Grams of } X = \frac{\text{Act.} \times \text{at.wt.}}{6.02 \times 10^{23} f(\sigma_{at}) \left(1 - e^{-\frac{0.693t}{t_{1/2}}}\right)}$$

where Act. is the activity (disintegrations/min); at.wt. is the atomic weight of isotope activated; f is the flux of neutrons (neutrons/cm² sec); (σ_{at}) is the atomic cross section of element—that is the isotopic cross section × percentage abundance of isotope (the cross section is a measure of the probability of activation for a particular isotope; a compilation of isotopic cross sections can be found in the literature); and $\left(1 - e^{-\frac{0.693t}{t_{1/2}}}\right)$ is the saturation factor which depends upon the ratio between length of bombardment and the length of half life of the isotope formed; t is the time of bombardment; and $t_{1/2}$ is the half life of isotope produced.

From this equation it can be seen that the activity for a given number of grams of material is directly proportional to the neutron flux. Furthermore, the number of grams of a material that gives a specified activity is inversely proportional to the flux. Thus the higher the neutron flux, the higher the sensitivity for detection of a given isotope.

Table 1 is a list of the average thermal neutron flux available from different sources of neutrons. A corollary to the fact that higher neutron fluxes give higher

Table 1. Neutron sources and usable flux (in n/cm² sec).

Ra-Be, 25 mg		$10^{8} - 10^{9}$
Sb-Be, 1 c		$10^{9} - 10^{10}$
Van de Graaf		$10^{11} - 5 \times 10^{12}$
Cyclotron		$10^{12} - 10^{13}$
Reactor		
Oak Ridge	X-10	5×10^{13}
Oak Ridge	LITR	1×10^{13}
Brookhaven	BNL	2×10^{13}
Chalk River	NRX	2×10^{13}
Idaho	MTR	2×10^{14}

sensitivities is the fact that lower level neutron sources can be used whenever lower sensitivities can be tolerated in an analysis. Hence even the portable low-level radium-beryllium and antimony-beryllium sources can be used for analysis on occasion (*19, 16*).

Order of magnitude sensitivities of various elements for activation analysis in the 5×10^{13} neutrons/cm² sec flux of the Oak Ridge X-10 reactor have been published (*7*). The sensitivities are shown in Figs. 1–4 (*7*). One additional chart could be made showing only europium and dysprosium with a sensitivity of 10^{-2} μg or less for the X-10 reactor. Values for higher flux reactors are indicated in the chart.

Comparison. Table 2 is a comparison of the sensitivities for the elements reported for activation analysis by Leddicotte and Reynolds (*9*) with those for five other typical trace analysis methods for solutions and soluble salts. These methods do not necessarily represent the five best methods for trace analysis but were chosen as typical methods in general use.

It was necessary to make a number of assumptions in order to make the comparison. Lists of sensitivity limits as published by an authority in the field were taken without any further individual evaluation. Higher sensitivities for one element or group of elements may not be possible because of recent perfection of new procedures. In addition sensitivities for an entire method might be increased by suitable improvements in technique. Neither of these possibilities has been taken into consideration in making these comparisons. Gross normalizing factors were introduced for each method to adjust the values to the same units as the activation analysis values.

The problem of interfering ions and the necessity, in some cases, for chemical separations before the indicated sensitivity can be obtained are completely neglected even though they play a very important practical role in the problem. The speed with which the analyses are made is not considered. Finally the problem of multiple-element analysis in a single sample is not considered.

Regardless of these limitations, the comparison is worth while in that it readily points out the few elements for which activation analysis is particularly well suited and also the few for which it is not. It also shows elements for which activation gives only relatively fair sensitivity but for which it is very useful because other methods are at present much less sensitive.

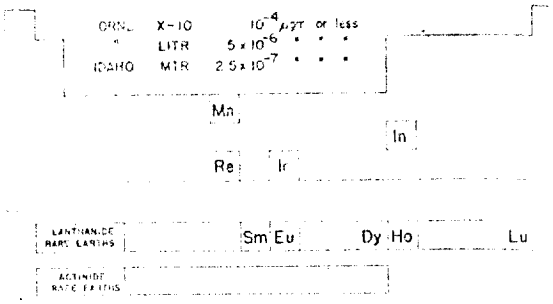

Fig. 1. Elements with thermal neutron activation sensitivity of 10^{-4} μg or less in the Oak Ridge X-10 reactor.

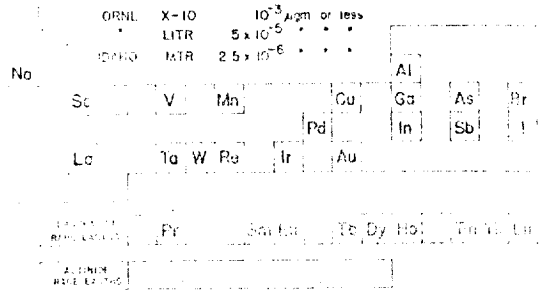

Fig. 2. Elements with thermal neutron activation sensitivity of 10^{-3} μg or less in the Oak Ridge X-10 reactor.

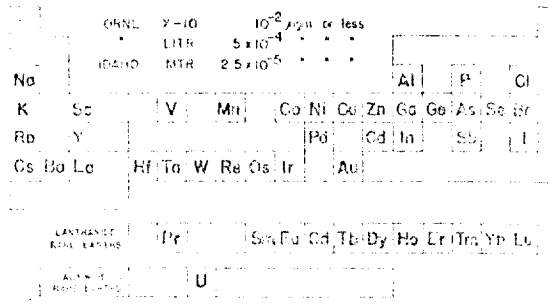

Fig. 3. Elements with thermal neutron activation sensitivity of 10^{-2} μg or less in the Oak Ridge X-10 reactor.

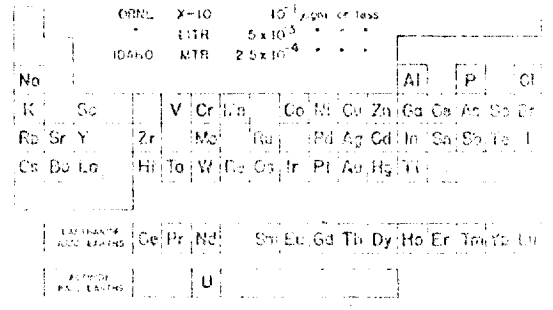

Fig. 4. Elements with thermal neutron activation sensitivity of 10^{-1} μg or less in the Oak Ridge X-10 reactor.

Table 2. Sensitivity comparisons (μg/ml).

Z	Element	Methods						
		Oak Ridge X-10 reactor	Oak Ridge LTR reactor	Copper spark	Graphite direct-current arc	Flame spectrophotometer	Sensitive color reaction	Amperometric titration
1	H							
2	He							
3	Li			0.002		0.02		
4	Be			.002		250.	0.01	
5	B			.1		10.		
6	C							
7	N							
8	O							
9	F			.1				0.25
10	Ne							
11	Na	0.007	0.00035	.1	20.	0.002		
12	Mg	.6	.03	.01	0.1	1.	.06	
13	Al	.001	.00005	.1	.2	20.	.002	200.
14	Si	1.	.05	.1	2.		.1	
15	P	0.02	.001	20.	50.		.01	15.
16	S	4.	.2					5.
17	Cl	0.03	.0015				.01	10.
18	A							
19	K	.05	.001	0.1		0.01		100.
20	Ca	3.8	.19	.1		.03		100.
21	Sc	0.002	.0001	.005				
22	Ti			.1		2.	.03	~10.
23	V	.001	.00005	.05		2.	.2	3.
24	Cr	.2	.01	.05	2.	1.	.02	1.
25	Mn	.0006	.00005	.02	0.2	0.1	.001	0.0001
26	Fe	9.	.45	.5	.2	2.	.05	2.
27	Co	0.02	.001	.5		10.	.025	100.
28	Ni	.03	.0015	.1	4.	10.	.01	0.5
29	Cu	.007	.00035		0.2	0.1	.03	10.
30	Zn	.01	.002	2.	20.	2000.	.016	10.
31	Ga	.007	.00035	1.		1.		
32	Ge	.04	.002				.08	
33	As	.002	.0001	5.	10.		.1	0.4
34	Se	.05	.0025					200.
35	Br	.003	.00015					
36	Kr							
37	Rb	.03	.0015	0.2		0.1		
38	Sr	.6	.03	.5		.1		
39	Y	.01	.0005	.01		50.		
40	Zr	.3	.015	.1			.13	
41	Nb	10.	.5	.2		20.	50.	
42	Mo	0.1	.005	.05		30.	0.1	5.
43	Tc							
44	Ru	.1	.005			10.	.2	
45	Rh					1.	.2	
46	Pd	.005	.00025	.5		1.	.1	
47	Ag	.11	.0055		0.1	0.5	.1	1.
48	Cd	.05	.0025	2.	4.	30.	.01	5.
49	In	.0001	.0000005	1.		1.	.2	100.
50	Sn	.2	.01		0.2	10.		2.
51	Sb	.001	.0002	5.	4.		.03	10.
52	Te	.1	.0005	0.5		100.	.5	
53	I	.002	.0001					1.
54	Xe							
55	Cs	.03	.0015	.5		1.		
56	Ba	.05	.0025	.1		3.		25
57	La	.002	.0001	.05		5.		
58	Ce	.1	.005	.5		20.	.25	~500.
59	Pr	.002	.0001	.2		100.		

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Z	Element	Methods: Oak Ridge X-10 reactor	Oak Ridge LPTR reactor	Copper spark	Graphite direct-current arc	Flame spectrophotometer	Sensitive color reaction	Amperometric titration
60	Nd	1	.005	.5		50.		
61	Pm							
62	Sm	.0016	.00033	.2		100.		
63	Eu	.00003	.000015	.02				
64	Gd	.02	.001	.1		10.		
65	Tb	.004	.0002					
66	Dy	.00003	.000015	.5		10.		
67	Ho	.0001	.00002	.2				
68	Er	[illegible]	[illegible]	.5				
69	Tm	[illegible]	[illegible]	[illegible]				
70	Yb	[illegible]	[illegible]	.1				
71	Lu	.0003	.000015	2.				
72	Hf	.02	.001	0.5				
73	Ta	.007	.00035	1.				
74	W	.005	.00015	0.5			0.1	
75	Re	.0005	.00003	2.			.05	
76	Os	.02	.001				1.	
77	Ir	.0003	.000015	5.			2.	
78	Pt	.1	.005	0.02			0.2	
79	Au	.002	.00015	.2		200.	.1	
80	Hg	.2	[illegible]	.5	2.	100.	.03	
81	Tl	.6	.05		0.2	1.		
82	Pb	2.	.1	0.05	.2	20.	.03	3.
83	Bi	~ 0.1	~ .02	.2	.2	300.	1.	300.
84	Po							
85	At							
86	Em							
87	Fr							
88	Ra			.1				
89	Ac							
90	Th			.2				
91	Pa			2.				
92	U	.01	.0005	1.		10.	0.7	

In Table 2, sensitivities that can be obtained by activation analysis with two different nuclear reactors are compared with sensitivities for the other methods. Sensitivities for each element and method are those reported in the compilations of data listed here. Certain gross normalizing factors, however, were used for each method to adjust the values to the common sensitivity basis of "micrograms per milliliter" and an error of about 10 percent. It was assumed that as much as 25 or 50 ml of the unknown solution was available.

The values listed in the table represent an *order of magnitude only*, since peculiarities of experimental procedure, interferences, and so forth, introduce considerable variables. For activation analysis they represent conservative values, although values for other methods may be somewhat optimistic. Several of the methods are capable of an increase in sensitivity of a factor of 10 or more by a change of equipment or the use of larger samples, and so forth, if they were to be pushed to the limit.

The table, although not complete, is presented as a basis for general comparisons of the methods or as a type of work sheet. Pertinent information on each method follows.

Oak Ridge X-10 reactor. Values are listed by Leddicotte and Reynolds (*8*) for activation analysis in the Oak Ridge X-10 reactor at a flux of 5×10^{11} neutrons/cm² sec to give 40 disintegrations/sec. Samples are activated for saturation or 30 days, whichever is shorter. It is assumed that a 1 ml sample is evaporated per analysis. The sensitivity of the method could be substantially increased by increasing the volume of sample used. The primary limitation is the effect of self-absorption of the bulky sample upon the radiations emitted by the radioisotope. If it were desired to increase the sensitivity further, samples with activity less than 40 disintegrations/sec might be accepted. With special techniques an activated isotope having about one-tenth of this activity could be determined.

Oak Ridge LPTR reactor. Values are given for activation analysis in the Oak Ridge LPTR reactor (or proposed university research reactors at full power) at a flux of 10^{13} neutrons/cm² sec. All other conditions and comments are identical to those given for the X-10 reactor.

Copper spark. The original sensitivity values from which these were derived are listed by Nachtrieb (*18*) for spectrographic analysis using the copper-spark method. It is assumed that for best results only 1/20 ml of sample can be evaporated on the electrode. If more sample could be evaporated without interfering seriously with the analysis, the sensitivity would be increased. Nachtrieb gives his data [illegible] and [illegible] percent. His values were [illegible] to convert to micrograms, multiplied by 20 to adjust to the basis of a 1-ml sample, and divided by 2 to allow for an error of ± 10 percent. The net result was to divide the literature values by 100 for the comparison.

Arc [illegible]. The original values are also listed by Nachtrieb (*18*) for spectrographic analysis using [illegible]. It is assumed that 1/20 ml of [illegible] is evaporated on the electrode. All comments on normalization given for the copper-spark method apply to this method. The literature values were divided by 100 for the comparison chart.

Flame spectrophotometer. The original sensitivity values for analysis using the flame spectrophotometer with an oxyhydrogen flame are given in Beckman Reprint R-56 (*19*). Values are given directly in micrograms per milliliter. The errors given for the reported sensitivities were ± [illegible] percent. [illegible] are [illegible] percent error in the other methods.

Sensitive color reaction. The original sensitivity values for spectrophotometric analysis using sensitive color reactions were taken from Sandell (*20*). The values are given in micrograms per [illegible] milliliter. It was assumed that a standard 1-cm cell was used in the spectrophotometer, although the 10-cm cell could be used to increase sensitivity by a factor of 10. As in the case of flame photometry, the original values were given with ± 10-percent error and were multiplied by a normalizing factor of 10 to convert to the prescribed basis.

Amperometric titration. The original values for amperometric titration are listed in a compilation made by Sargent and Co. (*21*). The values were not all converted to the same units in the compilation; some were given as micrograms, others as molarity, and so forth, with different bases of error. For this comparison, the sensitivity values were converted as nearly as possible to the common basis of the other methods considered. The normalized values, however, are much less certain than values presented for the other methods. Recent work on coulometric analysis may have superseded some of the lower sensitivity values for a number of the elements.

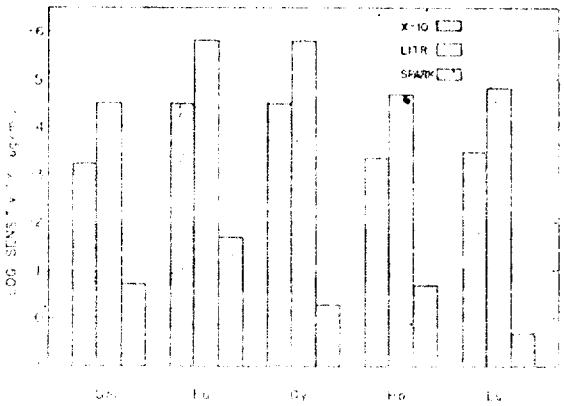

Fig. 5. Comparison graphs of some rare earth elements having high sensitivity for activation analysis.

Comparison graphs. Some means of the methods can be seen much more readily when they are presented in graphic form. Therefore methods for certain elements of importance have been compared graphically.

Certain of the rare earth elements show the highest sensitivity of any elements for activation analysis. These are shown in Fig. 5 compared with the values for spark spectroscopy. For europium and dysprosium truly fantastic sensitivities can be obtained. For each of the elements shown in this figure, analysis by activation so far overshadows other methods that it is possible to obtain sensitive analysis with neutron fluxes much lower than those of a reactor.

Manganese, indium, rhenium, and iridium are four other elements that show high sensitivity on the chart

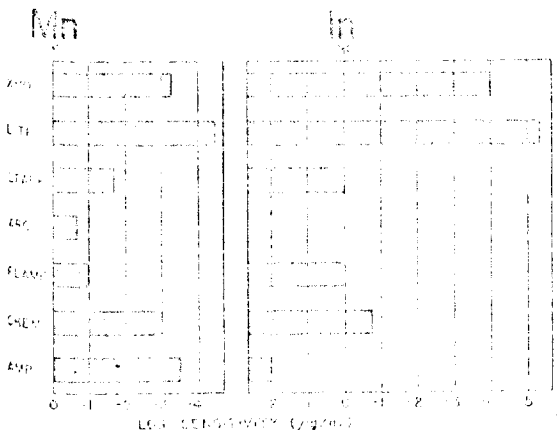

Fig. 6. Comparison graphs for manganese and indium.

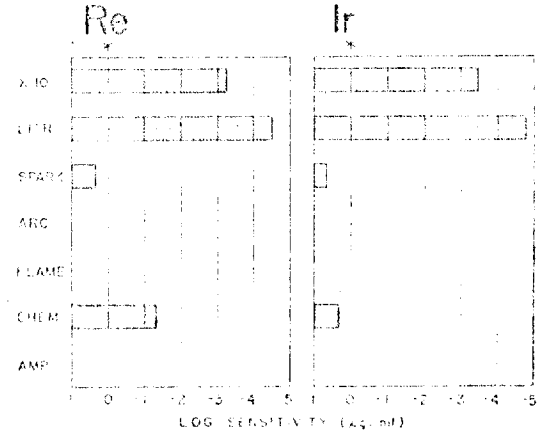

Fig. 7. Comparison graphs for rhenium and iridium.

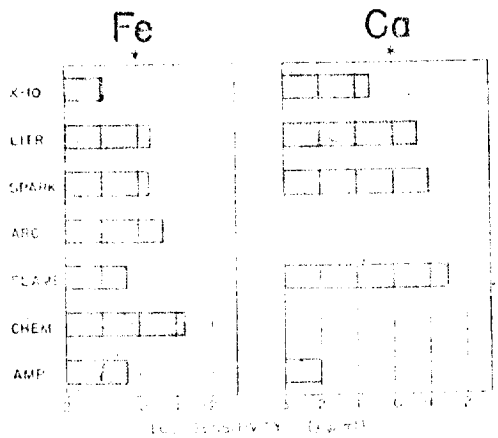

Fig. 8. Comparison graphs for iron and calcium.

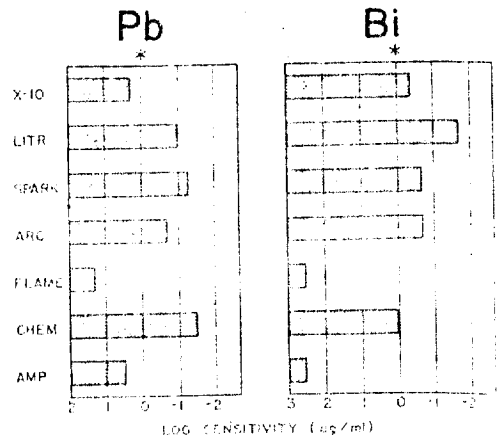

Fig. 9. Comparison graphs for lead and bismuth.

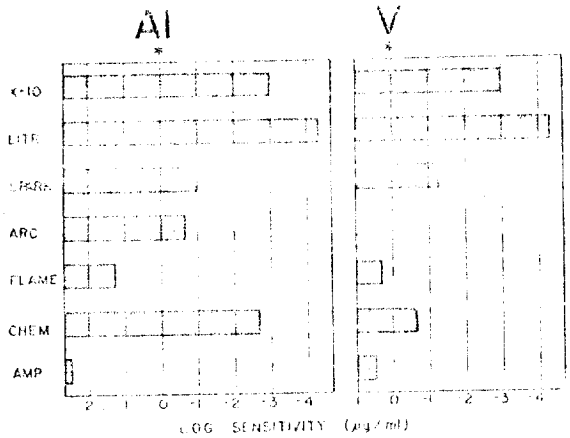

Fig. 10. Comparison graphs for aluminum and vanadium.

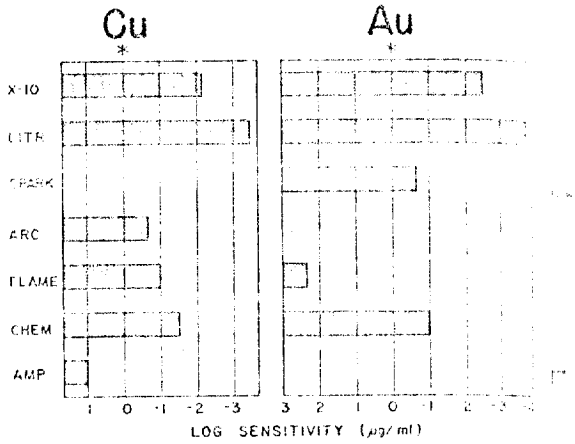

Fig. 11. Comparison graphs for copper and gold.

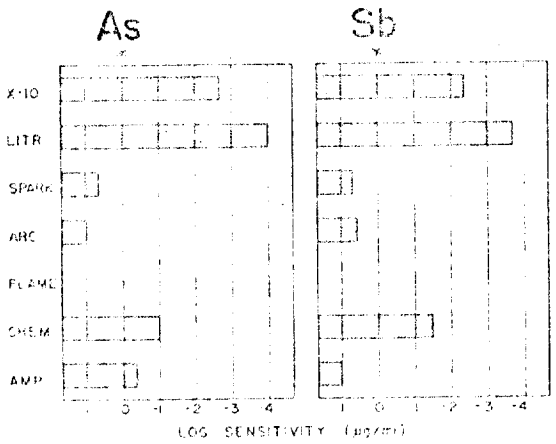

Fig. 12. Comparison graphs for arsenic and antimony.

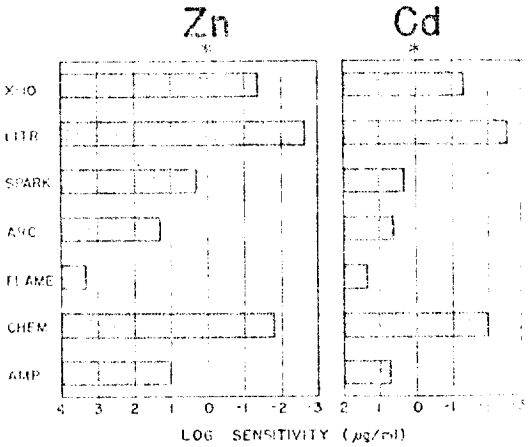

Fig. 13. Comparison graphs for zinc and cadmium.

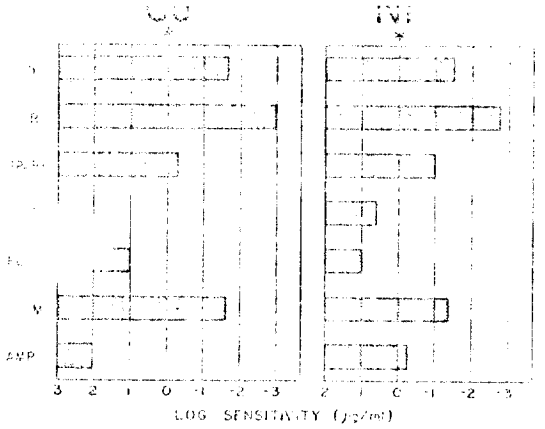

Fig. 14. Comparison graphs for cobalt and nickel

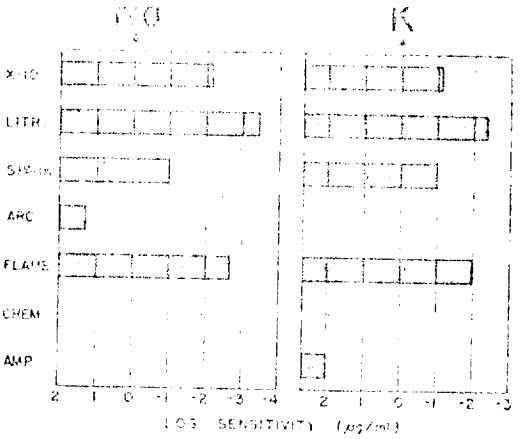

Fig. 15. Comparison graphs for sodium and potassium.

[illegible] 1. The sensitivities for these elements are [illegible]ed in Figs 6 and 7. The stars on the graph under [illegible] symbol of the element indicate the zero point on [illegible]log sensitivity scale. Although activation analysis [illegible] sensitive for manganese, the coulometric method [illegible] and the spectrophotometric method using [illegible] sensitivities. For aluminum, on the other hand, [illegible] methods are not as highly developed, and [illegible]ation would prove very useful. Activation would [illegible] prove invaluable for both rhenium and iridium, [illegible] other methods available at present prove unsatisfactory for high sensitivity.

[illegible]ures 8 and 9 show comparison graphs for iron,

Table 2. Reagents for high sensitivity color reactions [illegible]

Element	Reagent
Mn	4,4'-Tetramethyldiaminotriphenylmethane
In	8-Hydroxyquinoline
Re	Thiocyanate-stannous chloride (ether solution)
Ir	$IrCl_3$
Fe	o-Phenanthroline
Sn	
Pb	Dithizone (CCl_4)
Bi	Phenylthiohydrazolorthiol
V	Benzoylphenylhydroxylamine
Cu	Dithizone (CCl_4)
Au	o-Tolidine
As	Molybdate-hydrazine sulfate
Sb	Rhodamine B
Zn	Dithizone (CCl_4)
Cd	Dithizone (CCl_4)
Co	Nitroso-R-salt
Ni	Bromine-dimethylglyoxime
Na	
K	
Al	Oxine

calcium, lead, and bismuth, all of which show poor sensitivity for activation analysis. For analysis of these elements, it would be inadvisable to use activation analysis unless any special condition required it.

Figures 10, 11, and 12 show graphs for aluminum, vanadium, copper, gold, rhenium, and antimony, all of which show good sensitivity for activation analysis. Arc iron does not present enough advantage over conventional methods, to warrant its use for copper and aluminum, but for the others it may provide some advantage.

Figures 13, 14, and 15 show graphs of several other elements that are either neighbors in the periodic table or are in the same family. For each the activation sensitivity is only fair but in some cases the method would be advantageous.

Table 3 [illegible] the [illegible] are reported to [illegible] the sensitivities obtained for the sensitive color-reaction method on the graphs of Figs. 6–15.

Summary. Rough comparisons of the sensitivity of activation analysis with other standard methods give the investigator a feel for the elements for which activation can be used as a powerful tool. Although improvements in technique for an entire method or for an individual element will invalidate the figures given in Table 2, the over all picture will be changed only slightly. Furthermore, other considerations such as speed of analysis, interferences of other elements, and availability of analyzing instruments and facilities may indicate the use of one method in preference to another. The increasing availability of nuclear reactor facilities, however, means that analysis by activation is an important new tool for general use in trace analysis [illegible].

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THE EXPERIMENTAL PRODUCTION OF BASAL GANGLION SYMPTOMATOLOGY IN MACACUS RHESUS*

HUGO MELLA, M.D.

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BOSTON

With the recent, rapidly progressing interest in the basal ganglions one finds the literature on the histology, pathology and function of these structures becoming remarkably extensive. Experimental evidence, however, to support the theories of the functions of the basal ganglions, is lacking.

In Wilson's[1] comprehensive review in *Brain*, 1914, he not only gives the results of his experimental work but outlines the literature from the year 1667 when Thomas Willis considered the corpora striata to be the seat of the sensorium. This review is so complete and well known that it would be but a repetition to give another historical sketch here. It is evident, however, that in his experiments he did not produce the symptomatology now attributed to lesions of the basal ganglions. Possibly this was due to confusion of the picture by gross lesions in the corticospinal tracts, or to the fact that the lesions were too circumscribed and apparently failed to involve enough cells to produce a clinical disturbance.

It was my aim to produce, experimentally, symptoms like those of paralysis agitans. Focal injuries having failed to do this, it was thought that more diffuse lesions might be produced by special poisons administered to monkeys. This method would have the advantage of causing a gradual injury to the tissues; lesions in various stages of development could be studied, and it might produce more typical symptoms.

In attacking the problem at this point the report by Edsall, Wilbur and Drinker[2] that workmen employed in an atmosphere containing manganese dust frequently develop symptoms similar in many respects to those of the parkinsonian syndrome, seemed to give such a hopeful lead that it was deemed worth while to try manganese intoxication of

* From the Department of Neurology, Long Island Hospital, Boston, and the Department of Neuropathology, Harvard Medical School.

* Read before the Forty-Ninth Annual Meeting of the American Neurological Association, Boston, May 31, 1923.

1. Wilson, S. A. K.: An Experimental Research into the Anatomy and Physiology of the Corpus Striatum, Brain 36:427, 1914.

2. Edsall, D. L.: Wilbur, F. P., and Drinker, C. K.: The Occurrence, Course and Prevention of Chronic Manganese Poisoning, J. Indust. Hyg. 1:133, 1919.

monkeys. Consequently over a period of eighteen months manganese chlorid was administered to four monkeys at the Long Island Hospital, Boston.

METHOD OF INVESTIGATION

Six normal monkeys of the *Macacus rhesus* species were chosen. These monkeys had recently arrived from India and were kept under close observation for three months before commencing the experiments, in order to acclimate and accustom them to captivity and to a carefully prepared and balanced diet. The diet consisted of water, boiled rice, fresh carrots, boiled and baked potatoes, wheat bread, turnips, roasted peanuts, cracked corn, oats and bananas. Throughout the periods of observation and experimentation the monkeys developed no pulmonary or gastro-intestinal disturbances.

Beginning with the fourth month and extending over a period of eighteen months, gradually increasing doses of a sterilized solution containing one milligram of manganese chlorid to 1 c.c. of distilled water was administered intraperitoneally with a 50 c.c. Luer syringe to four of these monkeys, the other two being kept as controls. Every other day injections were given, warm, but not hot, after sponging the site of the injection with alcohol. Care must be taken that the needle penetrates the abdominal wall into the peritoneal cavity and that all utensils and syringes with needles are sterile. After the injection a small amount of air is forced through the needle to clear it of the solution, as it was found by previous experiments on cats, that if any escapes into the muscles or subcutaneous tissues a large indurated area or even a slough may result. The only immediate effect of the injection is a slight indisposition on the part of the animal; he leans against the side of the cage for from ten to fifteen minutes after which he resumes his normal activities.

PROTOCOLS OF EXPERIMENTS

Monkey 1. Laboratory No. E. N. 1, 1921. (Control).—

Aug. 15, 1921.—The animal is normal and is put on regular diet. The animal remained normal and no injections were given.

May 7, 1923.—Eats well. No abnormal movements. No disturbance of posture or gait.

June 17, 1923.—This morning animal drags self about cage. Apparently sick but eats carrots. Hind legs are dragged but stands on them occasionally. Respiration not accelerated.

June 18, 1923.—No change.

June 19, 1923.—No change.

June 20, 1923.—A little brighter. Uses legs better but still droops a great deal. Is very quiet and does not resent stroking. Bowels normal.

June 22, 1923.—About the same.

June 23, 1923.—About the same.

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June 18, 1923.—No change.

June 19, 1923.—No change.

June 20, 1923.—A little brighter. Uses legs better but still droops a great deal. Is very quiet and does not resent stroking. Bowels normal.

June 22, 1923.—About the same.

June 24, 1923.—Found lying on left side with respiration rate of 56. Does not respond when handled. Caffeine 0.20 gm. was given subcutaneously but animal did not improve.

June 25, 1923.—Animal died during the morning. The necropsy revealed nothing abnormal in the head, thorax or abdomen.

Histologic Examination.—No abnormalities were found in the cortex, basal ganglions, cerebellum, pons, spinal cord, or peripheral nerves. The pituitary, lungs, heart, spleen, pancreas, liver, kidneys and muscles were normal.

Manganese Content of Tissues

	Mg. Per 100 Gm. of Tissue
Liver	0.120
Lung	0.055
Brain	0.032
Kidney	0.155
Pancreas	trace
Spleen	0.100
Heart	0.070

Monkey 2.—Laboratory No. E. N. 2, 1921. (Control).—

Aug. 15, 1921.—Animal normal. Put on regular diet. Animal remained normal. No injections.

May 7, 1923.—Eats normally. There are no abnormal movements nor is there any disturbance of posture or gait. No injections have been given.

June 19, 1923.—The animal is not eating well and appears to be quite sick. The limbs are used freely but animal droops markedly. The respiratory rate is not accelerated. The bowels are normal.

June 22, 1923.—The animal is apparently well again.

June 25, 1923.—The activities are normal.

July 1, 1923 to Sept. 15, 1923.—The animal eats well and all activities appear normal. No abnormal movements are noted.

Monkey 3.—Laboratory No. E. N. 3, 1921.—

Aug. 15, 1921.—Animal normal. Put on regular diet. No injections given.

Nov. 15, 1921.—5 mg. of manganese chlorid was injected intraperitoneally.

Nov. 16, 1921.—The animal shows no ill effects from the injection. No abnormal movements have been observed. The dose of 5 mg. of manganese chlorid was administered every other day until July 21, 1922, when the dose was increased to 10 mg. every other day. Up to this time the animal showed no abnormal movements.

July 23, 1922.—10 mg. of manganese chlorid was injected intraperitoneally. No abnormal movements observed.

July 25, 1922.—The injection of 10 mg. was repeated. The monkey does not sit on the perch or swing, and slight choreo-athetoid movements are occasionally observed.

July 27, 1922.—The injection was increased to 15 mg. of manganese chlorid. The movements are now quite definite and the monkey is becoming slightly rigid.

July 29, 1922.—15 mg. of manganese chlorid was injected. The choreic movements have almost disappeared and a fine tremor of the hands is observed. The dose of 15 mg. was repeated every other day until Aug. 16, 1922. During this period the movements had not changed in character.

Aug. 17, 1922.—15 mg. of manganese chlorid was injected at m. The monkey continued to show fine tremors of the hands. At 7 p. m. the animal was found lying on its side apparently unconscious but breathing.

Aug. 18, 1922.—The animal is apparently dying, respirations very rapid and shallow. Died at 2 p. m.

Necropsy.—This was performed immediately after death. The brain and cord, in gross, appeared normal. The lungs only were examined and were reported "negative"! (This death and necropsy occurred during my absence and only the brain and part of the spinal cord were preserved.)

Histologic Report.—Spinal Cord: There is no cellular infiltration of the meninges and no perivascular infiltration. A normal number of cells is present in the white matter. The anterior horn cells appear normal, i. e., the cells are not swollen. The nuclei are central and the cells are not vacuolated (cresyl violet stain).

The meninges are normal, and the dorsal roots appear normal. There is no marginal gliosis and no evidence of neuroglia proliferation in any part of cord. Apparently there is a normal number of anterior horn cells present (phosphotungstic acid stain).

The dorsal and ventral roots, meninges, white and gray matter, and anterior horn cells appear normal. The blood vessels are normal and there is no perivascular exudate. No congestion of capillaries nor thrombosis is found (hematoxylin eosin stain).

Section of Medulla Oblongata and Portion of Cerebellum at the Level of the Olive: Occasionally a cell is observed in the medulla that seems to be slightly swollen, but these are rare. There is no definite cellular infiltration, but there appear to be a few more glia nuclei proportionately than one finds in the cord. The ependymal cells are normal, and a small section of the choroid is normal. As a whole the Purkinje cells appear normal (cresyl violet stain).

There is no neuroglia proliferation in the cerebellum although possibly there is a slight proliferation on the floor of the fourth ventricle. Otherwise the section is negative (phosphotungstic acid stain).

The cerebellum, medulla and meninges appear normal. No vascular changes are observed. There is no capillary congestion and the choroid is normal (hematoxylin eosin stain).

Section of Brain Through Hippocampus, Putamen, Pallidum, Internal Capsule and Thalamus: Very few ganglion cells are found in the putamen. Occasionally a large pallidal cell is found in this region. These are quite swollen, vacuolated, some of them showing chromatolysis, others being quite fragmented. The same picture appears in the pallidum. The cells of the thalamus and hippocampus are normal (cresyl violet stain).

There is no definite cellular infiltration, although the glia nuclei appear quite numerous. The blood vessels appear normal and there is no capillary congestion nor perivascular exudate (hematoxylin and eosin stain).

The neuroglia is quite dense in the putamen, and there is apparently an excessive amount in the region of the ansa lenticularis (phosphotungstic acid stain).

Manganese Content.—The brain contained 0.173 mg., the liver 4.906 mg. of manganese per 100 gm. of tissue.

Monkey 4.—Laboratory No. E. N. 4, 1921.—

Aug. 15, 1921.—Animal normal. Put on regular diet.

Nov. 15, 1921.—5 mg. of manganese chlorid was injected intraperitoneally.

Nov. 16, 1921.—The animal shows no ill effects from the injection. No abnormal movements have been observed.

Five milligrams of manganese chlorid was injected every other day until July 21, 1922, when the dose was increased to 10 mg. every other day. During this time the animal showed no abnormal movements.

July 27, 1922.—The animal appears to be normal. Dose increased to 15 mg. every other day.

This was continued until Nov. 15, 1922, when the dose was increased to 20 mg. of manganese chlorid, given every other day.

Nov. 19, 1922.—The monkey is beginning to show choreic movements; he does not sit on his perch, but prefers to sit on the floor of the cage.

Dec. 1, 1922.—The movements are now more of the choreo-athetoid type and the animal can remain on his perch only by grasping the side of the cage with both hands and both feet. The 20 mg. dose is continued every other day.

Dec. 30, 1922.—Condition practically the same as on Dec. 1, 1922. Same dose continued.

Jan. 17, 1923.—The movements are less noticeable, but the animal is becoming slightly rigid. Same dose continued.

Feb. 1, 1923.—The coarse movements have ceased and occasionally fine tremors can be seen in the fingers. The arms and legs are definitely rigid. Twenty milligram doses continued every other day.

March 2, 1923.—The fine tremors of the fingers are almost constantly present and the hands are contracting with the terminal phalanges of the fingers in extension. The monkey shows a gait which is very suggestive of propulsion.

This condition continued without change until April 19, 1923, when the monkey could not rise from his side nor take up food. The manganese injections were stopped, but the animal died on April 20, 1923.

Necropsy.—The brain, cord, trunk and abdominal organs, except the liver, showed no gross pathologic change. The liver presented large, very dark, reddish brown patches over its surface. These patches varied from 3 mm. to 2 cm. in diameter. On sectioning the liver the patches were found throughout the entire organ (Fig. 6).

Histologic Report.—Cortex.—As a whole the cortical ganglion cells appear quite normal, although the Nissl bodies do not stand out as sharply as they might. Eccentric nuclei are rarely found. The cells do not appear swollen. There is a questionable increase in glia nuclei. The ganglion cells are not disarranged. Occasionally one finds slightly vacuolated ganglion cells (cresyl violet stain).

The meninges appear normal and there is no edema of the pia. There is no evidence of perivascular infiltration or vessel change (hematoxylin-eosin stain).

There is no evidence of neuroglia proliferation in the gray or white matter. There is no marginal gliosis (phosphotungstic acid stain).

There is no evidence of myelin sheath degeneration (Van Heumann stain).

An occasional collection of fat droplets may be seen in the gray matter, but no fat droplets are found in the ganglion cells (Scharlach R. stain).

Section Through the Putamen, Pallidum and Internal Capsule: An average of about one half of the fibers running from the putamen to the pallidum show myelin sheath degeneration. The ansa lenticularis shows practically the same picture (Van Heumann stain).

Apparently there is a definite neuroglia proliferation in the region of the ansa lenticularis, which shows a breaking down in one or two areas. This area of destruction is quite dense, with neuroglia proliferation (phosphotungstic acid stain).

There is no evidence of any vessel change, nor of perivascular exudate (hematoxylin-eosin stain).

The cells of the putamen are narrow and shrunken. Some of the large pallidal cells appear large and swollen with eccentric nuclei and are scarce, some having extruded their nuclei (cresyl violet stain).

Section Through the Anterior Tip of the Caudate and Putamen Including a Small Section of the Cortex: Numerous bundles of fibers connecting the caudate and putamen have lost their myelin sheaths (Van Heumann stain).

The ependymal cells appear normal but are apparently pushed up by little hillocks of neuroglia about the caudate. There is no perivascular exudate. The blood vessels appear normal (hematoxylin-eosin stain).

There is a marked fibrillar proliferation under the ependyma beside the caudate. This appears more or less diffuse throughout the caudate (phosphotungstic acid stain).

The cells of the caudate are shrunken and pyknotic, and neuronophagia is present. Of the large pallidal cells some are shrunken and pyknotic, others are swollen and show chromatolysis, while others show marked neuronophagia. Many are vacuolated (cresyl violet stain).

Cerebellum: With the van Heumann stain and hematoxylin-eosin, the cerebellum appears normal. With cresyl violet the Purkinje cells appear normal, and with the phosphotungstic acid stain there is no evidence of neuroglia proliferation.

Pons: This region appears normal with the van Heumann stain.

The ganglion cells and nuclei appear normal as a whole, showing no chromatolysis. Occasionally one is found which is perhaps, slightly swollen (cresyl violet stain).

There is no evidence of hemorrhage or perivascular exudate; the meninges appear normal (hematoxylin-eosin stain).

The meninges and blood vessels appear normal. There is no evidence of neuroglia proliferation (phosphotungstic acid stain).

Muscle: The muscle fibers appear normal; there is no evidence of nuclear proliferation (eosin and methylene blue stain).

Lung: There is no evidence of pathologic change.

Spleen: This organ is normal.

Liver: There are numerous areas of necrosis accompanied by hemorrhages. The liver cells, to a great extent, are destroyed in these areas. Connective tissue proliferation is extensive in many of these areas (hematoxylin-eosin stain).

Manganese Content.—The brain contained 0.22 mg., the liver 5.58 mg. of manganese per 100 gm. of tissue.

Monkey 5.—Laboratory No. E. N. 5, 1921.—

Aug. 15, 1921.—The animal is normal. Put on regular diet. The animal remained normal. No injections were given.

Nov. 15, 1921.—Five milligrams of manganese chlorid was injected intraperitoneally.

Nov. 16, 1921.—The animal shows no ill effects from the injection. No abnormal movements have been observed.

Five milligrams of manganese chlorid was injected every other day until May 29, 1922.

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May 29, 1922.—The animal is slightly rigid and shows a tendency to fall forward in walking. There are definite choreo-athetoid movements in the neck and arms. Ten milligrams of manganese chlorid injected.

May 31, 1922.—The condition of the animal is the same as on May 29, 1922. The 10 mg. dose was repeated.

June 2, 1922.—There is no change in the condition of the animal. The 10 mg. dose was repeated.

June 4, 1922.—The animal remains the same. The dose was increased to 15 mg.

June 6, 1922.—The choreo-athetoid movements in the neck and arms are becoming very pronounced. Fifteen milligram dose repeated.

June 8, 1922.—The animal remains the same. The 15 mg. dose was repeated.

June 10, 1922.—The monkey accidentally became entangled in a climbing rope and died of asphyxia.

Necropsy.—The brain appeared normal in the gross. The heart and lungs were normal. The abdominal organs were all normal excepting the liver. The liver both on the surface and on sectioning showed several irregular patches of a deep reddish brown discoloration. These patches varied from 3 to 10 or 15 mm. in diameter.

Histologic Examination.—Spinal Cord: The meninges appear normal and there is no definite cellular infiltration. The ganglion cells appear normal and stain well. There is no evidence of chromatolysis (cresyl violet stain).

The meninges appear normal. The ventral and dorsal roots appear normal. There is no evidence of any neuroglia proliferation either marginal or throughout the cord (phosphotungstic acid stain).

The meninges appear normal. The gray and white matter is normal. The anterior horn cells are normal. Here and there one observes a congested capillary but this change is not remarkable (hematoxylin-eosin stain).

Section of Pons: The meninges are normal and there is no evidence of neuroglia proliferation (phosphotungstic acid stain).

The ganglion cells appear normal (cresyl violet stain).

Nothing abnormal is noted. There are no vascular changes or changes in the supporting structures (hematoxylin-eosin stain).

Cerebellum: The sections are faintly stained, but the Purkinje cells appear normal (cresyl violet stain).

The meninges are normal and there is no evidence of neuroglia proliferation (phosphotungstic acid stain).

The sections are stained quite faintly. The Purkinje cells are normal. There is no evidence of neuroglia proliferation, nor perivascular exudate (hematoxylin-eosin stain).

Cortex: The ganglion cells appear to be normal (cresyl violet stain).

There is no evidence of neuroglia proliferation (phosphotungstic acid stain).

The section is quite normal and there are no remarkable vascular changes (hematoxylin-eosin stain).

Basal Ganglions: The large pallidal cells are swollen and many show vacuolization as well as neuronophagia. Many of the smaller type cells are also swollen. Only very rarely are any pyknotic cells found (cresyl violet stain).

There is no perivascular exudate and the vessels appear normal (hematoxylin-eosin stain).

There is an increase in neuroglia nuclei but no fibrillar gliosis appears (phosphotungstic acid stain).

There is no evidence of any myelin sheath degeneration (van Heumann stain).

Liver: There are extensive patches of necrosis present in which a large percentage of the liver cells has been destroyed. There are many red blood cells in these patches. Connective tissue proliferation is active and many young cells may be seen (hematoxylin-eosin stain).

Manganese Content.—The brain contains 0.51 mg., and the liver 8.88 mg. of manganese per 100 gm. of tissue.

Monkey 6.—Laboratory No. E. N. 6. 1921.—

Aug. 15, 1922.—The animal is normal. Put on regular diet. The animal remained normal and no injections were given until Dec. 15, 1922.

Dec. 15, 1922.—Ten milligrams of manganese chlorid was injected intraperitoneally. This dose was repeated every other day with no change in the monkey's condition up to March 22, 1923.

March 22, 1923.—The dose was increased to 20 mg. and given every other day.

April 3, 1923.—The monkey has difficulty in picking up pieces of cracked corn. Twenty milligram dose continued.

April 5, 1923.—There are quite definite movements of a choreo-athetoid type in the arms.

April 7, 1923.—The movements are now also noticeable in the neck.

April 9, 1923.—The movements are diminishing slightly. The animal continued about the same until May 7.

May 7, 1923.—There are marked choreo-athetoid movements of the neck, arms, body and legs.

May 9, 1923.—The movements continue. Dose increased to 25 mg. given every other day.

May 19, 1923.—Fair choreo-athetoid movements in arms, neck and legs. There is a marked tendency to fall forward in walking.

May 30, 1923.—Fair movements continue. The animal is still alive.

EXPERIMENTAL RESULTS

A study of the protocols shows that in a typical experiment the sequence of events is somewhat as follows: The monkey at first develops movements which are choreic or choreo-athetoid in type, later passing into a state of rigidity accompanied by disturbances of motility; then appear fine tremors of the hands and finally contracture of the hands with the terminal phalanges extended (Fig. 1).

The typical histologic findings in three of these animals have been as follows:

Brain.—The cortex shows a slight but evenly distributed chromatolysis with rare vacuolated nerve cells, no gliosis and no myelin degeneration. There are no meningeal or vascular abnormalities. Scharlach R stain showed no fat droplets in the neurons of the cortex.

In the putamen and caudate the cells are shrunken and pyknotic, many having lost their nuclei, and occasionally neuronophagia is observed. The large pallidal cells are few in number, swollen, vacuolated and show eccentric nuclei. There are scattered areas of gliosis which are occasionally quite dense (Fig. 2). Many of the fibers connecting the caudate and putamen have lost their myelin sheaths.

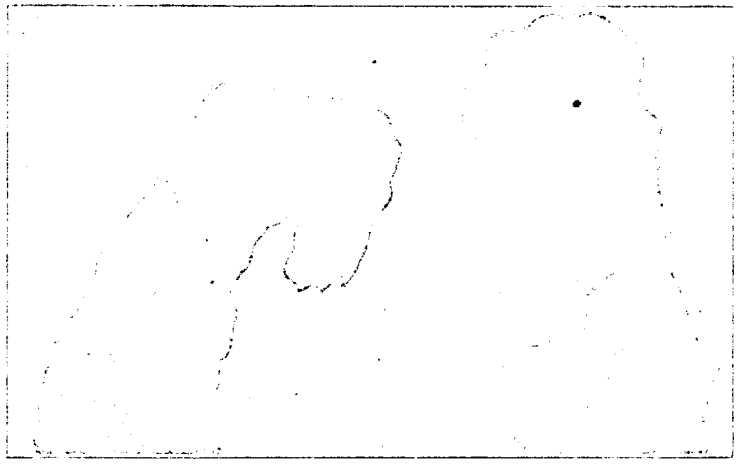


Fig. 1.—Typical paralysis agitans contracture of hands in Monkey 4.

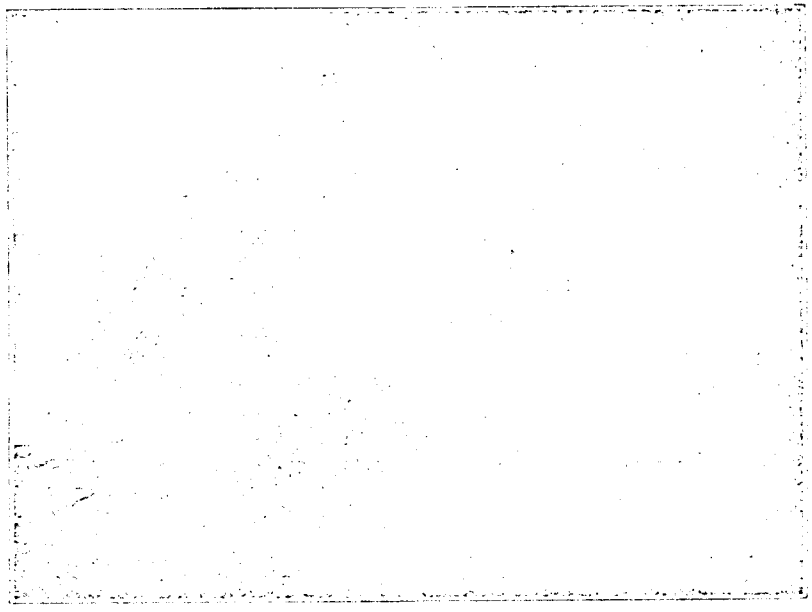


Fig. 2.—Section through anterior portion of the caudate showing gliosis. Phosphotungstic acid stain.

In the globus pallidus some of the large pallidal cells are shrunken and pyknotic (Fig. 3), others are swollen and show chromatolysis, others have eccentric nuclei and vacuoles. In one case a definite loss of myelin sheaths was observed in the ansa lenticularis. There were areas of gliosis present in one case.

The cerebellum is essentially negative, although occasionally the Purkinje cells do not stain well. The pons as a whole appears normal, although occasionally a swollen ganglion cell is observed. There is no gliosis and the blood vessels appear normal. The spinal cord is normal

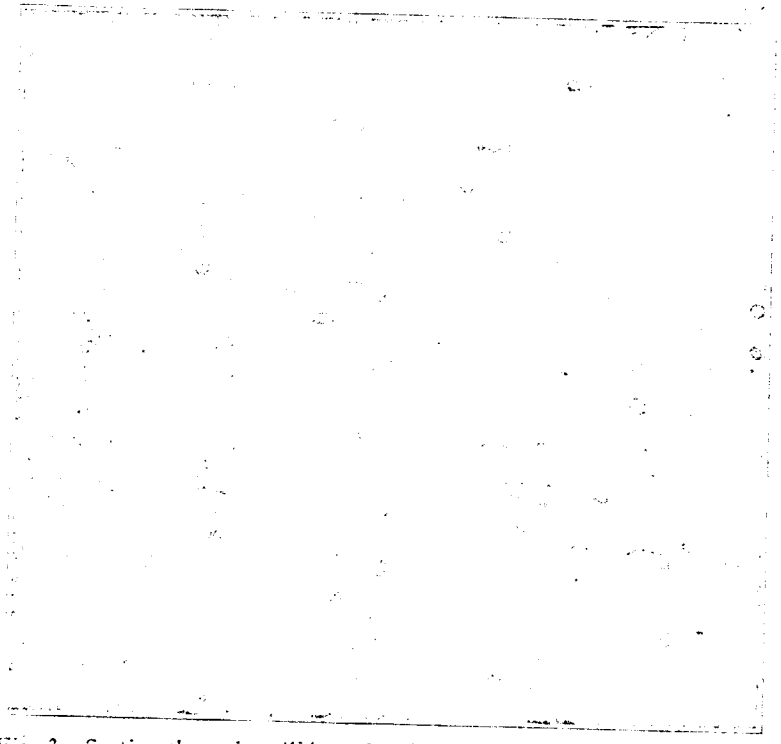


Fig. 3.—Section through pallidum showing shrunken and pyknotic cells with neuronophagia. Cresyl violet stain.

and the peripheral nerves show no Marchi degeneration. The muscle fibers appear normal and there is no evidence of nuclear proliferation. The lungs and spleen are normal.

The two livers show acute hepatitis, i. e., areas of necrosis (Fig. 4) with small hemorrhages and beginning fibrosis with scattered areas of chronic and more extensive fibrosis (Fig. 5).

The manganese content of the brain and trunk organs of all the injected animals was far above normal as is shown in the accompanying table. The brains contained about ten times the normal amount of

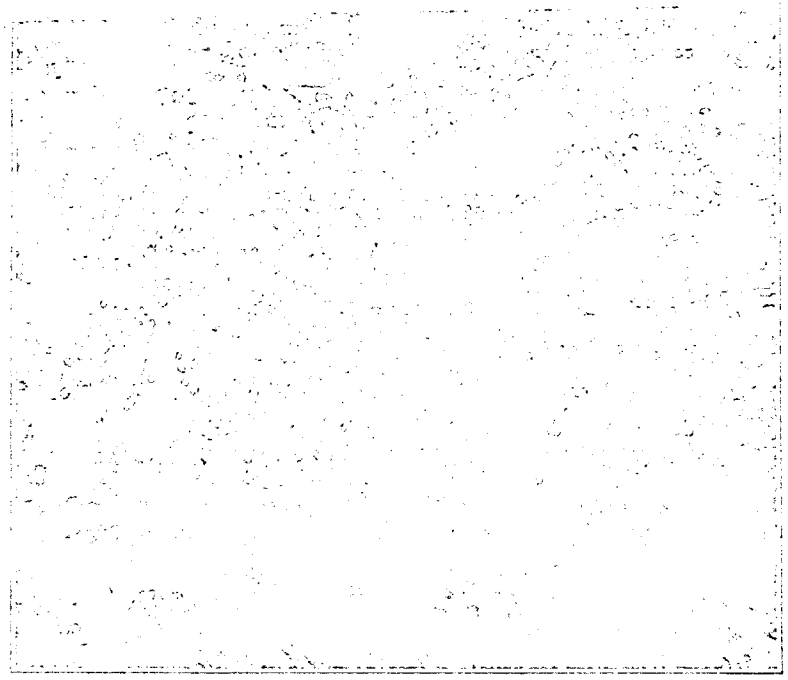
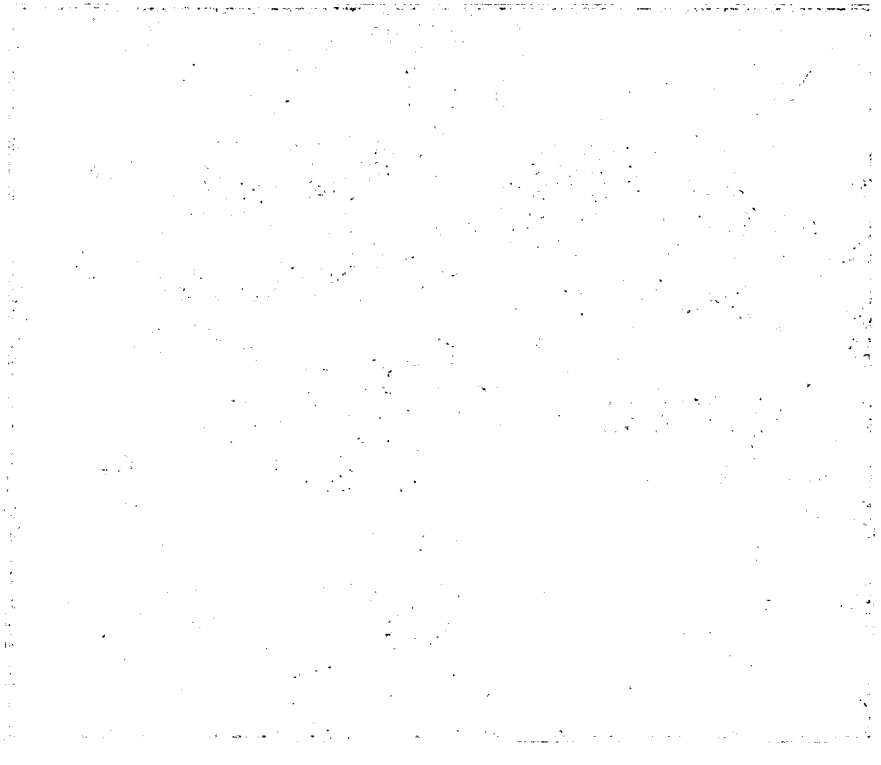


Fig. 4.—Liver showing area of acute hepatitis with hemorrhage. Eosin and methylene blue stain.



manganese and the livers about fifteen times. I am indebted to Miss Annie S. Minot of the Department of Applied Physiology of the Harvard Medical School by whom the manganese determinations were made.

*Manganese Content Per 100 Gm. of Tissue of Monkeys That Have Come to Necropsy and Normal Figures for Comparison**

Animal Number	Experimental Monkey		Normal Monkey		Normal Average for Cat, Dog and Rabbit		Normal Average for Man	
	Brain	Liver	Brain	Liver	Brain	Liver	Brain	Liver
Monkey 1 (control)	.....	.....	0.032	0.120	0.047	0.2523	0.028	0.170
Monkey 3	0.173	4.966	0.032	0.120	0.047	0.2523	0.028	0.170
Monkey 4	0.22	5.58	0.032	0.120	0.047	0.2523	0.028	0.170
Monkey 5	0.51	8.88	0.032	0.120	0.047	0.2523	0.028	0.170

* Averages for normal tissues were taken from Bohman and Minot, Journal of Biological Chemistry 42:[illegible] (June) 19[illegible].

COMMENT

I have already mentioned that Edsall, Wilbur and Drinker,[2] in examining a number of men employed in an atmosphere containing manganese dust, found that they frequently develop symptoms in many respects similar to the parkinsonian syndrome, but they also speak of the many points of similarity between their cases and Wilson's disease, progressive lenticular degeneration.

As one observes these animals passing into the stage of chronic poisoning, the rigidity and disturbance of motility, i. e., tendency to propulsion with choreo-athetoid movements, and later contractures of the fingers, it is striking how the symptoms simulate those of human cases now considered to be due to disturbances of the basal ganglions. Another striking fact, and one which is probably of great importance, is that we find pathologic changes in the liver (Fig. 6), but no abnormality in any other structure excepting the brain. As this work is but beginning I cannot say whether the liver changes precede the basal ganglion changes or vice versa. The relation between the liver and basal ganglion changes in Wilson's disease has not been worked out; possibly this method of experimentation will be a means of approaching that problem, as in these monkeys are found the dual lesions, namely, degenerative processes in the liver and basal ganglions.

The "shrinkage of the caudate" with gliosis, and marked degeneration of the lenticular fibers that run to the subthalamic nuclei and the red nucleus as described by Jelgersma[3] in Huntington's chorea, and

3. Jelgersma: Die anatomischen Aenderungen bei Paralysis Agitans und chronischer Chorea. Verhandl. d. Gesellsch. Deutsch. Naturforsch. u. Aerzte zu Köln, Leipzig, 1909.

the cell changes of the basal ganglions in paralysis agitans described by Ramsay Hunt,[4] Bielschowsky[5] and C. and O. Vogt,[6] are all to be seen in the brains of these animals to a greater or less extent. The value in this, I believe, is that the brains of the experimental animals may be studied at any selected stage of the clinical picture, whereas with human material one must usually wait until senile changes or some intercurrent fatal disease confuses the histologic picture, and thereby greatly diminishes the ultimate value of the study. The chief difficulty here arises that no method has yet been found whereby the tolerance of the monkey for the manganese can be determined, so that premature deaths may be avoided; but by administering smaller doses over a greater length of time this difficulty may be solved. The analyses shown in the accompanying table are not yet sufficiently numerous to justify any conclusions, but it is interesting to note that the liver is the organ

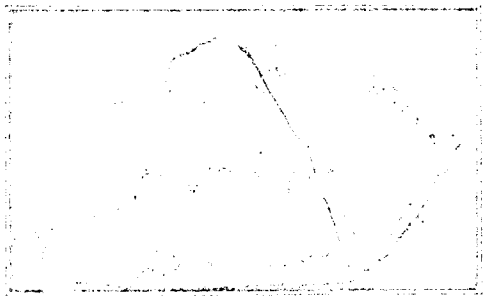


Fig. 6.—Section of liver in Kaiserling solution, showing patches of liver necrosis. From Monkey 4.

that is most heavily laden with manganese and that shows the most conspicuous pathologic changes.

CONCLUSIONS

From a study of these preliminary experiments it appears that in monkeys poisoned with manganese there were observed certain abnormal movements and disturbances of locomotion, which are usually attributed to lesions in the basal ganglions. The histopathologic picture in these animals shows definite changes, most pronounced in the striatum, pallidum and liver.

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Gigiyena Truda
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SOME IMPORTANT ASPECTS OF THE PATHOGENESIS AND THERAPY OF MANGANESE POISONING (A Review)

V. A. Mikhaylov

The pathogenesis of manganese poisoning with the symptoms of toxic parkinsonism characteristic of its last stage (amyostatic syndrome, akinesia, tremor and muscular rigidity) remains one of the most disturbing riddles of clinical medicine and neurophysiology (L. G. Okhnyanskaya and A. A. Komarova; Cotzias).

Clinical and experimental observations of recent years have thrown some light on the basic mechanisms of the toxic action of manganese. The aggregate of clinical, morphological, and experimental data indicates that the greatest changes take place in the basal ganglia in whose cells degenerative and dystrophic phenomena are more pronounced than elsewhere in the brain (Foss). The mass of anatomical and physiological connections of the caudate nucleus to the globus pallidus and through it to the thalamus opticus and cerebral cortex as well as to other subcortical nuclei suggest that the role of the basal ganglia is not limited to their participation solely in descending extrapyramidal functions. Nor is there any doubt that they play a part in the systemic impulses constantly created in the active brain at the thalamocortical level (V. A. Cherkes). Extirpation of the basal ganglia proved to be one of the most effective methods of treating parkinsonism in man. Thalamectomy produces a similar therapeutic effect (N. P. Bekhtereva). All this bears witness to the complex structure of the primary focus which appears to be situated not only in the basal ganglia but in other subcortical nuclei as well. The small-celled medial group of hypothalamic nuclei are the most sensitive to manganese (A. A. Levakovskaya). An experimental model of parkinsonism can be obtained by blocking the adrenergic reactive systems of the hypothalamus and reticular formation.

Research during the last 10 years has helped to elucidate the specific pathophysiological mechanisms of toxic parkinsonism of manganese etiology and the main causes of the pronounced selective effect of manganese on the subcortical formations. Manganese was shown to impair synaptic conduction. This effect is probably due to its ability selectively to be deposited in the mitochondria, its main intracellular depot (Cotzias et al.). The main group of nerve cell mitochondria is concentrated in the synapses. The synapses of the brainstem neurons contain many more mitochondria than do the cortical neurons. Not only mitochondria but synaptosomes and the acetylcholinereactive fraction of protein isolated from rat brain accumulate manganese quite intensively. Acetylcholine increases the accumulation of cesium, rubidium, manganese, and magnesium ions while decreasing the binding of lithium, potassium, and calcium ions (R. N. Glebov and A. N. Mezentsev). This is also shown by the fact that severe degenerative-dystrophic processes develop in the cortical synapses (M. S. Tolgskaya; V. A. Mikhaylov and G. V. Belobragina), spinal motor neurons, and peripheral myoneural synapses and muscle spindles (V. A. Mikhaylov et al.). The pathomorphological data are consistent with the results of electromyographic (L. G. Okhnyanskaya and A. A. Komarova) and chronaximetric (V. A. Mikhaylov, 1952; F. S. Trop) studies and with changes in the electrical activity and reflex excitability of the spinal cord and cerebral cortex (L. I. Belokon'; D. A. Ginzburg).

A comparison of these data with the demonstrated participation of mitochondria in the transmission of nervous excitation and in the synthesis of mediators of nervous excitation reveals that exposure to manganese may give rise to certain biochemical changes in the synapses characterized, specifically, by the accumulation of acetylcholine or similar substances.

In fact, we showed (V. A. Mikhaylov, 1969) that manganese is an acetylcholinesterase inhibitor. Manganese and copper are known to disrupt the conduction of excitation in the myoneural synapses, affecting the enzymic processes of acetylcholine synthesis and altering the sensitivity of the cholinoreceptors of postsynaptic membranes. Manganese acts as a cholinomimetic factor. We believe that two phases, readily detected in experiments on isolated organs, can be distinguished in the mechanism of the toxic action of manganese on the reactive systems of the cells. The first or "cholinergic" phase is marked by the predominance of cholinergic influences because of the simultaneous processes of injury to the adrenoreactive systems and depression of the enzymes that split acetylcholine. Then when the manganese concentration increases, a phase of areactivity sets in which is also caused by inhibition of the acetylcholinoreactive structures.

The increased acetylcholine concentration is obviously responsible for the subsequent block of transmission of excitation - asynapsia. This process is most pronounced in the basal ganglia because there is much more acetylcholine, acetylcholine acetylase, and acetylcholine esterase here than in the other portions of the brain. It was recently shown, however, that the cholinergic synapses are also found in the cerebral cortex, which emphasizes the significance of the parasympathomimetic direction of the action of manganese. It is interesting to note that the tremor of the extremities caused by the injection of acetylcholine into the region of the globus pallidus can be relieved with antiparkinson agents. This is also consistent with the results of experiments with the synthetic anticholinergic preparation tremorin (Achmed and Marshall). Chlorpromazine and neostigmine as well as reserpine (V. A. Mikhaylov, 1969) potentiate the toxic effect of manganous chloride in white mice. The increased concentration of manganese in brain tissue may play a part in the mechanism of this phenomenon

because experiments on monkeys showed that after peroral or intramuscular administration of chlorpromazine, the amount of manganese in the basal ganglia increases sharply. This correlates with the intensity of the clinical neurologic disorders (Bird et al.).

Therefore, in parkinsonism of any etiology, cholinergic influences are prominent, a fact that supports the assumption that a cholinergic mechanism is involved in the pathogenesis of the disease. It is further supported by the favorable results obtained with the use of belladonna preparations in the treatment of parkinsonism; the drugs are still of value today. The search for new anticholinergic preparations is continuing. One of the most interesting of these is benzotropin which combines anticholinergic with antihistaminic properties. Tropacin is sometimes successfully used with ATP or novocain and other anticholinergic agents.

However, the adrenergic mechanism of parkinsonism is by far the more significant. In the early 1950s Vogt observed that monamines are distributed unevenly in the brain. A very large amount of norepinephrine is concentrated in the hypothalamus, whereas 80% of all the dopamine in the brain is found in the basal ganglia, chiefly in the caudate nucleus. It was later demonstrated that there is a peculiar adrenergic "bridge" from the reticular formation to the cerebral cortex. Biochemical analysis of brain punctates taken from parkinsonism patients and determinations of dopamine in urine and in blood showed that the catecholamines, especially dopamine, decrease sharply in the basal ganglia as does its excretion with urine (Barbeau et al.; Bertler; Hornikiewicz; Ehringer and Hornikiewitcz; T. L. Storks; others). According to Steg, there exists an unusual dopaminergic reactive system in which dopamine functions not only as an epinephrine precursor but as a special serotonin-like inhibitory synaptic mediator, an acetylcholine antagonist.

The reasons for the decrease in concentration of the catecholamines, including dopamine, are still unexplained. It is assumed that the enzymic synthesis of dopamine in the substantia nigra is impaired. The catecholamine then passes through the nigrostriatal tract into the basal ganglia which serve as its main depot. The cause of impairment of dopamine synthesis may be the formation of a product of its pathological catabolism N acetyl-dopamine, which even in minute quantities can neutralize dopa-decarboxylase (I. A. Travenets). It is equally likely that the process of deposition of dopamine in the basal ganglia is disrupted. However, the issue is, on the whole, moot, although there is no doubt that the changes in concentration of the catecholamines are related to the development of symptoms of parkinsonism. A pharmacological model of parkinsonism can actually be obtained with a liberating agent, reserpine or the adrenolytic chlorpromazine. In monkeys, destruction of the substantia nigra caused contralateral hypokinesia. Injury to the cerebral peduncle gave rise to choreoid movements and lowered the serotonin level in the corpus striatum. But if these operations were combined at the same time with destruction of the tractus rubrospinalis, akinesia, tremor, etc. developed and the dopamine and serotonin levels dropped sharply. Similar phenomena occurred after chronic manganese dioxide poisoning of monkeys, and dopamine and serotonin decreased 1- to 1½-fold and 6-fold, respectively (Neff and Bartlett). Occupational and experimental manganese poisoning sharply decreased the amount of epinephrine in the adrenals and blood (V. A. Mikhaylov; V. M. Vereshchagina; A. A. Levakovskaya). Rats poisoned with manganous chloride exhibited a decrease in the norepinephrine content of the hypothalamus; the exhaustion of its reserve in the small cells of the medial group of hypothalamic nuclei was quite pronounced. All this shows that the mechanism of parkinsonism of manganese etiology is similar to that of parkinsonism due to other causes.

This disease is clearly characterized by marked disturbance of catecholamine and serotonin metabolism in the peripheral and central structures of the sympatheticoadrenal system and in the basal ganglia.

Note that manganese compounds are powerful oxidizing agents and that the catecholamines are oxidized almost instantaneously in their presence, possibl forming, among other things, a substance like leukoadrenochrome whose pronounced cholinomimetic properties have long been known. Manganese can also activate enzymes that split the catecholamines (V. M. Vereshchagina; E. N. Levina), MAO in particular, although under certain conditions it suppresses their activity. This may be promoted by injury to the protein structure of the granules in which the catecholamines are deposited in the cell.

However, it would be wrong to reduce the pathogenesis of parkinsonism solely to disturbances of catecholamine metabolism, which is only one of the elements in the complex and deeper impairments of the enzymic processes in the cells. Manganese disrupts the activity of enzymes and denatures the macrostructure of proteins, a likely possibility especially since manganese, unlike magnesium, is completely incorporated into proteins. Prolonged (9 months) intravenous injection of rabbits with manganous chloride produced a sharp decrease in the protein sulfhydryl groups of the blood (V. I. Davydova; Ye. M. Meizvestnova). Catecholamine, serotonin, acetylcholine, and histamine metabolism changes (Klawans) as does the content of RNA and purine bases not only in the nerve cells but in the neuroglia. This is particularly interesting in the light of the prevalent view that neurons and neuroglia form a single metabolic system.

The results of clinical and experimental studies on the therapeutic efficacy of MAO inhibitors, L-dopa the precursor of dopamine and α-methyldopa, have been remarkable. MAO inhibitors increase the concentration of catecholamines in the brain and do not relieve the clinical symptoms. However,

when administered simultaneously with L-dopa, they slightly intensify its favorable effect (Birkmayer and Hornikiewicz; Bernheimer et al.; others). α-Methyldopa for still obscure reasons may even induce symptoms of parkinsonism. L-dopa (50 to 150 mg) given to a group of 20 parkinsonism patients abolished symptoms of akinesia within 2 to 3 hours and the effect persisted for 24 hours (Bernheimer et al.). However, in 60 other patients (Cotzias et al.) L-dopa mitigated the clinical symptoms only slightly, giving rise at the same time to agranulocytosis, hyperkinesia, and choreoid movements. The prolonged administration of L-dopa is also known to cause intestinal bleeding, arrythmia, and mental disturbances. It follows, therefore, that, despite the undoubted participation of the adreno- and cholinoreactive systems in the pathogenesis of parkinsonism, its mechanism, including that of manganese etiology, is still unexplained. Further research is needed to throw light on the intimate mechanism of the metabolic disturbances occurring in the nerve cells and glia surrounding them in the autonomic centers of the hypothalamus and in the basal ganglia. It is particularly important to investigate the involvement of the hypothalamus whose role in the pathogenesis of manganese poisoning remains obscure.

Anticholinergic and adrenergic therapy and the attempts to remove manganese from the body by means of such complexing agents as EDTA and its derivatives (Eyble et al.), polyaminopolyphosphonic acid (O. G. Arkhipova), and I. A. Postovskiy's multiple complexing agent (V. A. Mikhaylov, 1969), despite some encouraging reports (Witlock et al.; Sarborick), did not produce the desired effect. This is understandable because, as Cotzias demonstrated not long ago, a high manganese content in the tissues is not a prerequisite of the development of pathological neurologic changes. Moreover, while an excess of manganese can be removed with EDTA, the clinical picture of parkinsonism remains virtually unchanged.

Thamine with lactate and glutamate with Versene are beneficial in the early stages of occupational manganese poisoning in humans and in experimental animals (V. A. Mikhaylov, 1969; Kh. A. Byso; N. V. Gorodnova). This clearly indicates that metabolic changes play a role at the molecular level in the sympatheticoadrenal system as a whole. However, the search should continue for new and more efficacious agents to normalize the metabolic processes and conduction of excitation in the synapses. The first steps have already been taken in this very ~~promising~~ encouraging direction. The findings to date are promising.

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УДК 616.24-003.669.743(017)

НЕКОТОРЫЕ АКТУАЛЬНЫЕ ВОПРОСЫ ПАТОГЕНЕЗА И ТЕРАПИИ МАНГАНОТОКСИКОЗА (Обзор)

GIG.TRUDA, 6: 14-19, 1971 *В. А. Михайлов* (Свердловск)

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(Поступила в редакцию 31/VII 1970 г.)

Патогенез манганотоксикоза с характерными для его последней стадии явлениями токсического паркинсонизма — амиостатический синдром, акинезии, тремор и ригидность мышц — остается одной из волнующих загадок клиники и нейрофизиологии (Л. Г. Охнянская и А. А. Комарова; Cotzias).

Клинические и экспериментальные наблюдения последних лет дают возможность приблизиться к пониманию основных механизмов токсического действия марганца.

Совокупность клинических, морфологических и экспериментальных данных позволяет предполагать очаг наибольших изменений в базальных узлах, в нервных клетках которых развиваются особо выраженные по сравнению с другими отделами головного мозга дегенеративно-дистрофические изменения (Foss). Богатство анатомо-физиологических связей хвостатого ядра с бледным шаром и через него со зрительным бугром и корой головного мозга, а также с другими подкорковыми ядрами дает основание полагать, что роль базальных узлов не исчерпывается их участием в исключительно нисходящих экстрапирамидных функциях. Несомненна их роль в системных импульсациях, постоянно создающихся в деятельном мозге на таламо-кортикальном уровне (В. А. Черкес). Удаление базальных узлов оказалось одним из эффективных способов лечения паркинсонизма у человека. Аналогичный лечебный эффект наблюдается и в результате таламэктомии (Н. П. Бехтерева). Все это свидетельствует о сложности структуры первичного очага, который, по-видимому, локализуется не только в базальных узлах, но одновременно в других подкорковых ядрах. Наиболее чувствительными к действию марганца оказались мелкоклеточные медиальные группы ядер гипоталамуса (А. А. Леваковская). Путем блокирования адренергических реактивных систем гипоталамуса и ретикуляр-

ной формации можно получить экспериментальную модель паркинсонизма.

В свете исследований последнего десятилетия становятся более понятными конкретные патофизиологические механизмы токсического паркинсонизма марганцевой этиологии, а также основные причины выраженного избирательного влияния марганца на подкорковые образования. Показано, что марганец нарушает синаптическую проводимость. Действие марганца на синапсы, вероятно, связано с его свойством селективно откладываться в митохондриях, являющихся главным его внутриклеточным депо (Cotzias и соавт.). Следует подчеркнуть, что в синапсах сосредоточена основная группа митохондрий нервных клеток, причем в синапсах стволовых нейронов содержится намного больше митохондрий, чем в нейронах коры. Особо интенсивно накапливают марганец не только митохондрии, но также синаптосомы, а равно ацетилхолинреактивная фракция белка, выделенного из мозга крыс. Ацетилхолин увеличивает накопление ионов цезия, рубидия, марганца и магния и уменьшает связывание ионов лития, калия и кальция (Р. Н. Глебов и А. Н. Мезенцев). Об этом свидетельствует также тот факт, что при воздействии марганца тяжелые дегенеративно-дистрофические процессы развиваются в синапсах коры (М. С. Толгская; В. А. Михайлов и Г. В. Белобрагина), мотонейронах спинного мозга, а также в периферических мионевральных синапсах и мышечных веретенах (В. А. Михайлов и соавт.). Патологоморфологические данные хорошо согласуются с результатами электромиографических (Л. Г. Охнянская и А. А. Комарова), хронаксиметрических (В. А. Михайлов, 1952; Ф. С. Троп) исследований и изменениями электрической активности и рефлекторной возбудимости спинного мозга и коры больших полушарий (Л. И. Белоконь; Д. А. Гинзбург).

Сопоставляя с этими фактами доказанное участие митохондрий в процессах передачи нервного возбуждения и в синтезе медиаторов нервного возбуждения, можно полагать, что при воздействии марганца в синапсах могут возникать определенные биохимические изменения, характеризующиеся, в частности, накоплением ацетилхолина или подобных ему веществ. Действительно, нами показано (В. А. Михайлов, 1969), что марганец является ингибитором ацетилхолинэстеразы. Известно, что марганец и медь нарушают проведение возбуждения в мионевральных синапсах, оказывая влияние на ферментативные процессы синтеза ацетилхолина и изменяя чувствительность холинорецепторов постсинаптической мембраны. Марганец при этом выступает как холиномиметический фактор. Мы полагаем, что в механизме токсического действия марганца на реактивные системы клеток следует различать две фазы, которые легко обнаружить в опытах на изолированных органах. Первая фаза — «холинергическая» — характеризуется преобладанием холинергических влияний, что связано с одновременными процессами повреждения адренореактивных систем и угнетением ферментов, расщепляющих ацетилхолин. В дальнейшем при нарастании концентрации марганца наступает фаза ареактивности, обусловленная торможением также ацетилхолинореактивных структур.

Нарастание концентрации ацетилхолина, очевидно, ответственно за последующую блокаду передачи возбуждения — асинапсию. Этот процесс достигает особого выражения в базальных узлах, ибо здесь содержится по сравнению с другими отделами мозга намного больше ацетилхолина, ацетилхолинацетилазы и ацетилхолинэстеразы. Недавно, однако, показано, что холинергические синапсы находятся также в коре головного мозга, что в свою очередь подчеркивает значение парасимпатомиметической направленности действия марганца. Представляет интерес, что тремор конечностей, вызванный введением ацетилхолина в область бледного шара, мог быть ликвидирован с помощью антипар-

кинсонических средств. С этим согласуются также опыты с синтетическим антихолинергическим препаратом треморином (Achmed и Marschall). Аминазин и прозерин, а также резерпин (В. А. Михайлов, 1969) потенцируют токсический эффект хлористого марганца у белых мышей. Возможно, что в механизме указанного явления имеет значение нарастание концентрации в мозговой ткани марганца, ибо в опытах на обезьянах показано, что после перорального или внутримышечного введения аминазина содержание марганца в базальных узлах резко увеличивается, что коррелирует с интенсивностью клинических неврологических расстройств (Bird и соавт.).

Следовательно, при паркинсонизме различной этиологии на первый план выступают холинергические влияния, что обосновывает предположение о холинергическом механизме патогенеза этого заболевания. Собственно это подтверждают также благоприятные результаты терапевтического применения препаратов белладонны при паркинсонизме, не потерявших значения до настоящего времени. Поиски новых антихолинергических препаратов продолжаются, среди них особого внимания заслуживает бензотропин, в котором удачно сочетаются антихолинергические и антигистаминные свойства. Иногда с успехом применяют тропацин в комбинации с АТФ или новоканном и другие антихолинергические агенты.

Однако несравненно бо́льшее значение имеет адренергический механизм паркинсонизма. Еще в начале 50-х годов Vogt обратила внимание, что моноамины распределяются в головном мозге неравномерно. Особенно много норадреналина содержится в гипоталамусе, тогда как 80% всего допамина, находящегося в головном мозге, находится в базальных узлах, преимущественно в хвостатом ядре. В последнее время доказано существование особого адренергического «моста» от ретикулярной формации ствола до коры головного мозга. Биохимические исследования пунктатов мозга, взятых у страдающих паркинсонизмом людей, а также определения содержания допамина в моче и в крови показали, что при паркинсонизме содержание катехоламинов, особенно допамина, в базальных узлах резко падает, уменьшается также выделение последнего с мочой (Barbeau и соавт.; Bertler; Hornikiewicz; Ehringer и Hornikiewitcz; Т. Л. Сторкс, и др.). По мнению Steg, существует своеобразная допаминергическая реактивная система, в которой допамин играет роль не только предшественника норадреналина, но особого, подобного серотонину тормозного синаптического медиатора, антагониста ацетилхолина.

Причины понижения концентрации катехоламинов, в том числе допамина, пока остаются невыясненными. Предполагают, что нарушается ферментативный синтез допамина в черной субстанции, откуда последний по нигростриальному тракту поступает в базальные узлы, служащие его основным депо. Может быть, причиной нарушения синтеза допамина является образование продукта его патологического катаболизма N-ацетилдопамина, который даже в минимальных количествах способен инактивировать допа-декарбоксилазу (И. А. Травенец). Не менее вероятно, что нарушается процесс депонирования допамина в базальных узлах. Однако в целом вопрос этот остается открытым, хотя связь изменений концентрации катехоламинов с возникновением явлений паркинсонизма представляется несомненной. Действительно, фармакологическую модель паркинсонизма можно получить с помощью высвобождающего агента — резерпина или адренолитика — аминазина. У обезьян разрушение черной субстанции вызывало контралатеральную гипокинезию. Повреждение церебральной ножки обусловливало появление хореоподобных движений и понижение уровня серотонина в стриарных телах. Но если эти операции сочетались с одновременным разрушением рубросегментоспинального тракта, то развива-

лись явления акинезии, тремора, уровень допамина и серотонина резко понижался. Аналогичные явления возникали и при хроническом отравлении обезьян двуокисью марганца. При этом в базальных узлах содержание допамина снизилось в 1—1½ раза, а серотонина — даже в 6 раз (Neff и Bertlett). При профессиональном, а также экспериментальном манганотоксикозе в надпочечных железах и в крови резко снижается содержание адреналина (В. А. Михайлов; В. М. Верещагина; А. А. Леваковская). У крыс, отравленных хлористым марганцем, понижается содержание норадреналина в гипоталамусе, причем особо выражено истощение его запасов в мелких клетках медиальной группы ядер гипоталамуса. Все это сближает механизмы паркинсонизма марганцевой этиологии и паркинсонизма, возникающего в результате действия других причин. Несомненно, что при этом заболевании наступают существенные нарушения обмена катехоламинов и серотонина в периферических и центральных структурах симпатико-адреналовой системы и в базальных узлах.

Следует учитывать, что соединения марганца относятся к числу сильных окислителей, в их присутствии наступает почти мгновенное окисление катехоламинов, возможно образование в числе прочих такого продукта, как лейкоадренохром, выраженные холиномиметические свойства которого давно известны. Марганец может активировать также ферменты, расщепляющие катехоламины (В. М. Верещагина; Э. Н. Левина), в частности МАО, хотя при определенных условиях он угнетает ее активность. Этому может способствовать повреждение белковой структуры гранул, в которых депонированы в клетке катехоламины.

Но было бы неверным сводить сущность патогенеза паркинсонизма исключительно к нарушениям катехоламинового обмена, являющимся лишь одним из звеньев сложных, более глубоких нарушений ферментативных процессов в клетках. Марганец нарушает активность ферментов, оказывает денатурирующее влияние на макроструктуру белков, что тем более вероятно, поскольку в отличие от магния марганец полностью включается в белки. Продолжительное, в течение 9 месяцев, внутривенное введение хлористого марганца кроликам вызвало резкое снижение содержания белковых сульфгидрильных групп в крови при нормальном уровне остаточных (В. И. Давыдова; Е. М. Неизвестнова). Изменяется обмен катехоламинов, серотонина, ацетилхолина и гистамина (Klawans), изменяется содержание РНК и пуриновых оснований не только в нервных клетках, но также в нейроглии, что особенно интересно, поскольку, по современным представлениям, нейроны и нейроглия составляют единую метаболическую систему.

Примечательны результаты клинических и экспериментальных исследований лечебной эффективности ингибиторов МАО, прекурсора допамина Л-допа и α-метилдопа. Ингибиторы МАО, повышая содержание катехоламинов в мозгу, не устраняют клинических явлений; их одновременное введение с Л-допа, впрочем, несколько усиливает благоприятное влияние последнего (Birkmayer и Hornikiewicz; Bernheimer и соавт., и др.). α-Метилдопа при каких-то еще неясных обстоятельствах может даже вызвать явления паркинсонизма. Л-допа у группы, состоявшей из 20 больных паркинсонизмом, уже через 2—3 часа после введения 50—150 мг устранял явления акинезии, эффект сохранялся в течение суток (Bernheimer и соавт.). Но в наблюдениях других авторов (Cotzias и соавт.), проведенных на 60 больных паркинсонизмом, Л-допа вызывал лишь незначительное ослабление клинических явлений, одновременно развились агранулоцитоз, гиперкинез, хореоподобные движения. Известно также, что продолжительное введение Л-допа вызывает кишечные кровотечения, аритмию и расстройства психики. Отсюда следует, что, несмотря на несомненное участие адрено- и холинореактивных систем в патогенезе паркинсонизма, его механизм,

в том числе и при паркинсонизме марганцевой этиологии, остается еще невыясненным, необходимы дальнейшие исследования, которые могли бы пролить свет на интимный механизм нарушений обмена веществ в нервных клетках и окружающей их глии в вегетативных центрах гипоталамуса и в базальных узлах. Особенно актуальны исследования участия гипоталамуса, роль которого в патогенезе манганотоксикоза продолжает оставаться неясной.

Антихолинергическая и адренергическая терапия и попытки выведения марганца из организма с помощью таких комплексонов, как ЭДТА и ее производные (Eyble и соавт.), полиаминополифосфоновые кислоты (О. Г. Архипова), поликомплексон И. А. Постовского (В. А. Михайлов, 1969), несмотря на отдельные обнадеживающие сообщения (Willock и соавт.; Sarborick), не принесли желаемого эффекта. Это понятно, ибо, как недавно это продемонстрировал Cotzias, повышенное содержание марганца в тканях не является обязательным условием возникновения патологических неврологических сдвигов. Более того, избыток марганца может быть удален с помощью ЭДТА, однако клиническая картина паркинсонизма остается практически без изменений.

На ранних стадиях профессионального манганотоксикоза у людей, а также в эксперименте на животных оказывают благоприятное влияние тиамин с лактатом и глютамат с версеном (В. А. Михайлов, 1969; Х. А. Эйсо; Н. В. Городнова). Это, несомненно, указывает на значение обменных сдвигов, развивающихся на молекулярном уровне в симпатико-адреналовой системе в целом. Однако поиски новых, еще более эффективных веществ, нормализующих процессы обмена веществ и проведения нервного возбуждения в синапсах, должны продолжаться. В этом многообещающем направлении сделаны еще первые шаги. Полученные материалы свидетельствуют о перспективности дальнейших исследований.

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SOME URGENT PROBLEMS OF PATHOGENESIS AND THERAPY OF MANGANOTOXICOSIS (SURVEY)

V. A. Mikhailov

Summary

The paper carries information contained in literature sources and the author's personal data concerning pathogenesis and therapy of manganotoxicosis. An inference is made on the need for continued investigations aimed at clarifying metabolic disorders occurring in the cells at a molecular level. It appears to be promising to look into new agents influencing the function of the adrenergic and cholinergic systems so as to be able to treat toxic parkinsonism developing under the effect of manganese.

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The Determination of Manganese in Urine by Neutron Activation Analysis*

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A rapid and simple method for the determination of manganese in urine using neutron activation analysis is described. The procedure consists of radiochemical separation followed by measurement of the radioactivity induced in manganese by multichannel pulse-height analyses. The excretion of manganese in urine of normal human subjects was found to range from 7·7 to 16·7 μg for a 24-hr period. After administration of EDTA, excretion of manganese was significantly higher.

LE DOSAGE DU MANGANESE DANS L'URINE PAR L'ANALYSE A ACTIVATION NEUTRONIQUE

On décrit une méthode rapide et simple pour le dosage du manganèse dans l'urine employant l'analyse à activation neutronique. Le procédé consiste en une séparation radiochimique suivie de la mesure de la radioactivité induite dans le manganèse, au moyen des analyses multicanulaires de hauteur d'impulsion. On trouva que l'excrétion du manganèse dans l'urine des sujets humains normaux s'étendait de 7,7 a 16,7 μg, à travers une période de 24 h; suivant l'administration de l'EDTA, l'excrétion du manganèse subit une augmentation significante.

ОПРЕДЕЛЕНИЕ МАРГАНЦА В МОЧЕ ПОСРЕДСТВОМ АКТИВАЦИИ НЕЙТРОНАМИ

Описан простой и быстрый метод определения марганца в моче посредством анализа активации нейтронами. Процедура состоит в радиохимическом разделении и последующем измерении радиоактивности сообщенной марганцу, употребляя многоканальный анализ амплитуд импульса. Было найдено, что выделение марганца в моче здоровых людей колеблется от 7,7 до 16,7 μгр за период в 24 часа. После введения в организм этилен-диамино-тетра-уксусной кислоты, выделение марганца значительно повышается.

DIE BESTIMMUNG VON MANGAN IM HARN MITTELS NEUTRONEN AKTIVIERUNGSANALYSE

Eine schnelle und einfache Methode zur Bestimmung von Mangan im Harn mittels Neutronenanalyse wird beschrieben. Das Verfahren besteht in der radiochemischen Trennung mit darauf folgender Messung mittels Vielkanal Impulshöhenanalyse der Mangan Aktivität. Die Abscheidung von Mangan in dem Harn normaler Menschen wurde in dem Bereich von 7,7–16,7 μg in 24 Stunden befunden. Nach Verabreichung von EDTA war die Abscheidung des Mangans bemerkenswert höher.

* This investigation was made in the Nuclear Chemistry Department, Soreq Research Establishment, Atomic Energy Commission, Yavne, Israel, in co-operation with the Metabolic Unit, Rogoff Medical Research Institute, Beilinson Hospital, Petah-Tikvah, Israel. The project was supported by a grant from the Federation of Jewish Labour, Histadruth.

INTRODUCTION

Most of the analytical procedures for determining manganese are based upon volumetric methods in which the manganese is first oxidized to permanganate and then reduced.[1] Colorimetric methods which are also in common use, employ agents such as periodate, persulphate, bismuthate or lead dioxide.[2,3] However, these and all other analogous analytical methods are not easily applied to the analysis of trace amounts of manganese in complex matrices, such as biological materials. It has been noted[4,5] that although there are many methods reported for determining manganese in biological materials, the published data are rather limited. On the other hand, the method of neutron activation analysis provides high specificity, precision, high sensitivity and rapidity, and is quite easily applied to manganese determination in biological samples.[4-8]

Investigation of the renal excretion of manganese derived from interest in the biological role of manganese. It is known from metabolic studies with Mn^{56} and Mn^{52} that the excretion of manganese in man and in animals is primarily fecal.[4,9] The amount of manganese in urine is extremely small—often too small for detection by standard chemical methods[10]. Manganese in urine had been determined previously only by a conventional method of chemical concentration followed by spectrographic analysis.[10-13] The sensitivity of that method was limited and the manganese concentration in some of the urine samples from normal subjects was below the detectable limit. Therefore, the calculated mean was only a rough estimation.

Characteristic nuclear properties of manganese suggested the suitability of neutron activation analysis for determination of the small amounts of manganese in urine. The most important characteristics are the large cross section for thermal neutron capture by Mn^{55} and the γ-radiation emitted in the subsequent decay of Mn^{56}. The main advantages of this method are its specificity, high sensitivity and freedom from reagent contamination. Because elements such as Na, Cl and K will become intensely radioactive and will mask the Mn^{56}, the manganese is separated chemically after adding a manganese carrier to the sample. The Mn^{56} radioactivity is measured by a multichannel analyser using the 0·845 MeV photopeak.

Determination of manganese in 10 urine samples from normal human subjects is carried out in this study. Administration of the chelating agent, EDTA, causes an increase in the amount of manganese excreted in urine.

METHODS AND RESULTS

Samples

Urine was collected from human subjects who received the same specific known diet. Each sample consisted of the urine excreted during a 24-hr period.

Irradiation

Samples were weighed into polyethylene vials 25 mm high and 23 mm diameter, which were heat-sealed before irradiation. All the samples were irradiated for 5 min in the pneumatic transfer tube of the IRR-1 (The Israel Research Reactor) at Nahal Soreq. At the power level of 1·6 MW which was used for all the experiments, the thermal neutron flux was about $7{\cdot}13 \times 10^{12}$ n/cm^2/sec and the ratio of thermal-to-fast neutron fluxes was 3:1. The main nuclear reaction is Mn^{55} (n, γ) Mn^{56}.

Chemical separation

Because of the large amounts of sodium ($\sim$5 mg/ml), potassium, chlorine and other constituents of urine, it is very difficult to determine manganese by a direct non-destructive spectrometric technique. Therefore, a chemical separation is carried out—precipitating the manganese as the dioxide by boiling the 5 ml sample with nitric acid and potassium chlorate, and then adding 40 mg of manganese carrier. The precipitate is then washed four times with boiling water and alcohol and is ether-dried. The chemical yield of MnO_2 precipitate is between 70 and 95 per cent.

This procedure takes 5–6 min and removes the Mn^{56} from most of the other constituents of the sample. Small amounts of Na^{24} remain in the precipitate, but its interference is small and can be subtracted.

Measurement of radioactivity

The manganese dioxide precipitate is placed near a NaI crystal connected to a multichannel

pulse-height analyser. The Mn^{56} radionuclide is evaluated quantitatively by comparing the 0·85 MeV photopeak activity of the sample with standards[11].

TABLE 1. Calibration for manganese analysis

Manganese content (μg)	cpm at $T = 20$ min in $E = 0{\cdot}85$ MeV corrected for yield	cpm for 0·01 μg Mn
0·00992	70	70·6
0·02480	180	72·6
0·04960	348	70·2
0·07440	530	71·2
0·09920	713	71·9
0·14880	1068	71·8
0·19840	1412	71·2

Arithmetical mean 71·4 ± 0·8
71·4 ± 1·1 (S.D.).

The decay of the 0·85 MeV photopeak activity in all irradiated urine samples (MnO_2 precipitate), showed a single decaying component with a half-life of 2·58 hr, in good agreement with the published value[12].

The net γ-activities measured in samples containing known amounts of manganese varied linearly with the manganese content (Table 1). The total Mn^{56} radioactivity measured at $T = 0$ min was 71·4 ± 0·8 cpm/0·01 μg Mn. The minimum amount of manganese detectable in urine under the conditions of this experiment was 0·005 μg.

Urine samples were determined three times during a 3-month period and reproducibility is presented in Table 2. Fluctuations from the mean value did not exceed 4 per cent. Known amounts of manganese ("spikes") were added to known 5-ml urine samples and their manganese content was determined (Table 3). The findings agreed very well with the estimated quantity which was calculated as the sum of the amounts present in and added to the samples.

The manganese content of urine samples collected during a 24-hr period ranged from 7·7 to 16·7 μg Mn (see Table 4). After administration of EDTA (3 g/day), the manganese content of urine was significantly higher (see Table 5).

DISCUSSION

Although much physiological, biochemical, and clinical information has been published, surprisingly little has been ascertained about the normal metabolism of manganese. Some of the difficulty arises from the inadequacies of the quantitative chemical methods used to analyse biological material for manganese. In general, they lack sensitivity or accuracy and therefore are not suitable for determining changes in the pathways of manganese absorption and excretion in man and animals. Furthermore, urine contains elements which interfere with the assay for manganese. Therefore, the neutron activation analysis technique was used in this study to determine precisely the amount of manganese in the urine of normal human subjects.

The overall accuracy of this method for determination of manganese in urine is approximately ±5%. According to BOWEN[6], the principal limitation to accuracy is the error in the final determination of yield, i.e. in weighing

TABLE 2. Reproducibility of manganese determination in urine samples

Sample	Volume of urine in 24 hr (ml)	Creatinine in 24 hr (mg)	Mn^{56} content in 5 ml sample (cpm) corrected for yield	Mn^{56} content, mean value (cpm)	Mn excreted in 24 hr, mean value (μg)
AI AII AIII	2100	844	269 250 255	258 ± 11	15·12 ± 0·63
BI BII BIII	1900	801	199 183 197	193 ± 10	10·64 ± 0·55

TABLE 3. Determination of known amounts of manganese in urine

Manganese present (μg)	Manganese added (μg)	Manganese calculated* (μg)	Manganese found (μg)	Deviation from the calculated manganese (%)
0·0360 ± 0·0016	0·00992	0·04592	0·04770	3·871
0·0360 ± 0·0016	0·04960	0·08560	0·08668	1·261
0·0360 ± 0·0016	0·07410	0·11040	0·10950	0·821
0·0275 ± 0·0011	0·09920	0·12670	0·13198	4·161
0·0275 ± 0·0011	0·14880	0·17630	0·18102	2·671

* The sum of the amount present in 5 ml urine plus the amount added.

TABLE 4. Manganese determination in urine samples

Sample	Volume of urine in 24 hr (ml)	Creatinine in 24 hr (mg)	Manganese excreted in 24 hr (μg)	Mn/ml urine (mg)
1	900	672	15·30	0·0170
2	2100	844	10·08	0·0051
3	1900	801	9·50	0·0050
4	2010	1775	7·75	0·0038
5	1900	889	15·20	0·0080
6	1700	1802	14·96	0·0088
7	1740	1949	9·04	0·0052
8	2100	1974	13·86	0·0066
9	2000	844	16·00	0·0080
10	2140	862	16·69	0·0078

TABLE 5. Manganese determination in urine before and after EDTA treatment†

Sample	Date	Treatment	Volume of urine in 24 hr (ml)	Creatinine in 24 hr (mg)	Manganese excreted in 24 hr (μg)	Mn/ml urine (mg)
Z.	12 June 1964		1950	1209	13·63	0·0070
	14 June 1964	EDTA	2050	1189	38·55	0·0188
	15 June 1964		2300	1234	18·19	0·0079
	16 June 1964	EDTA	3100	1364	44·10	0·0142
	17 June 1964		3120	1217	29·35	0·0094
B.	29 June 1964		1510	946	8·70	0·0057
	30 June 1964	EDTA	1030	899	42·81	0·0396
	1 Jul. 1964		1100	797	8·12	0·0074

† Intravenous administration of EDTA (3 g/day).

the manganese dioxide precipitate. Although the precipitate has large absorbent capacity, the homogeneous precipitation of manganese dioxide is the best single-step separation from other elements.[1,7]

Although neutron bombardment of urine produces Mn^{56} as a consequence of the thermal neutron reaction Mn^{55} (n, γ) Mn^{56}, two other fast reactions must be accounted for: Co^{59} (n, α) Mn^{56} and Fe^{56} (n, p) Mn^{56}. One can calculate the contribution of these reactions to the observed Mn^{56} under the conditions of activation used in this study. The amount of Co^{59} in normal urine is not precisely known. PERRY[10] found qualitatively and quantitatively all the metals of the first transition series except

Co in most human urine, but SPECTRO[14] reported that urine contains 5 $\mu g/l$ of Co^{59}. On the basis of the latter, the transformation of Co^{59} into Mn^{56} contributes not more than 1×10^{-5} per cent to the Mn^{56}. The amount of Fe^{56} in urine is about 60 $\mu g/l$[15]; therefore, the transformation of Fe^{56} into Mn^{56} contributes not more than 1×10^{-4} per cent to the Mn^{56}.

The full sensitivity of the method was not utilized here since it was not needed in this study. However, the sensitivity can be easily increased by increasing the irradiation time.

This procedure is rapid (5 min of irradiation and 5-6 min for chemical separation) and there is only a small lapse of time from the end of the neutron irradiation until the time when the sample may be counted for its manganese radioactivity. Thus, since the counting rate is higher, the Mn^{56} measurements are more accurate. This procedure is now being used routinely in analysing the urine of patients and normal subjects of manganese.

SUMMARY

Neutron activation analysis is used for the determination of manganese in urine. The procedure, initial radiochemical separation followed by measurement of the radioactivity induced in manganese by multichannel pulse-height analysers is simple, short and sensitive. Manganese concentration was measured in the urine of normal human subjects, and after a treatment with EDTA.

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Selective Depletion of Caudate Nucleus Dopamine and Serotonin During Chronic Manganese Dioxide Administration to Squirrel Monkeys[1]

Prolonged inhalation of dust containing manganese produces mental and extrapyramidal disturbances in man. The latter may resemble Parkinson's disease, Wilson's disease, or a mixture of both[2]. Attempts to produce an experimental animal model of this condition have been successful. Repeated administration of $MnCl_2$ to Rhesus monkeys produced disturbances of extrapyramidal motor system function associated with pathological changes in the basal ganglia resembling the pathological changes seen in man[3]. Chronic inhalation of MnO_2 dust by monkeys results in degeneration of Purkinje cells and granule cells of the cerebellum. These changes are associated with incoordination and fine intention tremor[4]. Repeated i.m. injection of MnO_2 in olive oil into Rhesus monkeys produced astroglial proliferation and neuronal degeneration primarily in the subthalamic nucleus and medial segment of the pallidum[5]. These animals had impaired coordination and flexion posture of the extremities.

Parkinson's disease is characterized by variable degrees of rigidity, tremor, and bradykinesia. The major biochemical deficit is depletion of brain monoamines, chiefly in the striatum[6,7]. Many of the features of Parkinson's disease are ameliorated by treatment with L-3,4-dihydroxyphenylalanine, DOPA[8,9]. This drug is also beneficial in chronic manganese intoxication[5].

The similarities between Parkinson's disease and chronic manganese poisoning prompted a study of the biogenic amine concentrations in monkey brain after chronic administration of MnO_2.

Methods. MnO_2 was finely ground in a mortar and olive oil added to form a suspension containing 200 mg MnO_2/ml. A total of 1 ml of suspension was injected s.c. at several sites in 15 squirrel monkeys (*Saimiri sciureus*). 5 control animals received 1 ml of olive oil. All animals received a s.c. injection of 50,000 units of potassium penicillin G following either MnO_2 or the oil vehicle. Animals were injected on 11 August 1967 and 19 September 1967. 2 weeks following the first injection 4 control and 4 MnO_2 treated monkeys died. Gross and microscopic examination of liver, kidney and brain tissue failed to reveal abnormalities. During November 1967, 5 of the remaining treated monkeys developed signs of toxicity. These monkeys (Group A) and 2 control animals were sacrificed by decapitation on 30 November 1967. The brains were divided midsagittally: half studied for monoamine content and half for histological changes. The remaining 6 test (Group B) and 2 control monkeys were given a final injection on 30 November 1967 and were sacrificed on 30 January 1968.

The caudate nucleus, cerebrum and brain stem specimens exclusive of the cerebellum from Group A were assayed for norepinephrine and dopamine[10]. The caudate nuclei and cerebrum of the Group B monkeys were homogenized in 0.01 N HCl; norepinephrine and dopamine were assayed in half the homogenate and serotonin in the other half[11].

Brain tissue for histological examination was fixed in 10% neutral formalin and sections stained with hematoxylin-eosin (HE) and with phosphotungstic acid-hematoxylin. Liver and kidney sections were fixed and stained with HE.

Results and discussion. All monkeys in Group A exhibited signs of muscular rigidity, flexion posturing of the extremities, or fine rapid tremors of the distal extremities manifest on intention. 2 of the test monkeys were unable to climb about the cage when provided. 1 exhibited an exaggerated startle reaction, hitting its head upon the cage top when approached. Several monkeys exhibited obstinate progression which occasionally reached near sommersault proportions.

Unlike the first group, Group B monkeys did not show uniform toxic features. 2 appeared normal, 3 showed slight hand tremor, and only 1 showed signs of gross toxicity. The latter animal had pronounced hand tremor and impaired equilibratory coordination function.

Tables I and II summarize the results of the chemical analysis of the 3 groups. The average norepinephrine and dopamine content of the control monkeys in both groups were computed together. The concentration of caudate dopamine was significantly reduced after chronic MnO_2 administration, whereas the cerebral and brain stem norepinephrine content was not affected. Moreover, dopamine depletion appeared related to the degree of toxicity, i.e. Group A monkeys exhibited the greatest disturbance of motor function and also showed the largest reduction in caudate nucleus dopamine. Caudate nucleus concentrations of serotonin were also reduced in association with MnO_2 treatment (Table II).

Cerebral cortex, caudate nucleus, pallidum, thalamus and hypothalamus, hippocampus, subthalamic nucleus, substantia nigra and cerebellum were examined as well as other areas of the lower brain stem. There were no changes in neurones or glial elements nor in the vascular supply in any of the regions studied. Variable, often mil-

Table I. Concentrations of norepinephrine and dopamine in brain after MnO_2 treatment

	Caudate nucleus Dopamine (µg/g ± S.E.)	Cerebrum Norepinephrine (µg/g ± S.E.)	Brain stem Norepinephrine (µg/g ± S.E.)
Control (10)	2.96 ± 0.28	0.17 ± 0.01	0.52 ± 0.06
Group A (5)	1.29 ± 0.33[a]	0.18 ± 0.01	0.49 ± 0.04
Group B (6)	2.17 ± 0.13[b]	0.22 ± 0.03	0.55 ± 0.01

Number of observations in brackets. [a] $P < 0.01$, or [b] $P < 0.05$ when compared with control animals.

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[illegible] changes in liver cells were observed, however, [illegible] changes were noted in both test groups and did not [illegible] with the severity of clinical symptoms.

[illegible] finding that 2 conditions (one spontaneous and [illegible], the other experimental) with manifestations [illegible] extrapyramidal motor dysfunction are associated with [illegible] of dopamine and serotonin in the caudate [illegible] that this deficiency may play a significant role [illegible] producing this dysfunction. In the present studies, the [illegible] of norepinephrine and serotonin in cere[illegible] and brain stem appeared essentially normal after [illegible] treatment. Therefore, failure of the enzyme [illegible] responsible for their formation and destruction [illegible] was probably not the primary reason for depletion. Furthermore, it is important to recognize that clinical and biochemical abnormalities may appear before histopathological changes in brain can be demonstrated, and that the presence of histological changes in specific locations may not explain the clinical manifestations[12].

[illegible] II. Concentrations of serotonin in caudate nucleus and [illegible] after MnO_2 treatment

	Caudate nucleus [illegible]	Cerebrum [illegible]
[illegible] (2)	0.77 (0.66–0.88)[a]	0.19 (0.18–0.20)
[illegible] (6)	0.12 ± 0.05[b]	0.19 ± 0.03

[illegible] of observations in brackets. [a] Mean (range). [b] Mean ± S.E.

Riassunto. Scimmie scoiattolo trattate cronicamente con MnO_2 presentano disturbi del sistema extrapiramidale ed una riduzione delle concentrazioni di serotonina e dopamina nel nucleo caudato. L'intensità dei disturbi extrapiramidali e la riduzione della concentrazione delle amine nel caudato appaiono correlate.

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Manganese Citrate in Therapeutics

by Dr. LEON NORMET

Chief Doctor for The Colonial Army

Having previously described (Presse Médicale, January 10, 1925), the particular therapeutic use of metallic ions in citric acid, we here propose to summarize the observations of six months experience at the military hospital at Hué (Indochina), which provided us information on this subject and in particular on the action of manganese citrate.

We first used the formula already described in our previous article and which we reproduce below:

Citrate of soda	.52 g
Magnesium citrate	10 g
Ferric-potassium tartrate	3 g
Manganese citrate	1 g
Water	1,000 g

Of the different salts used in this formula, manganese citrate is entirely unknown in therapeutics. Our experiments on animals have demonstrated its absolute innocuousness in the indicated doses, but its use in man has revealed important properties which we will describe here.

At the dosage of a 1/4 cc per kilogram, an intravenous injection of the solution given above, after one hour caused a temperature elevation with chills and profuse sweating. The effect was much more pronounced in

very thin individuals. After this reaction, the temperature progressively returned back to normal six to seven hours after injection, even in the most feverish patients. This thermal reaction persisted if the dosage on manganese citrate was diminished, as long the dose did not go below 0.40 g p. 1000. The reaction weakened and disappeared completely below 0.20 g. p. 1000. We suppose that this is due to the energy oxidation phenomenon produced by the combination of ferric-potassium tartrate and manganese citrate. Dangerous symptoms were never produced, and the patient was generally not discomfited except in cases of severe anemia in which the treatment was poorly tolerated. We then varied the amount of manganese citrate in our formula according to the disorder to be treated, and we discovered that in anemia where the morphogenic action of citrates is a main concern, we could, without difficulty, lower the amount of manganese citrate to 0.05 p. 1000. At this level, no reaction is discernible, but the tiny trace of the injected metal (0.0001 g approximately) seems to have a useful effect on the formation of hemoglobin.

This effect was also seen in the treatment of infections where manganese plays an extremely important role. Its action works on certain toxins affecting the nervous system. It is very clear in cases of influenza, cerebrospinal meningitis, measles, and icterohemoglobinuria, where we have had the opportunity to observe it. The effect is less great in cases of typhoid fever, where we obtained a lowering of the temperature of short duration, without other influence on the course of the disease.

How can the manganese get rid of the neuronal toxins which are already present? We are left for the moment with unverified hypotheses. It seems

that the phenomena produced in vivo by these cells are comparable to those which Ramon obtained in vitro with formol on the diphtheria toxin. It is likely that certain toxins are completely changed by the loss or addition of some molecules of oxygen and, that this is the secret of the therapeutic action which in a number of cases has brought about remarkable results.

Whatever it is, these results can only be obtained with sufficient doses of manganese which have the disadvantage of bringing accompanying acute reactions which sometimes distress the patient or his family. We have tried to avoid this distress, and we have succeeded very simply by adding to the solution 1/20 of its volume of neutral glycerine. The glycerine acts to slow down the ionization and oxidation of the salts. It does not hinder at all the therapeutic effects which take place normally. It is a kind of regulator which counteracts the action of the abrupt injection into the vein of the energetic oxidizing agent.

The formula thus modified gives us constant results in the disorders previously mentioned, without any reaction. We regret that we did not have the chance to try it in other cases where the therapeutic measures we used were insufficient or had no effect at all (tetanus, poisoning from mushrooms, etc.). We propose to try it in every case where a neurotropic toxin is present in the nerve cell, and where the usual therapeutic measures have no effect.

An epidemic of influenza gave us a good field for experimentation, and it was with this disease that we were best able to study the effects of our formula. Below, we summarize the observations of a case of broncho-pneumonic influenza.

Observation --

Phu, 25 years old. Entered the hospital April 12 with chills fever and respiratory distress. Nothing clearly heard with a stethoscope. Diagnosis: Influenza.

April 14 -- source of bronchopneumonia, the base of the left lung: numerous fine crackling rales and tubular whistles, temperature 39.6°, severe dyspnea. Scarified air-holes, diuretics, fever-reducers.

April 16 -- Temperature 39.3°, patient very breathless, pressure above and below the sternum; a second site of bronchopneumonia at the middle and base of the right lung.

April 17 -- Persistance of the same symptons, coma, very serious condition. Injection of 10 cc of the citrate solution at 10 a.m. By the afternoon, the temperature fell to 37°.

April 18 -- No fever, the patient spent a good night. Dyspnea substantially diminished. On the left: subcrackling rales in place of the fine crackling rales; the tubular whistle was barely perceptible. The right lung was nearly clear with still a few subcrackling rales at the base. Patient was wide awake and asked to eat.

April 19 -- No more fever, no more dyspnea, there remained a small source of subcrackling rales in the right base. The patient ate.

April 20 -- Normal temperature, normal pulse, no more symptoms discernible through stethoscopic examination. Cure.

LE CITRATE DE MANGANÈSE EN THÉRAPEUTIQUE

PAR

Le Dr Léon BERNARD
Médecin [illegible]

Ayant indiqué antérieurement (*Paris médical*, 10 janvier 1925) le parti que l'on pouvait tirer en thérapeutique des propriétés très particulières que présentent les ions métalliques mis à l'étude chimique, nous nous proposons de résumer ici les conclusions que six mois d'expérience dans un service d'hôpital à [illegible] nous ont permis d'acquérir sur ce sujet et en particulier [illegible].

Nous avons d'abord utilisé la formule déjà publiée dans notre article précédent et que nous reproduisons pour rappeler :

Citrate de soude	52 grammes
Citrate de manganèse	10 —
Tartrate ferrico-potassique	3 —
Citrate de magnésie	1 —
Eau Q. S. p.	1 000

Des différents sels entrant dans cette formule, le citrate de manganèse était tout à fait inconnu en thérapeutique. Nous avons vu sur les animaux nous avoir démontré son innocuité absolue à de fortes doses, mais son emploi chez l'homme nous a révélé des propriétés importantes que nous allons indiquer ici.

A la dose d'un quart de centimètre cube par kilogramme, l'injection intraveineuse de la solution ci-dessus provoque une heure après la piqûre une élévation thermique avec frisson et transpiration abondante. L'élévation thermique est plus ou moins forte chez les sujets. Elle l'est beaucoup plus chez les individus très anémiques. A cette réaction succède un abaissement progressif de la température qui descend à la normale même chez les sujets fébriles au bout de sept heures après la piqûre. Cette réaction thermique persiste si l'on indique la dose de citrate de manganèse tant que cette dose ne dépasse pas un dixième de [illegible] par kilogramme. Au-dessus de [illegible] il est [illegible] et disparaît complètement [illegible] 0,20 [illegible]. Nous avons vu que cette réaction est due à des phénomènes d'oxydation [illegible] produits par le complexe tartrate ferrico-potassique et citrate de manganèse. Elle ne s'accompagne jamais de phénomène dangereux et n'inquiète généralement pas le malade, sauf dans les anémies graves où elle est mal supportée. Nous avons donc été amené à faire varier la dose de citrate de manganèse dans notre formule selon les affections à traiter et nous avons reconnu que dans l'anémie où l'on veut obtenir uniquement l'action morphogène des citrates on pouvait sans inconvénient abaisser la dose de citrate de manganèse à 0,05 p. 1 000. A ce taux on ne constate plus aucune réaction, et cependant la trace infime de métal injectée (0,05 environ par kilogramme) exerce une action utile sur la formation de l'hémoglobine.

Il n'en va pas de même dans le traitement des infections, où le manganèse joue un rôle extrêmement important. Son action porte sur certaines toxines fixées sur le système nerveux. Elle est très nette dans la grippe, la névralgie cervico-brachiale, la sciatique, l'ictère hémolytique [illegible] et l'angine de la convalescence. Elle est peu intéressante dans la fièvre typhoïde, où nous avons obtenu un abaissement de température de courte durée sans influence sur la marche de la maladie.

Comment le manganèse peut-il débarrasser le neurone des toxines qu'il a déjà fixées? Nous en sommes réduits à des hypothèses inévitables pour le moment. Il semble que le phénomène produit *in vivo* par ce corps soit comparable à celui que Ramon a obtenu *in vitro* avec le formol sur la toxine diphtérique. Il est probable que certaines toxines sont entièrement modifiées par la perte ou l'addition de quelques molécules d'oxygène et que c'est là tout le secret d'une action thérapeutique qui donne dans de nombreux cas des résultats remarquables.

Quoi qu'il en soit, ces résultats ne peuvent être obtenus qu'avec des doses de manganèse suffisantes et qui présentent l'inconvénient de s'accompagner d'une réaction assez vive, de nature à impressionner quelquefois le malade ou son entourage. Nous avons essayé d'éviter cet inconvénient et nous avons pu y parvenir très simplement en ajoutant à la solution un vingtième de son volume de glycérine neutre. La glycérine agit en ralentissant l'absorption et l'oxydation des sels. Elle n'entrave nullement les effets thérapeutiques, qui se développent normalement. Elle est en quelque sorte un régulateur qui s'oppose au coup de fouet de l'injection lorsque dans la veine d'un oxydant énergique.

La formule ainsi modifiée nous a donné des résultats constants dans les affections précédentes

[illegible] citées sans aucune réaction. Nous regrettons de n'avoir pas eu l'occasion de l'essayer dans d'autres cas où elle nous [illegible] très indiquée et où nos moyens [illegible] sont insuffisants ou nuls (tétanos, intoxication par les champignons, etc.). Nous nous proposons de l'essayer dans tous les cas où une toxine neurotrope se trouve fixée sur la cellule nerveuse, les moyens [illegible] restant sans action [illegible].

Une épidémie de grippe nous ayant donné un bon champ d'expérimentation c'est dans cette affection que nous avons pu étudier le mieux ses effets. Nous donnons ci-dessous l'observation résumée d'une broncho-pneumonie grippale guérie par une seule injection.

[illegible] pital le 12 *avril* avec frisson, fièvre, gêne respiratoire. Pas de signes stéthoscopiques nets. Diagnostic : grippe.

14 *Avril*. — Foyer de broncho-pneumonie à la base du poumon gauche : [illegible] râles crépitants fins et sous le [illegible], température [illegible], dyspnée intense. Ventouses scarifiées, diurétiques, antithermiques.

16 *Avril*. — T. [illegible], [illegible] très oppressé, [illegible] et sous-sternal ; un deuxième foyer de broncho-pneumonie a fait son apparition à la partie moyenne et à la base du poumon droit.

17 *Avril*. — Persistance des mêmes symptômes, stupeur, état très grave. Injection de 20 centimètres cubes de [illegible] solut. [illegible] à [illegible] du matin. Dans l'après-midi, la température tombe [illegible].

18 *Avril*. — Pas de fièvre, le malade a passé une bonne nuit. La dyspnée a bien diminué. A gauche : râles sous-crépitants, à la place des râles crépitants fins ; le souffle [illegible] est à peine perçu. Le poumon droit est presque complètement dégagé, avec encore quelques râles sous-crépitants à la base. Malade bien éveillé, demande à manger.

19 *Avril*. — Pas de fièvre, plus de dyspnée, il reste encore un tout petit foyer de râles sous-crépitants à la base droite. Le malade mange.

20 *Avril*. — Température normale, pouls normal, plus de signes stéthoscopiques. Guérison.

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Neutron Activation Analysis: The Determination of Manganese*

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It has been noted elsewhere (1, 2) that the large number of methods for determining manganese in samples of biochemical interest has produced limited amounts of published data. This essential micronutrient, being primarily intracellular, occurs in extremely low concentrations in such extracellular pools as serum and plasma. Conversely, these fluids are enriched with elements which are apt to hinder the assay for manganese or for many other "trace" constituents. Hence such systems seemed ideal for testing the potentiality of neutron activation analysis, particularly after Bowen (3) had shown the high sensitivity of this procedure.

In order for this approach to be effective it should meet the following requirements. It should be inherently simple. Its accuracy and precision should be within ±5% for a mean of the order of 1×10^{-10} g, so that tissue fluids can be assayed sequentially, even in a small animal such as the mouse.

The present communication shows how these requirements have been met by means of neutron activation analysis and gives examples of the usefulness of this approach in biochemical work. Before this study, it has been thought that the concentration of manganese is characteristic of a given organ rather than of the species from which the organ was obtained. The present data indicate that this conclusion may have to be revised.

EXPERIMENTAL PROCEDURE

Materials and Methods

1. Instruments—The reactor used was the B.N.L. Graphite Research Reactor, which operated between 17 and 20 megawatts, yielding a total neutron flux of 1.07×10^{13} neutrons per cm² per second, with a "Cadmium ratio"[1] of about 38 at the facility used. The ambient temperature was less than 90°.

The gamma ray spectrometer unit included a thalium-activated cylindrical NaI crystal (diameter, 12.75 cm; height, 10.20 cm; shielded and mounted to a 12.75-cm DuMont Photomultiplier Tube). This was connected to a preamplifier, a linear amplifier (Cosmic Radiation Laboratories), a power supply (Beva Laboratory model 300), and a Penco model PA-4 100 channel pulse-height analyser. (Conventional apparatus might be used if desired.)

The Coleman Universal spectrophotometer, model 14 with cuvette No. C, was used. Filtration apparatus (Millipore Company, catalogue No. XX10 025 00) was provided with white millipore paper (catalogue No. HAWP02500). Quartz pipettes calibrated to deliver 50 and 100 μl of water were used. The demineralizer used was the Bantam model BD-1 from the Barnstead Company.

The tube holder is shown in Fig. 1. Its essential feature was that it kept the tubes slightly bent so that the sample remained localized in the middle during drying. A simple rack, holding one or two polyethylene tubes (Clay-Adams, Inc. PE 390), can be constructed by sealing a glass rod across the top of a 30-ml beaker and covering it with a 250-ml beaker.

The Dolecknum Heat Sealer (model No. DS-150-W), needles (Yale, disposable, plastic hub, 18-20 gauge), and aluminum planchets, I.D. 3.1 cm and O.D. 4.0 cm, were used.

2. Reagents—Water, triple distilled and demineralized, was a reagent used, as was distilled nitric acid, Baker's Reagent Grade concentrated HNO_3, which was handled as recommended by Thiers (4).

The manganese standard was 3.07682 g of Baker and Adamson spectrographically pure $MnSO_4 \cdot H_2O$, dried to constant weight and dissolved in 1000 ml of the demineralized water to provide 1 mg of Mn per ml (Solution A). One milliliter of this was diluted to 1000 ml with the demineralized water (Solution B). Fifty microliters of this solution contained 0.050 μg of Mn. Solution B was considered as unstable.

The oxidizing solution (5) was a mixture of 75 g of $HgSO_4$ (Baker and Adamson) dissolved in 400 ml of the concentrated nitric acid (Baker's), 200 ml of distilled water, and 200 ml of concentrated phosphoric acid (Baker and Adamson, 89%). After the addition of 3 mg of $AgNO_3$, the solution was made up to 1000 ml with distilled H_2O. Of this, 5 ml were diluted to 100 ml with distilled H_2O and ammonium persulfate (Baker and Adamson, 1.5 g) was added with a calibrated spoon, for the oxidation to be described.

Tetraphenylarsonium chloride powder (K & K Laboratories, Inc.) was used in a 1% solution in H_2O for the precipitation of MnO_4^-. A 0.1% solution of this salt was used for the washing of the precipitated tetraphenylarsonium permanganate.

3. Principles of Procedure—The isotope of manganese encountered in tissues is the stable Mn^{55}. Upon neutron bombardment this becomes Mn^{56}, which decays with a half-life of 2.58 hours by emission of energetic beta and gamma rays. If activa-

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[1] A measure of the ratio between the thermal and the resonance neutron flux (22). It is defined by the ratio of the neutron intensities recorded by the same device in the absence and in the presence of cadmium shielding.

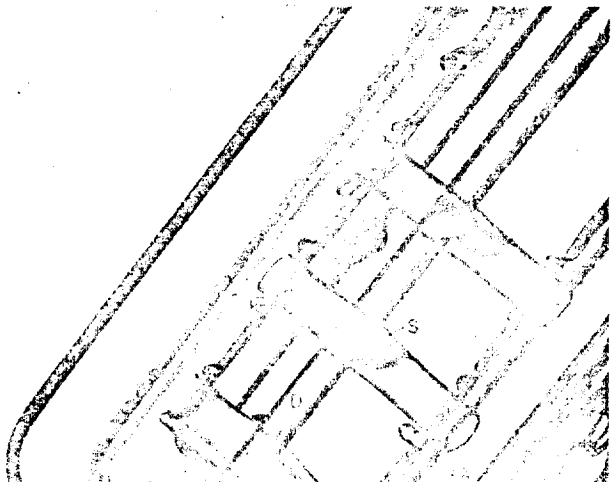

FIG. 1. Tube holder. *A*, tray; *B*, cover; *C*, rack; *D*, rod; *E*, tube (4.0 × 0.6 cm); *S*, sample.

tion conditions are constant, this radionuclide is produced in amounts proportional to the parent element Mn^{55}. To control these conditions, a standard consisting of a measured quantity of manganese is activated with the sample for periods of time sufficient to yield statistically significant amounts of Mn^{56} in both. Because elements such as K, Na, Cl, and Br will become intensely radioactive and will mask the Mn^{56}, the latter is separated chemically from the mixture of isotopes. This separation is facilitated by the addition to the sample of a carrier for manganese. Use of a carrier *is not valid* unless it be proven that the carrier becomes and remains mixed with the radionuclide throughout all the chemical steps, a property referred to as "true carrier." This point is very important when dealing with transition group elements such as manganese which may be multivalent whereas the carrier used for their separation is divalent and, therefore, possibly not a true carrier. Multichannel pulse-height analyzers provide resolution and automatic recording of gamma energy spectra, thus offering higher precision, accuracy, and convenience than do the conventional assemblies.

4. Method—This consisted of collection of the sample, activation in the reactor, addition of carrier, wet digestion to destroy organic matter, oxidation of manganese to permanganate, precipitation of the latter, and, finally, determination of its radioactivity and of its recovery. These steps were duplicated with standard.

Collection—Contamination is of concern only until activation has been completed (6). It was prevented by (*a*) rinsing the apparatus with the distilled nitric acid and triple distilled water, (*b*) rinsing the skin with alcohol and ether, (*c*) using hypodermic needles which yield blood containing the same amount of manganese as did platinum cannules, (*d*) using quartz instruments for sampling the solid tissues, and (*e*) conducting the preparatory steps under dust-free conditions.

Samples were placed in the middle of the tubes (Fig. 1) and coagulated by drying at 85° for 1 hour. The tubes were then heat-sealed at both ends, and were placed in a container with similarly treated standard (= 50.0 μl of Solution B).

Activation—The container was introduced into the reactor for 2 hours for the analyses of extracellular pools and for 30 minutes in the case of solid tissues. After this time, the polyethylene tubes were removed and rinsed externally with dilute nitric acid followed by distilled water. Both ends were then cut off with scissors and the sample was flushed through with 20 ml of hot nitric acid into a 250-ml Erlenmeyer flask containing 1.5 ml of Solution A. The location of the sample in the middle of the tube facilitated this process.

Wet Digestion and Oxidation to MnO_4^-—To the flask was added 0.2 ml of 30% H_2O_2. It was then heated on a hot plate until the contents were almost dry. This step was repeated with an additional 5 ml of nitric acid plus 0.2 ml of H_2O_2. Thereafter, 100 ml of the oxidizing solution were added and brought to boiling for 2 minutes. This was proven by direct experimentation to oxidize the manganese fully to permanganate.

Separation of Permanganate—After the flask was allowed to cool, 5 ml of the 1% tetraphenylarsonium solution were added. This resulted in the immediate specific precipitation of tetraphenylarsonium permanganate. The contents of the flask were filtered and the precipitate was then washed with 100 ml of the 0.1% phenylarsonium solution. The filter paper was removed, the white border was discarded, and the purple portion was placed on a planchet for assay of the radioactivity. Losses of precipitate were of little consequence up to this point, but they became important thereafter.

Determination of Manganese—The gamma spectrum was recorded by the pulse-height analyzer under constant geometry. The radioactivity represented by the entire energy peak of Mn^{56} at 0.845 m.e.v. was determined from the recorded spectrum with a graphical method (7–9). To determine recovery, the filter paper was placed in the Erlenmeyer flask and the oxidation was repeated.

The ensuing permanganate was made up to 100 ml with the oxidizing solution, and its concentration was determined by reading the optical density at 522 mμ against a blank solution containing all the reagents except for manganese.

The standard was treated identically. Activation of empty tubes showed that no correction for radioactivity "blanks" was necessary.

The amount of manganese recovered as permanganate was read from calibration curves (10) made by diluting Solution A serially. Oxidation was carried out as described. These calibration curves obeyed the Beer-Lambert law and were checked every few weeks.

The millimicrograms of manganese present in the original sample were computed from the following formula:

$$M = \frac{W \times S_1}{S_2}$$

in which M = mμg of Mn in the sample, W = mμg of Mn in the standard, S_1 = specific activity of the sample, and S_2 = specific activity of the standard.

Exchange of Added Carrier with Endogenous Manganese—In order to test the validity of the radiochemical procedure re-

ported here, a rat was injected with Mn^{54} and allowed to metabolize the tag normally for 50 days. During that time, the tracer must have entered the various physiological compartments in which manganese might exist as a multivalent element (11–14). The animal was then killed, and twelve homogenates of various strengths were prepared from liver, brain, lung, kidney, and muscle. The concentration of the tracer was determined in each. The aliquots were submitted to all the steps listed under "Materials and Methods" with the exception of the neutron activation. The Mn^{54} radioactivity recovered corresponded to the chemical yield of the carrier manganese within 5%, thus proving that the added carrier was a true carrier of tissue manganese.

Control of Losses—That there was no loss of manganese during drying and the other steps preceding activation was shown with serum samples to which carrier-free $Mn^{54}Cl_2$ was added *in vitro*.

The precipitation of carrier-enriched Mn^{54} as the tetraphenylarsonium permanganate resulted in losses ranging from 5% to 12%, inherent in the precipitation and washing of the permanganate. Nonetheless, the corresponding losses of the added carrier have also ranged from 5% to 12%. Hence, the specific activity (c.p.m.Mn^{54}/mgMn^{55}) remained constant within ±3%. This proved that the carrier method of correcting for the small amount of tissue manganese lost during these radiochemical steps was valid.

Recovery of Manganese Added to Serum—These experiments were performed in order to assess the linearity of the data yielded by the present method. Fig. 2 summarizes experiments with one pool of serum, aliquots of which were supplemented with known additions of manganese. The added metal was recovered within a total variance of ±3% from a line with a slope of +1, fitted by eye to the data. As can be seen, this line intercepted

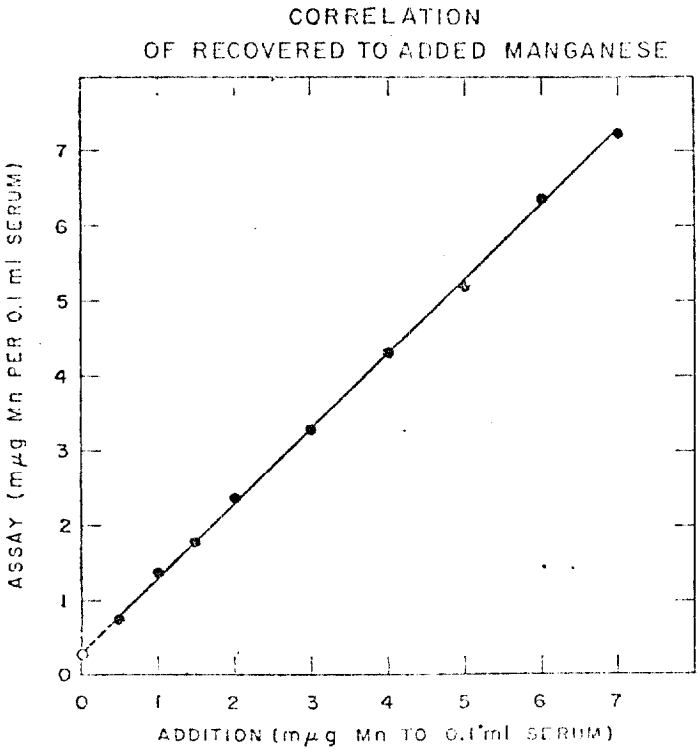

FIG. 2. *Solid circles* correspond to serum samples to which manganese was added; the *open circle* depicts the Mn concentration in that serum.

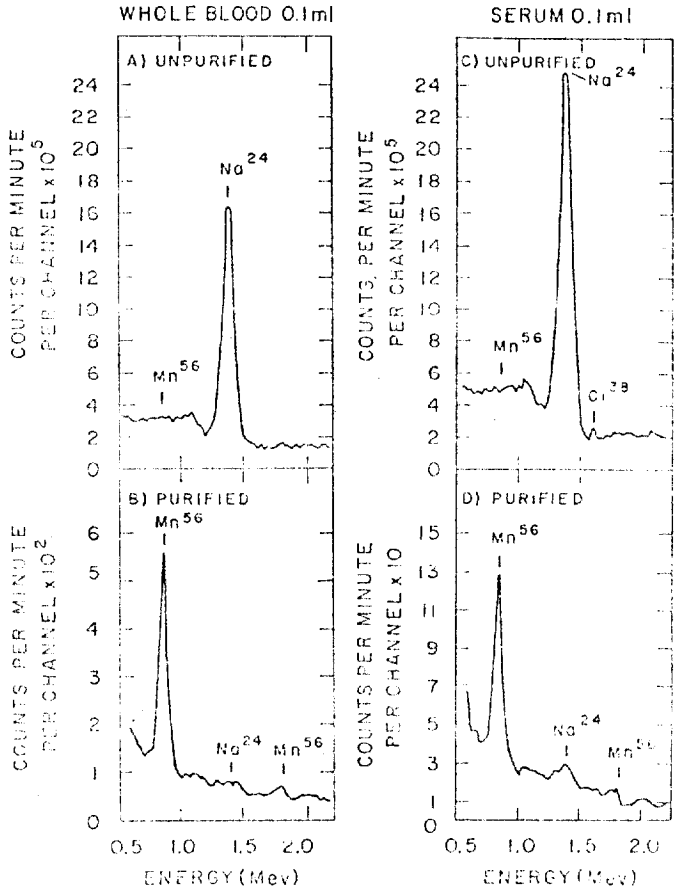

FIG. 3. The gamma ray spectra of 100 μl of blood and of serum are shown at the *top*. The corresponding purified radioactivities are at the *bottom*.

the ordinate at the value of the experimentally determined concentration of serum manganese. This linearity continued, at least up to 400 mμg of Mn per sample.

Radiological Purity of Mn^{56}—Fig. 3 shows the energy spectra of precipitated permanganate from neutron-activated serum and blood as compared to those of the untreated samples. The high resolution of the energy spectrum of manganese as a result of the purification is evident.

This purification procedure diminished the contaminating Na^{24} to a maximum of 4×10^{-6} parts of its original concentration. In most instances, Na^{24} could not be detected in the precipitate. Still, resolution of the gamma energy spectrum is recommended in practice, because there can be no certainty of such purification without this proof.

Alternate Procedural Steps—Earlier in this work, serum and blood were collected on low ash filter paper for easier combustion in the Thomas-Schöniger apparatus (15, 16). The manganese concentration of this paper was about 2 mμg per sheet and could not be sufficiently lowered by washes with water, nitric acid, and ethylenediaminetetraacetate solutions, or by electrophoresis. However, this procedure might still prove to be convenient for manganese-rich samples.

Others (17, 18) have recommended separation of tetraphenylarsonium pertechnetiate and, by analogy, of permanganate (19) by extracting the salt from water with chloroform. This procedure is not recommended here because it suffers from too many drawbacks, which could not be corrected by purification of the

chloroform or by substitution of this solvent by carbon tetrachloride, ether, mineral oil, chlorobenzene, pyridine, or xylene.

It was essential to oxidize manganese to permanganate *precisely* as defined here, because alternative combinations of acids or of oxiding agents proved ineffective.

RESULTS

The precision of this measurement was investigated on a sample of normal human serum, eleven aliquots of which showed 0.261 ± 0.009 mμg of manganese per 100 μl of serum. The variance was ±3.4%, indicating that this procedure was applicable to the determination of the manganese concentration in some tissues and in some reagents which are of interest in biochemical studies of this element.

It is evident from Table I that the manganese concentration in samples of human serum, plasma, or blood varied within a rather narrow range. These samples were obtained by venipuncture. This is significant because samples, obtained by pricking the skin, showed a range of 4.5 to 13.0 μg per liter of serum (eleven samples from five individuals). The larger variance and the higher mean are attributable to introduction of tissue manganese into these sera as a consequence of tissue trauma. This constitutes a less readily recognized type of contamination than that caused by extrinsic sources and might explain some discrepancies alluded to below.

The narrow range of the manganese concentrations in blood and plasma led to a test of their stability after metabolic loads with manganous sulfate. For this, three rabbits were injected intravenously with the amounts of manganese stated in the table, whereas one animal was not. Two weeks later, the plasma manganese concentration was quite similar in all four animals, whereas the blood of the injected animals contained increasingly more manganese. This indicated that it is the intracellular manganese level that might fluctuate, whereas the extracellular level seemed less prone to do so.

TABLE I

Material	Grade	No. of samples	No. of analyses	Manganese	
				Mean	Range
				μg	
Human serum		16	28	2.50	2.05– 2.97[a]
Human plasma		7	9	2.69	2.10– 3.02[a]
Rabbit plasma		4	4	1.01	0.91– 1.11[a,b]
Human blood		7	8	11.60	9.01– 14.50[a]
Rabbit blood		4	4	6.85	4.12– 9.70[a,b]
Rat bile		5	5	66.30	29.80–190.00[a]
Rabbit liver		4	4	1.69	1.65– 1.71[c]
Mouse liver		3	3	1.14	1.00– 1.26[c]
Rabbit diaphragm		2	2	0.19	0.16– 0.21[c]
Mouse diaphragm		3	3	0.27	0.23– 0.31[c]
Ethylenediamine-tetraacetate	Reagent	2	2	51.5	49.50– 54.00[d]
Ascorbate	Reagent	2	2	25.5	15.00– 35.00[d]
$K_2S_2O_8$	Reagent	2	2	6.3	4.90– 7.70[d]
Sucrose	Reagent	1	1		5.30
NaCl	Reagent	1	1		9.40
NaCl	Optical crystal	3	3		$<6 \times 10^{-10}$[d]

[a] Per liter.

[b] Animals injected with 0, 2.5, 10.0, and 20.0 μg of Mn^{++} 2 weeks earlier.

[c] Per g of wet tissue.

[d] Per mole.

Because this element is excreted presumably via the bile (1, 2), its concentration was studied in rat bile obtained by catheterization of the common bile duct. These experiments revealed (*a*) that sequential analyses for this trace element could be performed on samples from a small body pool, and (*b*) that the manganese concentration in bile varied markedly from one animal to the next in a manner characteristic of an excretory pool.

Analyses of the solid tissues showed concentrations similar to those found by others (2) but, contrary to expectation (1, 2), the variance among species may be marked in some instances.

Analyses of the reagents listed on the table showed that significant contamination could occur during studies of sequestration, oxidation, or reduction of manganese in tissues, tissue fractions or extracts, because natural ligands tend to concentrate the metal.

DISCUSSION

A common error in activation analysis is to assume that the radionuclide which one determines has originated solely from the element which one studies. In this instance, production of Mn^{56} during neutron bombardment of biological materials occurs overwhelmingly as a consequence of the reaction: $Mn^{55}(n, \gamma)Mn^{56}$. Nonetheless, two additional reactions need be accounted for, namely, $Fe^{56}(n, p)Mn^{56}$ and $Co^{59}(n, \alpha)Mn^{56}$ (20–22). One can calculate the contributions to Mn^{56} by these reactions for the conditions of activation and detection used here, according to formulations established by others (20–22). These calculations were omitted for the sake of brevity. They showed that, in the case of blood, iron contributed 4 to 5% of the Mn^{56}. In the case of plasma and tissues, neither iron nor cobalt contributed significant amounts of this nuclide.

Although the evidence presented is characterized by a high degree of internal consistency, it is not always in agreement with published information. Bowen's samples (3) are concordant with the ones obtained here after pricking the skin, but both sets are higher than the present ones for venous blood because of the reasons already indicated. The discord becomes pronounced indeed when our own data for venous blood are compared with those of others (23–25); our values are lower by factors ranging from 2 to 10 and so is the individual variance. Such significant discrepancies could not be dismissed because our tissue analyses were similar to the reliable ones of Fore and Martin (26): the concentration of manganese is more readily amenable to quantitation in tissues than in blood or plasma, because the abundance of this element in tissues may be higher than in blood or plasma by three orders of magnitude. Hence there had to be developed a confirmatory spectrographic procedure. This suffered from the disadvantages indicated by Thiers (4), which limited its desirability as a routine procedure, but not as a method of comparison. As will be reported elsewhere,[2] its results coincided with those of neutron activation analysis as recommended here.

SUMMARY

Criteria pertaining to biochemical applications of neutron activation analysis were formulated and dealt with experimentally.

[2] J. Forrest and H. L. Finston, to be published.

An ultramicromethod was described which permits the determination of the essential microconstituent, manganese, in biological samples, including small pools with low concentrations.

Samples containing, on the average, 0.261 mμg of manganese were analyzed with a variance of ±3.4%.

The mean concentration of manganese in human plasma was 2.69 μg per liter. The plasma concentration remained constant in the rabbit after injections of manganous sulfate whereas that of whole blood increased.

Manganese concentrations were determined in some tissues and in some reagents.

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DISTRIBUTION OF MANGANESE IN FOODS*

By

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Within the last few years it has been observed that a number of elements, although frequently occurring in only minute amounts, are universally present in plant and animal materials. Their universal occurrence can hardly be a matter of chance and, consequently, numerous investigators have attempted to ascertain what functions these elements perform in plants and animals. One of these elements, manganese, has received considerable attention from the standpoint of both plant and animal nutrition.

In 1913 Osborne and Mendel (1) observed that better growth of rats fed on "artificial protein-free milk" was secured when traces of iodine, fluorine, aluminum, and manganese were included in the ration. Daniels and Hutton (2), in 1925, reported that rats on a milk diet are able to produce five generations of normal young if small amounts of manganese, fluorine, and aluminum are added along with sodium silicate. The beneficial effects noted in these experiments, however, could hardly be attributed with certainty to the presence of manganese, since this element was only one of several added. Other investigations, especially in recent years, have been made in which manganese alone was added to various rations, some of which probably were inadequate from standpoints other than manganese deficiency. Levine and Sohm (3) reported that manganese produced a favorable effect upon rats as indicated by their appearance and activity as well as by a more rapid growth of their offspring. Richet, Gardner, and Goodbody (4) demonstrated that small amounts of manganese when administered to dogs every three days enhanced growth, whereas the reverse was true if the element was given more frequently. That manganese exerts a favorable effect upon the growth of mice and rats has been observed by Bertrand and Nakamura (5) and McHargue (6) respectively. McCarrison (7) likewise found that the element accelerated growth if given to the extent of 0.009 mg. per rat daily, but if the intake of manganese was increased

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to 0.56 mg. a definite retardation of growth followed. That the element is stored throughout life, but especially prior to maturity, and that the rate of storage can be markedly increased by the addition of manganese to either a complete or incomplete ration has been demonstrated by Skinner, Peterson and Steenbock (8).

With the discovery by Hart, Steenbock, and associates (9) that copper functions in hemoglobin formation, a new field of investigation was opened for the study of manganese as well as of various other elements. Titus and Hughes (10, 11) and Beard and Myers (12, 13) reported that manganese, like copper, is an active agent in the regeneration of hemoglobin, but the work of Hart, Steenbock, and associates (14, 15), Krauss (16), and Lewis and associates (17) throws the weight of experimental evidence against this conclusion.

Regardless of the final conclusion concerning the function of manganese in hemoglobin regeneration, most investigators are agreed that the element does play some important rôle in the animal organism. Therefore, in order that a sufficient amount of the element may be included in the human diet, a knowledge of its occurrence in various food materials is highly desirable. A previous publication from this laboratory (18) gave the manganese content of a number of our common food materials. More recently several investigators (19, 20, 21, 22) have contributed to the knowledge of the occurrence of this element in foods.

In this paper are presented the data for 83 food materials, the greater number of which have not been analyzed previously for manganese. The data given herein, together with those in a previous publication, have been combined so as to summarize concisely the occurrence of manganese in the common classes of foodstuffs.

Experimental

Since the method of preparation of the samples has been given in a previous publication (23), it need not be described here. The manganese determinations were run by the periodate method in which, with one exception, phosphoric acid was used for extraction of the ash. The procedure given by Davidson and Capen (24) was followed in the analysis of samples containing 0.1 mg. or more of manganese, which included mainly the vegetable materials. Foodstuffs containing very small amounts of the element were analyzed according to the modification used by the authors (25) in the determination of manganese in animal materials. The high chloride content of olives did not permit a determination of manganese by either of these procedures. Instead, the ash was extracted with dilute H_2SO_4, and

after the addition of 5 cc. of concentrated HNO_3 the chlorine was removed by evaporating until SO_3 fumes came off. To insure complete removal of the chlorine this step was repeated twice. The solution was then oxidized to permanganate and the color compared with the standard in the usual manner. In most instances, triplicate determinations, one of which involved the recovery of a known amount of added manganese, were made on each sample. If a recovery of 90 per cent or better was not obtained, the analysis was repeated.

Since it was the aim of this investigation to supplement the data previously reported, duplication of analyses was avoided. In two instances, however, it seemed desirable to check some of the results obtained in the previous investigation. In the former publication certain materials, *e.g.*, fish, were reported to contain either no manganese or only traces of the element. With a more sensitive method available, it seemed probable that the actual amounts of manganese in these materials could be detected and, accordingly, some of the samples have been reanalyzed. At the other extreme was a sample of blueberries which appeared unusually high in manganese. The original sample was reanalyzed and on more thorough oxidation was found to be even higher in manganese (299 mg. per kg., dry basis) than was previously reported (122.4 mg.). A new sample was therefore procured and was found to be still higher (314.9 mg. per kg.). From these results it appears that blueberries are normally high in manganese.

In Table I are given the moisture and manganese content, calculated on both the fresh and dry basis, of 83 representative food materials. It will be noted that in no case was the manganese concentration too low to warrant a numerical value, thus substantiating our belief that the more sensitive method would permit detection of the minute amounts present. On the fresh basis the concentration of manganese ranged from 0.028 mg. per liter of milk to 49.9 mg. per kilo. of bran flakes. Of the total number of foodstuffs analyzed, thirty-six were found to have a concentration of less than 1 mg., twenty-five from 1 to 5 mg., and only twenty-two more than 5 mg. of manganese per kilo of fresh material. On the dry basis the manganese content varied from 0.091 mg. in bacon to an exceptionally high concentration of 314.9 mg. per kilo. in blueberries. The six samples of milk varied from 0.017 to 0.048 mg. of manganese per liter. Three of these milks were produced by groups of the University herd on three different rations and three samples were obtained from herds in other states. Variations in manganese were less extensive among milks from the groups of the University herd than among milks from herds in other states.

TABLE I

MANGANESE CONTENT OF FOODS (EDIBLE PORTION)

Food*	Moisture	Manganese Content	
		Dry basis (100°)	Fresh material
	per cent	mg. per kg.	mg. per kg.
Almonds	3.9	20.2	19.4
Artichoke	84.4	23.2	3.6
Bacon	13.7	0.091	0.078
Beans, string (2)	91.4	28.2	2.4
Beef, casings	81.1	1.3	0.25
Beef, lung	80.3	2.7	0.52
Beef, pancreas	80.0	3.3	0.65
Beefsteak, T-bone	74.5	0.65	0.16
Blueberries	85.9	314.9	44.4
Brains, calf	80.7	2.3	0.44
Bran flakes	6.5	53.4	49.9
Brazil nuts	4.3	9.6	9.2
Bread, white	35.0	4.7	3.1
Butter	13.0	0.45	0.39
Cantaloupe	94.6	7.7	0.42
Cauliflower	93.0	25.2	1.7
Celery cabbage	95.0	23.3	1.2
Cheese, American	31.6	0.77	0.53
Cheese, cottage	75.0	2.1	0.53
Cheese, Swiss	37.5	3.1	1.6
Chestnuts, Italian	34.5	56.0	36.7
Chocolate, bitter	5.0	32.2	30.5
Cocoa	4.5	37.0	35.3
Coconut	46.4	25.0	13.1
Corn, sweet	68.0	4.7	1.5
Corn flakes	6.2	0.52	0.49
Corn-meal, white, prepared	6.2	1.6	1.5
Corn meal, yellow, prepared	6.0	3.1	2.8
Cranberries	88.1	24.9	3.0
Cream of Wheat	7.4	4.8	4.5
Cucumbers	96.9	48.4	1.5
Dandelion greens	82.4	19.2	3.4
Eggplant	92.2	14.6	1.1
Eggs	72.8	1.1	0.30
Egg yolk	49.5	2.3	1.1
Endive	93.6	35.0	2.2
Filberts	2.0	42.8	41.7
Fish and sea foods			
Cod	80.7	0.63	0.12
Crab	77.1	1.3	0.30
Lobster	84.3	2.5	0.39
Mackerel	72.6	0.87	0.24
Oyster	90.0	20.8	2.1
Red snapper	79.2	0.63	0.13
Shrimp	71.0	1.0	0.29

TABLE I (*continued*)

Food*	Moisture	Manganese Content	
		Dry basis (100°)	Fresh material
	per cent	mg. per kg.	mg. per kg.
Flour, buckwheat	13.8	24.2	20.9
Flour, Graham	12.7	49.4	42.8
Flour, patent	12.0	4.5	4.0
Flour, rye	6.4	20.7	19.4
Hominy	9.7	1.2	1.1
Honey	12.6	0.36	0.31
Lamb chops	69.2	1.2	0.37
Lemons	88.9	3.6	0.40
Lettuce, leaf	93.8	167.0	10.4
Milk, whole			
Maximum		0.37	0.048**
Minimum		0.13	0.017**
Average (6)	87.5	0.22	0.028**
Milk, colostrum	85.1	0.28	0.044**
Milk, whole, powder	5.0	0.19	0.18
Molasses	25.7	5.5	4.2
Mushrooms	88.1	6.9	0.82
Oatmeal	9.9	31.0	27.9
Olives, green, canned	77.0	2.4	0.55
Oyster plant	72.0	12.4	3.5
Peas, split	9.5	30.7	27.7
Pecans	2.3	35.7	34.8
Peppers, green	92.5	19.1	1.4
Plums	88.0	6.2	0.74
Pork chops	63.1	1.6	0.59
Poultry			
Duck	43.7	0.58	0.33
Goose	57.0	1.2	0.52
Turkey, dark meat	72.2	1.6	0.45
Turkey, light meat	72.1	1.1	0.31
Puffed Rice	10.9	8.4	7.3
Puffed Wheat	8.6	30.2	27.2
Pumpkin	91.7	4.5	0.37
Radishes	96.2	13.7	0.52
Raspberries	88.9	45.9	5.1
Rhubarb	95.3	30.6	1.5
Rice, polished	13.3	12.9	10.8
Rutabagas	86.6	9.9	1.3
Shredded Wheat	8.1	25.9	23.9
Tomatoes	94.2	24.0	1.4
Turnips	92.0	5.4	0.43
Veal chops	78.5	1.3	0.28
Watercress (2)	92.2	69.1	5.4

* When more than one sample was analyzed, the number of samples is indicated by the figure in parentheses.

** mg. per liter.

A survey of the concentration of the element in classes of food materials is obtained from Table II. In this table are listed 12 major classes of foodstuffs, arranged in descending order of their manganese content, together with the minimum, maximum, and average for each group. This table, as mentioned above, has been compiled from the data in Table I and those in the previous publication (18) wherever numerical values were obtained for the manganese concentrations.

TABLE II

GROUPS OF PRINCIPAL FOODSTUFFS ARRANGED IN DESCENDING ORDER OF MANGANESE CONTENT—FRESH BASIS (EDIBLE PORTION)

Class of food	Number of samples	Manganese		
		Minimum	Maximum	Average
		mg. per kg.	mg. per kg.	mg. per kg.
Nuts	10	6.3	41.7	22.7
Cereals and their products	23	0.49	91.1	29.2*
Dried legume seeds	4	10.7	27.7	20.0
Green leafy vegetables	18	0.76	12.6	4.5
Dried fruits	7	1.5	6.7	3.3
Roots, tubers, and stalks	12	0.35	9.2	2.1
Fresh fruits, excluding blueberries	25	0.18	10.7	2.0
Fresh fruits, including blueberries	26	0.18	44.4	3.7
Non-leafy vegetables	5	0.82	2.4	1.5
Animal tissue	13	0.078	3.8	1.0
Poultry and poultry products	6	0.30	1.1	0.50
Dairy products	7	0.028	1.6	0.47
Fish and sea foods, excluding oysters	6	0.12	0.39	0.25
Fish and sea foods, including oysters	7	0.12	2.2	0.51

Since the first three classes of foodstuffs listed in Table II, nuts, cereals, and dried legume seeds, are mainly dry matter, the manganese content is found to be correspondingly high. Leafy vegetables, which rank fourth in a comparison of materials in their native state, easily lead if the comparison is made on the dry basis. Since blueberries contain such an enormous amount of the element, the average for fresh fruits has been given both with and without the blueberry value. The average exclusive of blueberries is much more nearly representative of this class of foodstuffs and ranks the group in seventh place. The last three groups, poultry and poultry products, dairy products, and fish and sea foods are seen to be very low in manganese; all average about 0.5 mg. per kilo. of fresh material. In the group of fish and sea foods is found one material, oysters, which is much higher in manganese than the other members of the group. The average

manganese concentration of animal tissue (1 mg. per kg.) is doubtless greater than the average for meat actually consumed in the human diet since liver, a material containing a relatively large amount of the element, represents almost one-fourth of the samples analyzed, whereas it represents a much lower proportion of the meat consumed by an individual.

Variations within a given group were very great in many instances. The greatest regular variation was exhibited by the cereals and their products, in which group the maximum concentration (wheat bran) was 186 times as great as the minimum (corn flakes). Non-leafy vegetables were most uniform, with only a three-fold variation.

From the point of view of practical nutrition, the question arises as to what can or does supply the greatest share of manganese in the diet. Although nuts contain the highest concentration of the element, the intake of these is so low as to make them of little importance as sources of manganese for man. On the other hand, since the cereals and their products form such a large portion of the human diet, and since they also have a high concentration of the element, they probably contribute most to the supply of manganese in the diet. If calculated on the basis of food materials served in typical American diets as given by Rose (26, 27), the cereals and their products contribute about 38 per cent of the total manganese in two representative family menus. On the same basis of calculation, fruits and vegetables furnish 32 and 14 per cent respectively.

Only when the specific function or functions of manganese in the animal body have been uncovered and the amount needed daily for proper nutrition ascertained, can it be said that a given diet is adequate or deficient in this element. In view of the fairly high concentration of the element in cereals and their products as well as in fruits and vegetables, it appears unlikely that ill effects from inadequate amounts of manganese will result so long as these classes of food materials contribute their present share to the total food intake.

Summary

The manganese content of 83 representative food materials is given. The concentrations range from 0.028 mg. per liter of milk to 49.9 mg. per kilo. of bran flakes. Thirty-six of the materials contained less than 1 mg., twenty-five from 1 to 5 mg., and only twenty-two more than 5 mg. per kilo. of fresh material.

Arranged in descending order with respect to their manganese content, 12 classes of foods, representing 138 food materials, appear as follows: nuts; cereals and their products; dried legume seeds; green leafy vegetables; dried fruits; roots, tubers, and stalks; fresh fruits; non-leafy vegetables; animal tissue; poultry and poultry products; dairy products; and fish and sea foods.

Marked variations in manganese content were found among the several members of a given group.

In typical American diets cereals and their products contribute the largest proportion of the manganese intake.

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Influence of Manganese on Immunology

by C.-E. Pico

In a paper by Walbum and Murch[1] on the influence of manganese and other metals on the elevation of the strength of antitoxin serums, these authors came to the conclusion that manganese causes an over-production of antitoxins. A. Sordelli has done a series of experiments demonstrating the facts pointed out by these authors. We pursued these experiments, developed them further, and we want to publish this article as a preliminary report.

Anti-diphtheria serum. After the immunization of a horse, we waited for a week, then injected for three consecutive days, 10 cc of a solution $\frac{M}{2}$ of $MnCl_2$, intravenously.

Blood tests done before and after (about one hour) the treatment with manganese, enabled us to determine the strength of the antitoxin.

The results were the following:

Horse	Before the injection March 10, 1924	After the treatment March 13, 1924	Increase p. 100
998	300 Antitoxin Units	500	40
918	900 Antitoxin Units	1200	25
927	600 Antitoxin Units	800	25
933	900 Antitoxin Units	1100	18.18
936	600 Antitoxin Units	700	14.28
914	500 Antitoxin Units	500	0
929	400 Antitoxin Units	400	0
917	400 Antitoxin Units	500	20
942	1100 Antitoxin Units	1500	26.66
926	1000 Antitoxin Units	1200	20
932	900 Antitoxin Units	1000	10
916	900 Antitoxin Units	1000	10

[1] Annales, Institut Pasteur, 1923, p. 396.

In summary, it can be said that the manganese raises the level of the antitoxin by approximately 17%. Naturally, the increase noted above does not depend on the elevation seen in the customary curve because after 8 days, the control horses showed no increase in the antitoxin value of their serum.

Antitetanus serum. As was the case with other investigators, Sordelli could not obtain results with this serum. (Experiment done on 12 horses: numbers 743, 855, 844, 772, 776, 702, 759, 737, 750, 554, 741, and 742.)

Antiophidica serum (Lachesis alternatus). In experiments done with this serum, we could not verify an increase in antibodies:

Blood test before the injection of Mn (March 17, 1924)

Serum in cc	Venom in mgr.	Results
0.5	2.5	Death after 2 minutes
1	2.5	Death after 7 minutes
1.6	2.5	Death after 20 minutes
1.8	2.5	Survived
2	2.5	Survived
2.2	2.5	Survived
2.5	2.5	Survived

Blood test after the injection of Mn (March 28, 1924)

Serum in cc	Venom in mgr.	Results
0.5	2.5	Death after 2 minutes
1	2.5	Death after 8 minutes
1.6	2.5	Death after 20 minutes
1.8	2.5	Death after 25 minutes
2	2.5	Death after 35 minutes
2.2	2.5	Survived
2.5	2.5	Survived

The dosage consisted of a mixture of serums coming from 9 immunized horses (numbers 845, 873, 875, 879, 880, 881, 882, 885, and 889).

Anti-meningococci serum. The same technique was used here.

We found some interesting facts relating to the increase of the fixative power of alexin, but no clear results with agglutination as the following tables demonstrate:

Alexin fixation, June 24, 1924

Horse	Blood test before Mn injection Strength of total fixation	Blood test after Mn injection
693	800	800
707	800	800
698	200	300
705	1000	1000
696	400	600
758	600	800
Mixture	400	800

But, since the curve showing the increase of antibodies in anti-infectious serums is not yet precisely established, we cannot draw any definite conclusions from these experiments because they would be premature. It is thus necessary to make further observations and to undertake other experiments under more adequate conditions.

Agglutination (June 26, 1924)

Horse	Blood tests before the Mn injections (June 16)				Blood tests after the Mn injections (June 23)			
	1500	2000	2500	3000	1500	2000	2500	3000
693	3	3	3	3	3	3	3	3
707	3	3	3	3	3	3	3	3
705	3	3	3	3	3	3	3	3
696	3	3	3	2	4	3	4	4
758	4	5	4	3	4	4	4	3
Mixture	2	2	2	-	2	2	2	-

Precipitant serums. The experiments were negative with a mixture of human precipitant serums.

Precipitant serums. The experiments were negative with a mixture of human precipitant serums:

June 17, 1924 - Blood test. Rabbits 196, 155, 187: 1/7.000 liter.

June 20, 21, and 23, 1924 - Injections of $\frac{M}{2}$ solution of $MnCl_2$ 0.10 cc.

June 26, 1924 - Blood tests. Same results.

Hemolytic serums (anti-sheep). Rabbits 166 and 55 received on May 16 and 21, 1924, 0.10 cc of $\frac{M}{2}$ $MnCl_2$ solution: strength 1/5.000. The strength was not changed.

Anaphylaxis. First, we studied whether the injection of Mn favored the development of sensitization. We injected intra-sensitizing doses, previously determined. After several days, we repeatedly injected manganese; we let a certain time pass, then injected a very strong dose of shock antigen.

We contend that 1 cc of an equine solution of 1/100,000 (of which about 1 millionth is protein) is not sensitizing. The guinea-pigs thus under-sensitized received new doses of manganese, and then we tried to produce shock.

Influence du manganèse sur la sensibilisation anaphylactique.

Cobaye	2 mai 1924	7 mai	8 mai	17 mai	Résultat
193	sous sensibilisés	Reçoit 1 c.c. de Cl^2Mn $\frac{M}{100}$	1 c.c. Cl^2Mn $\frac{N}{200}$	1 c.c. de sérum équin	Choc (1)
184		» Cl^2Mn $\frac{M}{200}$	»	»	Pas de choc
129		Témoin	»	»	»
104		»	»	»	»

Cobaye	22 mai 1924	28 mai	31 mai	2 juin	3 juin	10 juin	Résultat
292	sous sensibilisés	0,5 Cl^2Mn $\frac{M}{100}$	Id.	Id.	Id.	1 c.c. de sérum équin	Pas de choc
285		»	»	»	»	»	Choc
2[illegible]		»	»	»	»	»	Pas de choc
206		»	»	»	»	»	»
282		»	»	»	»	»	»
287		»	»	»	»	»	»
202		Témoin	»	»	»	»	»
224		»	»	»	»	»	»

(1) Influence of manganese on anaphylactic sensitization.

(2) Guinea-pig

(3) May 2, 1924 (dates: May and June)

(4) Under-sensitized

(5) Result

(6) Received 1 cc of $\frac{M}{100}$ $MnCl_2$

(7) 1 cc. of equine serum

(8) Shock

(9) No shock[1]

(10) Control

After having found that in the guinea pig the injections of manganese brought about a state of depression bordering on shock, we studied whether the immediately caused shock was diminished or eliminated. We thought that the Mn, being likely to initiate the vascular endothelium, could at the same time, create a state of hyposensibility.

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[1]Autopsy showed a pulmonary pseudo-tuberculosis.

The experiment done on 35 sensitized male guinea-pigs gave the following result: 35 sensitized guinea-pigs received an injection of 0.5 cc of a solution of $MnCl_2$ at 4 p. 1,000, immediately followed by an injection of shock antigen of 1 cc of serum from a horse; 12 survived having shown no shock or having had only very slight manifestations of hypersensibility; 23 experienced shock, followed by death.

Conclusions

1. $MnCl_2$ is capable of appreciably increasing the antitoxin strength of the serum of immunized horses (antidiphtheria serum).

2. Our experiences showed that $MnCl_2$ produced no change in the strength of the tetanus antitoxin, nor in that of the precipitant serums, the hemolytic serums, or the antiophidica serums (Lachesis alternatus).

3. It seemed to raise the strength in the fixation of the complement in the case of anti-meningococci serum, but it had no effect on agglutination.

4. A third of the sensitized guinea-pigs were almost completely protected by an injection of $MnCl_2$, which immediately preceded (from 1 to 4 minutes), the injection of a strong dose of shock antigen.

(Bacteriological Institute of the National Department of Hygiene, Buenos Aires)

Influence du manganèse sur les phénomènes de l'immunité,

par C.-E. Pico.

Dans une communication de Walbum et Mürch (1*), au sujet de l'influence du manganèse et d'autres métaux sur l'élévation du titre des sérums antitoxiques, ces auteurs arrivent à la conclusion, que le manganèse donne une surproduction d'antitoxines. A. Sordelli a entrepris une série d'expériences tendant à démontrer les faits signalés par ces auteurs. Nous avons poursuivi ces expériences, les développant davantage, et nous désirerions publier cette note comme communication préliminaire.

Sérum antidiphtérique. — Après l'immunisation du Cheval, nous attendons une semaine, puis on injecte, pendant 3 jours consécutifs, 10 c.c. d'une solution $\frac{M}{2}$ de Cl^2Mn, par voie intraveineuse.

Des saignées exploratrices, pratiquées avant et après (1 heure environ), le traitement par le manganèse, ont permis de déterminer le titre antitoxique.

Les résultats ont été les suivants :

(1*) *Annales Institut Pasteur*, 1923, p. 396.

Cheval	Avant l'injection 10 mars 1924	Après le traitement 14 mars 1924	Augmentation p. 100
998	500 U. A.	500	40
918	900 »	1200	25
927	600 »	800	25
933	900 »	1100	18,18
936	600 »	700	14,28
914	500 »	500	0
929	400 »	400	0
917	400 »	500	20
942	1100 »	1500	26,66
926	1000 »	1200	20
932	900 »	1000	10
916	900 »	1000	10

En résumé, on peut dire que le manganèse élève le taux de l'antitoxine dans la proportion de 17 p. 100 environ. Naturellement, l'augmentation signalée ci-dessus ne dépend pas de l'élévation de la courbe habituelle car, après 8 jours, les Chevaux témoins n'ont présenté aucune augmentation de la valeur antitoxique de leur sérum.

Sérum antitétanique. — Comme d'autres expérimentateurs, Sordelli n'a pas pu obtenir de résultats avec ce sérum. (Expérience faite sur 12 Chevaux : n°s 743, 855, 844, 772, 776, 702, 759, 737, 750, 554, 741 et 742).

Sérum antiophidique (Lachesis alternatus). — Dans les expériences faites avec ce sérum, nous n'avons pas constaté l'augmentation d'anticorps :

Saignée exploratrice avant l'injection de Mn (17 mars 1924).

Sérum en c.c.	Venin en mgr.	Résultat
0.5	2,5	Mort au bout de 2 minutes.
1	»	Mort au bout de 7 minutes.
1.6	»	Mort au bout de 20 minutes.
1,8	»	Survie.
2	»	Survie.
2,2	»	Survie.
2,5	»	Survie.

Saignée exploratrice après l'injection de Mn (28 mars 1924)

Sérum en c.c.	Venin en mgr.	Résultat
0.5	2,5	Mort au bout de 2 minutes.
1	»	Mort au bout de 8 minutes.
1,6	»	Mort au bout de 20 minutes.
1,8	»	Mort au bout de 25 minutes.
2	»	Mort au bout de 35 minutes
2,2	»	Survie.
2,5	»	Survie.

Le dosage fut pratiqué avec un mélange de sérums provenant

de 9 Chevaux immunisés (n°s 845, 873, 875, 879, 880, 881, 882, 885 et 889).

Sérum antiméningococcique. — La même technique a été employée ici.

Nous avons trouvé des faits intéressants se rapportant à l'augmentation du pouvoir fixateur de l'alexine, mais aucun résultat net avec l'agglutination, comme le démontrent les tableaux suivants :

Fixation de l'alexine, 24 juin 1924.

Cheval	Saignée avant les injections de Mn	Saignée après les injections de Mn.
	Titre de fixation total.	
693	800	800
707	800	800
698	200	300
705	1000	1000
696	400	600
758	600	800
Mélange	400	800

Mais, comme la courbe d'augmentation d'anticorps dans les sérums anti-infectieux n'est pas encore parfaitement établie, nous ne pouvons tirer de ces expériences, aucune conclusion définitive, car elle serait prématurée. Il est donc nécessaire de faire de nouvelles observations et de réaliser d'autres expériences dans des conditions plus adéquates.

Agglutination (26 juin 1924).

Cheval	Saignée avant les injections de Mn (16 juin)				Saignée après les injections de Mn (23 juin)			
	1500	2000	2500	3000	1500	2000	2500	3000
693	3	3	3	3	3	3	3	3
707	3	3	3	3	3	3	3	3
705	3	3	3	3	3	3	3	3
696	3	3	3	2	4	3	4	4
758	4	5	4	3	4	4	4	3
Mélange ..	2	2	2	—	2	2	2	—

Sérums précipitants. — Les expériences sont négatives avec un mélange de sérums précipitants humains :

17 juin 1924. — Saignée exploratrice. Lapins 196, 155, 187 : titre 1/7.000.

20, 21 et 23 juin 1924. — Injections d'une solution de $Cl^2Mn \frac{M}{2}$: 10 c.c.

26 juin 1924. — Saignée exploratrice. Même titre.

Sérums hémolytiques (anti-Mouton). — Les Lapins 166 et 55

reçoivent le 16 et le 21 mai 1924, 0,10 c.c. d'une solution de $Cl^2Mn \frac{M}{2}$: titre 1/5.000. Le titre n'est pas modifié.

Anaphylaxie. — Premièrement, nous avons recherché si l'injection de Mn favoriserait la production de la sensibilisation. On injecte des doses intra-sensibilisantes, préalablement déterminées. Au bout de quelques jours, et à plusieurs reprises, on injecte du manganèse ; on laisse s'écouler un certain temps, puis on injecte une très forte dose déchaînante.

Nous constatons que 1 c.c. d'une solution de sérum équin au 1/100.000 (donc 1 millionième de protéine environ), n'est pas sensibilisant. Les Cobayes ainsi sous-sensibilisés reçurent de nouvelles doses de manganèse, puis nous essayâmes de produire le choc.

Influence du manganèse sur la sensibilisation anaphylactique.

Cobaye	2 mai 1924	7 mai	8 mai	17 mai	Résultat
193	sous sensibilisés	Reçoit 1 c.c. de $Cl^2Mn \frac{M}{100}$	1 c.c. $Cl^2Mn \frac{N}{200}$	1 c.c. de sérum équin	Choc (1)
184		» $Cl^2Mn \frac{M}{200}$	»	»	Pas de choc
129		Témoin	»	»	»
104		»	»	»	»

Cobaye	22 mai 1924	28 mai	31 mai	2 juin	3 juin	10 juin	Résultat
292	sous sensibilisés	0,5 $Cl^2Mn \frac{M}{100}$	Id.	Id.	Id.	1 c.c. de sérum équin	Pas de choc
285		»	»	»	»	»	Choc
229		»	»	»	»	»	Pas de choc
206		»	»	»	»	»	»
282		»	»	»	»	»	»
287		»	»	»	»	»	»
202		Témoin	»	»	»	»	»
224		»	»	»	»	»	»

Après avoir constaté que les injections de manganèse déterminaient, chez le Cobaye, un état de dépression qui était voisin du choc, nous avons recherché si le choc immédiatement provoqué était diminué ou aboli. Nous avons pensé que le Mn, étant susceptible d'irriter l'endothélium vasculaire, pourrait, en même temps, créer un état d'hyposensibilité.

L'expérience faite sur 35 Cobayes mâles sensibilisés, a donné le résultat suivant : 35 Cobayes sensibilisés reçoivent l'injection de 0,5 c.c. d'une solution de Cl^2Mn à 4 p. 1.000, suivie immé-

(1) L'autopsie montre une pseudo tuberculose pulmonaire.

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diatement d'une injection (déchaînante) de 1 c.c. de sérum de Cheval : 12 survivent, n'ayant pas présenté de choc, ou n'ayant eu que de très légères manifestations d'hyper-sensibilité : 23 ont présenté un choc, suivi de mort.

Conclusions. — 1° Le Cl^2Mn est capable d'augmenter sensiblement la richesse antitoxique du sérum des Chevaux immunisés (sérum antidiphtérique).

2° Nos expériences ont montré que le Cl^2Mn ne produit aucune modification du titre de l'antitoxine tétanique, ni de la valeur des précipitines, hémolysines et du sérum antiophidique (*Lachésis alternatus*).

3° Il semble élever le titre dans la fixation du complément, dans le cas du sérum antiméningococcique, mais il reste sans action sur son pouvoir agglutinant.

4° Un tiers des Cobayes sensibilisés a été presque complètement protégé par une injection de Cl^2Mn, qui précédait immédiatement (de 1 à 4 minutes), l'injection d'une forte dose de l'antigène déchaînant.

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Z. Krebsforsch. 52: 32-36, 1942

From the Institute of Biology of the University of Sofia (Bulgaria)

Director: Prof. Dr. Methodi Popoff

On the effect of chemical stimulants on the growth of mouse tumors

Commonication II

by M. Popoff

With one text illustration

(Received on 16 May, 1941)

In my publication, "On the effect of some chemical stimulants on the growth of cancerous tumors" [Acta Cancrologica 2, No. 2, 173-180 (1936)], I discussed the theoretical considerations suggesting that it must be possible, through improvement of the general cell functions of the body and its incretory glands by means of stimulants, to return the chemical transformations and the growth correlations to normal and to check the proliferation of the tumor cells. In support of this concept I have cited the results of experiments with $MgCl_2$, $MgSO_4$, MgBr, KBr, glycerine, $MnSO_4$, KJ, and $NaSO_4$, not only in external use on cancerous lesions, but also in subcutaneous injections. They exhibited a distinct retardation of the growth of the tumor, but without damaging the vitality of the tumor cells to such an extent that the tumor discontinued its growth entirely. Accordingly, there must have still remained in the body certain functional changes which favored the life of the tumor cells and made that life possible. It was not possible through these chemical effects to return the regulatory processes of the body completely to their normal course. Based on these findings, I have attempted further to strengthen the observed growth restrictions on the tumor cells by varying the application

of different chemical stimulants not only with respect to their concentration, but also their composition. As the results to be reported here will show, I succeeded in actually achieving a significantly stronger inhibitory effect on growth; this suggested that there are, and must indeed exist, chemical actions capable of exerting extensive influence on tumor growth, and that consequently it would be worthwhile to pursue research in this direction. But this time, too, I failed to achieve a complete cessation of tumor growth.

My method of operation is outlined below:

After many experiments with varyingly differing chemical stimulant solutions, the following combination produced for me relatively the best retarding effects. The principles of the stimulant combinations are described fully in my book, "Cell stimulation and its use in horticulture and medicine"* (Paul Parey Verlag, Berlin, 1931, p. 375).

0.5 g $MgCl_2$ + 200 mg $MnSO_4$ ---- Subsequent to earlier, exhaustive research by the author, these two chemicals have been generally recognized as having a cell-stimulating effect; similarly, also, glycerine -- 2 ccm; calcium glycerophosphoricum --- 15 drops; and iodine --- 3 drops regular tincture of iodine. Likewise, as general cell stimulants, LiCl and LiJ were included in the solution --- 3 ccm of each from a 5% solution. In particular, the lithium salts were included for the additional reason that according to the investigations of HERBST with sea urchin eggs and larvae, lithium exerts a regulatory effect upon the growth processes and thus to some extent further the organic formative stimuli of the body. In order to increase the activity of the reproductive glands in their capacity as important incretory glands, there was also added to the above-named

* Title translated.

stimulants, a small amount -- 2 ccm -- of a 1% yohimbine solution. All of the above was dissolved into 40 ccm clear-filtered soil decoction. It has been learned from culture experiments with protozoa that the humus acids have a favorable effect on the normalization of cell functions. Next, in order to enrich the solution thus compounded, with vitamins, I added 5 ccm of an extract from hot, red peppers. This extract was produced in the following way; 2 g air-dried peppers were finely grated and then extracted in 50 ccm water at a temperature of 60°-70° for 20 minutes in an enamel container. After settling, 5 ccm of this extract was added to the solution. Many repeated experiments using injections of pepper extract alone have resulted in a certain degree of inhibition of tumor growth. It is possible that in this case the capsaicine plays a certain role. Investigations oriented in this direction have not yet been concluded.

The solution thus compounded was freshly made up each time before application.

The experiments were conducted as follows: Each experiment series consisted of 15-20 white mice, which had been transplanted with a small portion of the same tumor. After 3 to 4 days, when the tumor had begun to grow, the tumor-transplant mice were divided into two equal groups corresponding to the size of the tumor. One group was continued without treatment as control, the other was injected subcutaneously with the stimulant solution (1/2 ccm), each time on the side of the body opposite the tumor. The injections were given once daily for two or three days consecutively, then one day intermission, then a further injection, and so on. In all, 8 to 10 injections were given. After this period had elapsed, considerable differences could already be ascertained in tumor growth as

between the controls and the test animals. These differences were often very marked, as the photograph (Fig. 1) shows. These experiments were undertaken with about 300 mice; growth inhibition could be constantly observed. The tumors of the treated mice constituted, as the case might be, 1/3 to 1/5 the tumor size of the control mice.

The inhibitory effect on growth continued as long as the injection were kept up. As soon as the latter ceased, growth recommenced, a fact indicating that the tumor cells were inhibited in their division by the injected solution, but not damaged to such an extent that their growth had been fully suspended.

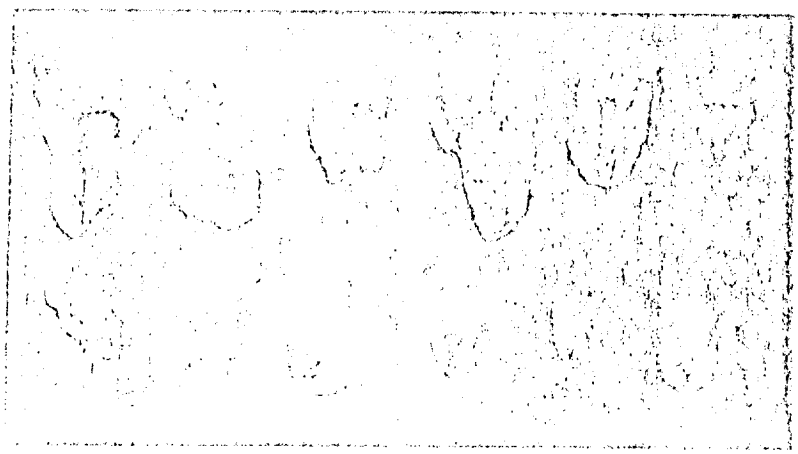

Fig. 1. Upper row: untreated mouse tumor control. Lower row: treated mouse tumors from the same test series.

As a result, the treated mice outlived the controls at the most by one to three weeks and then perished likewise from the tumors that had commenced growing again. The mice did not tolerate injections repeated too

often, for if enployed over a longer period, the solution gradually exhibited damaging effects upon the animal.

These experiments show that success can be achieved in inhibiting extensively the growth of a tumor for a certain length of time -- in fact, just as long as the injections continue. These results, if still incomplete at this time, give reason to hope that following this course of action, through improvement of the injection solutions, it could be possible to further increase the growth-inhibitory effect and to make it longer-lasting. In this effort, the goal must be to replace the metallic salts by organic Mg and Li compounds. According to my experience hither to, it seems to me that the magnesium, manganese, and espicially, the lithium salts exert a distinct inhibitory effect on tumor growth, and, what is more, not only via injections, but also by oral intake, through direct feeding.

The experiments along this line include about 200 mice; they were conducted in test series of 14 to 20 mice each, whose tumors in this instance also derived from an initial tumor. Here, too, one half of the mice were left untreated as control and the other half used as research animals. The feeding of both groups was strictly controlled in quantity of foodstuffs used: the controls as well as the test mice received the same amount of boiled milk, bread, and oats. The sole difference was that the research mice received an addition of lithium and manganese salts: per 100 ccm milk, 1 ccm of a 10% LiCl solution and 0.3 ccm of a 10% $MnSO_4$ solution. On every third day an addition was made to the Li-Mn milk of one drop each of tincture of iodine. The mice that received this nutrition displayed a distinct inhibition of tumor growth; in comparison to the control mice, tumor growth attained 1/3 to 1/4 of the normal size. The

inhibitory effect was apparent. Since the mice did not like the Li-Mn milk as a steady diet, a day of recreation, using regular milk, was inserted, as the case might be, after three to four feeding days. But here too, through the summation of the Li-Mn action, a damaging effect was finally brought about, as occurred in the injection experiments. The result was that the author had a test-mice mortality of 30% to 40%. This high mortality the author sought to lessen by a reduction of the Li-Mn concentration; the effort succeeded, but simultaneously, the favorable, inhibitory effect of the Li-Mn salts upon tumor growth diminished considerably.

The mice which survived the treatment and showed a reduction in tumor growth outlived the control mice by only one to three weeks. They gradually died, one after another.

It was noteworthy in these experiments, also, that the tumors displayed only one growth inhibition; the vitality of the tumor cells was retained, since following cessation of the treatment, tumor growth recommenced and finally, as in the control mice, even with a delay of two to three weeks, the ulcerative stage was reached and the death of the mice followed as a result.

These experiments also demonstrated that it is essential to achieve a reduction of the damaging effect of the Li-Mn salts solutions; so that the growth-inhibiting effect of the Li-Mn compounds may be better exploited.

Possibly, relevant organic compounds will become significant in this connection.

SUMMARY

In this second communication [the first communication appeared in Acta Cancrologicaa 2, No. 2 (1936)], the experiments are discussed which demonstrate the effect of chemical stimulants on the growth of mouse tumors. Solutions of $MgCl_2$, $MnSO_4$, Ca - Glycerophosphate, Yohimbine, iodine and capsaicine were used as stimulants in the form of subcutaneous injections. Using the percentage composition of these solutions as set forth in the text, it was possible to observe a distinct inhibitory effect upon tumor growth. An inhibitory effect on tumor growth was also achieved through feeding with LiCl and $MnSO_4$. In both instances, however, growth of the tumor later recommenced-after cessation of treatment. Accordingly, the tumor cells are only delayed in their proliferation, but are not permanently damaged.

The administration of all these chemicals in the optimally effective stimulation dosage was intended to normalize and promote the vital functions of the body cells and the activity of the incretory glands and in this way return the body tissues to orderly growth correlations. The experiments along the course outlined herein will continue to be conducted.

(Aus dem Biologischen Institut der Universität Sofia [Bulgarien].
Direktor: Prof. Dr. *Methodi Popoff.*)

Über die Wirkung chemischer Stimulationsmittel auf das Wachstum von Mäusetumoren.

(II. Mitteilung.)

Von

M. Popoff.

Mit 1 Textabbildung.

(Eingegangen am 16. Mai 1941.)

In meiner Publikation „Über die Wirkung einiger chemischer Stimulationsmittel auf das Wachstum von Krebstumoren" [Acta Cancrologica 2, H. 2, 173—180 (1936)] habe ich die theoretischen Erwägungen besprochen, welche dahin weisen, daß durch eine Hebung der allgemeinen Zellfunktionen des Körpers und seiner innersekretorischen Drüsen durch Stimulationsmittel möglich sein muß, die chemischen Umsetzungen und die Wachstumskorrelationen des Körpers wieder zu normalisieren und auf diese Weise das Wachstum und die Vermehrung der Tumorzellen einzudämmen. Zur Stützung dieser Auffassung habe ich die Resultate von Versuchen mit $MgCl_2$, $MgSO_4$, MgBr, KBr, Glycerin, $MnSO_4$, KJ und $NaSO_4$ sowohl bei äußerer Anwendung auf Krebswunden als auch bei subcutanen Einspritzungen angeführt. Sie zeigten eine deutliche Hemmung des Tumorwachstums, ohne aber die Vitalität der Tumorzellen so weit zu schädigen, daß der Tumor sein Wachstum vollständig einstellte. Demnach müßten im Körper noch Funktionsumstimmungen geblieben sein, die das Leben der Tumorzellen begünstigten und möglich machten. Die Wachstumsregulationsprozesse des Körpers haben durch diese Einwirkungen nicht ganz nach der normalen Richtung umgestimmt werden können.

Von diesen Konstatierungen ausgehend habe ich versucht, durch Variieren verschiedener Stimulationsmittel sowohl nach Konzentration als auch nach Zusammensetzung die beobachteten Wachstumshemmungen auf die Tumorzellen weiter zu verstärken. Wie die hier mitzuteilenden Ergebnisse zeigen werden, gelang es mir, in der Tat eine bedeutend stärkere Hemmung des Wachstums zu erreichen, was darauf hinleitet, daß [illegible] Einwirkungen gibt und geben muß, die imstande sind, das Tumorwachstum weitgehend zu beeinflussen und daß es folglich [illegible] in dieser Richtung [illegible] ein vollständiges [illegible] des Tumorwachstums [illegible] nicht.

Nachfolgend die Arbeitsweise:

Nach vielen Versuchen mit verschiedentlich variierten Stimulationslösungen hat mir die folgende Zusammenstellung verhältnismäßig die besten Heilungserfolge gegeben. Die Leitgedanken der Stimulationszusammenstellungen sind in meinem Buch „Die Zellstimulation und ihre Anwendung in der Pflanzenzüchtung und Medizin" (Verlag Paul Parey, Berlin, 1931, S. 375) ausführlich dargelegt.

0,5 g $MgCl_2$ — 200 mg $MnSO_4$. — Diese zwei chemischen Mittel sind nach früheren eingehenden Untersuchungen von mir als allgemein zellstimulierend wirkend [illegible] worden, desgleichen auch das Glycerin — 2 ccm, das Calcium [illegible] — 15 Tropfen und das Jod — 3 Tropfen normale [illegible]. Ebenfalls als allgemeine Zellstimulationsmittel wurden das LiCl und das KJ in der Lösung aufgenommen, und zwar zu je 3 ccm von einer 5proz. Lösung. Speziell die Lithiumsalze wurden außerdem noch aus dem Grunde aufgenommen, da nach den Untersuchungen von *Herbst* an Seeigeleiern und Seeigellarven das Lithium eine regulationsfördernde Wirkung auf die Wachstumsprozesse ausübt und so gewissermaßen die organischen formativen Reize des Körpers fördert. Um die Tätigkeit der Geschlechtsdrüsen als wichtige, sekretorische Drüsen zu heben, wurden den obigen Stimulationsmitteln noch eine geringe Dosis — 2 ccm einer 1proz. Johimbinlösung zugesetzt. Das Ganze wurde in klar filtrierte 40 ccm Erdabkochung aufgelöst. Aus Züchtungsversuchen an Protozoen ist bekannt geworden, daß die Humussäuren eine günstige Wirkung auf die Normalisierung der Zellfunktionen ausüben. Um nun die so zusammengesetzte Lösung vitaminhaltig zu gestalten, habe ich 5 ccm von einem Extrakt von scharfen, roten Pfefferschoten zugesetzt. Dieser Extrakt wurde auf folgende Weise hergestellt: 2 g luftgetrocknete Pfefferschoten wurden fein verrieben und in 50 ccm Wasser bei Temperatur von 60—70° 20 min lang in emailliertem Gefäß extrahiert. Nach dem Absetzen wurden von diesem Extrakt 5 ccm der Lösung zugegeben. Vielfach wiederholte Versuche mit Injektionen von Pfefferextrakt allein haben auch eine gewisse Hemmung auf das Tumorenwachstum ergeben. Möglicherweise hat hier das Capsaicin eine gewisse Rolle gespielt. Die Untersuchungen in dieser Richtung sind noch nicht abgeschlossen.

Die so zusammengesetzte Lösung wurde jedesmal vor der Anwendung frisch hergestellt.

Die Versuche wurden folgendermaßen ausgeführt: Jede Versuchsserie bestand aus 15—20 weißen Mäusen, die subcutan mit Stückchen [illegible] Tumor transplantiert worden waren. Nach 3—4 Tagen, wenn der Tumor zu wachsen begonnen hatte, wurden die Tumormäuse in zwei [illegible] der Tumormäuse gleiche Teile geteilt. Der eine [illegible] ohne Behandlung als Tumorquelle weitergeführt. Der andere

Teil wurde mit Stimulationslösung ($^1/_2$ ccm) subcutan injiziert, und zwar jedesmal auf der dem Tumor entgegengesetzten Körperhälfte. Die Injektionen wurden 2—3 Tage nacheinander einmal täglich unternommen, dann einen Tag ausgesetzt, wieder injiziert usw. Es wurden im ganzen 8—10 Injektionen gegeben. Nach Ablauf dieser Zeit konnten schon erhebliche Unterschiede im Tumorwachstum zwischen den Kontrollen und den Versuchstieren festgestellt werden, die vielfach sehr auffallend waren, wie die Photographie (Abb. 1) zeigt. Diese Versuche wurden an etwa 200 Mäusen unternommen, die Wachstumshemmung

Abb. 1. Obere Reihe: unbehandelte Mäusetumoren-Kontrolle. Untere Reihe: behandelte Mäusetumoren von der gleichen Versuchsserie.

war stets zu beobachten. Die Tumore der behandelten Mäuse bildeten je nachdem $^1/_3$ bis $^1/_5$ der Tumorgröße der Kontrollmäuse.

Die Wachstumshemmung dauerte so lange an, als die Injektionen fortgesetzt wurden. Nach Einstellung derselben fing das Wachsen von neuem an, was dafür spricht, daß die Tumorzellen in ihrer Teilung durch die injizierte Lösung gehemmt waren, nicht aber so weitgehend geschädigt, daß sie ihr Wachstum vollständig eingestellt hätten. Infolgedessen überlebten die behandelten Mäuse die Kontrollen mit höchstens 1—3 Wochen und gingen dann an den nachwachsenden Tumoren ebenfalls zugrunde. Zu oft wiederholte Injektionen vertrugen die Mäuse nicht, denn längere Zeit angewandt, zeigte die Lösung allmählich schädigende Wirkungen für das Tier.

Diese Versuche zeigen, daß es gelingt, für eine gewisse Zeit das Tumorwachstum weitgehend zu hemmen — und zwar so lange, wie die Injektionen dauern. Diese, wenn auch zur Zeit noch unvollkommenen Versuche geben aber der Hoffnung Raum, daß es nach dieser Richtung,

durch Verbesserung der Injektionslösungen möglich sein könnte, die wachstumshemmende Wirkung noch weiter zu steigern und dauerhafter zu machen. Es muß dabei angestrebt werden, die Metallsalze durch organische Mg- und Li-Verbindungen zu ersetzen. Nach meinen bisherigen Erfahrungen scheint es mir, daß die Magnesium-, Mangan- und besonders die Lithiumsalze eine deutlich hemmende Wirkung auf das Tumorwachstum haben, und zwar nicht nur auf dem Wege der Injektionen, sondern auch durch Einnahme per os, durch direkte Verfütterung.

Die Versuche nach dieser Richtung behaufen sich auf etwa 200 Mäuse, durchgeführt in Versuchsserien von je 11—20 Mäusen, deren Tumoren auch hier jedesmal von einem Ausgangstumor abstammten. Auch hier wurde die eine Hälfte der Mäuse als Kontrolle unbehandelt gelassen und die andere Hälfte als Versuchstiere verwendet. Die Fütterung beider Gruppen war streng abgemessen nach der Menge der Nahrungsmittel, sowohl die Kontrollen als auch die Versuchstiere bekamen dasselbe Quantum abgekochter Milch, Brot und Hafer. Der einzige Unterschied bestand darin, daß die Versuchsmäuse in die Milch einen Zusatz von Lithium- und Mangansalzen erhielten, und zwar auf 100 ccm Milch je 1 ccm von einer 2proz. LiCl-Lösung und 0,3 ccm von einer 10proz. $MnSO_4$-Lösung. An jedem dritten Tag wurde außerdem der Li-Mn-Milch auch je 1 Tropfen Jodtinktur zugesetzt. Die Mäuse, die diese Nahrung bekamen, zeigten eine deutliche Hemmung des Tumorwachstums, dieses erreichte im Vergleich zu den Kontrollmäusen $^1/_3$ bis $^1/_4$ der normalen Größe. Die Hemmungswirkung war augenscheinlich. Da die Mäuse die Li-Mn-Milch auf die Dauer nicht gerne nahmen, so wurde je nachdem nach 2—4 Fütterungstagen ein Erholungstag mit normaler Milch eingeschaltet. Durch die Summierung der Li-Mn-Wirkung kam es aber auch hier wie bei den Injektionsversuchen schließlich zu einer schädigenden Wirkung, so daß ich eine Sterblichkeit der Versuchstiere von 30—40% hatte. Diese hohe Sterblichkeit habe ich versucht durch Herabsetzung der Li-Mn-Konzentration zu vermindern, was mir auch gelang, gleichzeitig damit aber nahm auch die günstige, hemmende Wirkung der Li-Mn-Salze auf das Tumorwachstum beträchtlich ab.

Die Mäuse, welche die Behandlung überstanden und eine Herabsetzung des Tumorwachstums zeigten, überlebten die Kontrollmäuse mit nur 1—3 Wochen — sie starben allmählich eine nach der anderen ab.

Bemerkenswert auch bei diesen Versuchen war, daß die Tumoren nur eine Wachstumshemmung zeigten: die Lebensfähigkeit der Tumorzellen blieb erhalten, da nach dem Aufhören der Behandlung das Wachstum des Tumors von neuem anfing, um schließlich wie bei den Kontrollmäusen, wenn auch mit einer Verspätung von 2—3 Wochen, das Stadium der Ulceration zu erreichen und den Tod der Mäuse herbeizuführen.

Diese Versuche zeigten ebenfalls, daß es notwendig ist, zu einer Herabminderung der schädigenden Wirkung der Li-Mn-Salzlösungen zu kommen, um die wachstumshemmende Wirkung der Li-Mn-Verbindungen besser ausnützen zu können.

Möglicherweise werden hier diesbezügliche organische Verbindungen von Bedeutung werden.

Zusammenfassung.

In dieser zweiten Mitteilung [die erste Mitteilung in Acta Cancrologica 2, H. 2 (1936)] werden die Versuche besprochen, welche die Einwirkung von chemischen Stimulationsmitteln auf das Wachstum von Mäusetumoren zeigen. Als Stimulationsmittel werden die Lösungen von $MgCl_2$, $MnSO_4$, Natriumglycerophosphat, Adrenalin, J und Capsaicin angewandt, und zwar als subcutane Injektionen. Bei der im Text angegebenen prozentualen Zusammensetzung dieser Lösungen konnte eine deutlich hemmende Wirkung auf das Tumorwachstum beobachtet werden.

Eine hemmende Wirkung des Tumorwachstums wurde auch erzielt bei Verfütterung von LiCl und $MnSO_4$. In beiden Fällen fing aber das Wachstum des Tumors später von neuem an — nach der Aufhebung der Behandlung. Also werden die Tumorzellen in ihrer Verzehrung nur aufgehalten, aber nicht dauernd geschädigt.

Die Darreichung aller dieser chemischen Mittel in der optimal wirkenden Stimulationsdosis hatte den Zweck, die Lebensfunktionen der Körperzellen und die Tätigkeit der innersekretorischen Drüsen zu normalisieren und zu heben und auf diese Weise das Körpergewebe wieder zu geordneten Wachstumskorrelationen zurückzuführen. Die Versuche in der hier angegebenen Richtung werden weiter fortgeführt.

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THE DETERMINATION OF MANGANESE IN ORGANIC MATERIAL CONTAINING LARGE AMOUNTS OF CALCIUM AND CHLORIDES

THE DETERMINATION OF MANGANESE IN THE MOUSE AND IN MILK

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Studies on the metabolism of manganese in the mouse frequently require knowing an analysis of this element in the ash of the whole animal. The large amounts of calcium and chloride present in tissue present a special problem in the quantitative determination of manganese. The chlorides must be removed, but in doing this one must avoid forming insoluble calcium salts, because these would be difficult to remove by washing from materials such as silica, sulfate and calcium phosphate.

A modification of the Davidson and Cannon method is used with success. The Davidson and Cannon method is suitable for analysis of manganese in plants which contain relatively small amounts of chlorides, but to apply it to ash high in chlorides it was found that special modifications had to be introduced. The present method consists in oxidizing the manganese to permanganic acid with potassium persulfate and then determining the intensity colorimetrically. Complete conversion of manganese to permanganic acid requires the removal of chlorides. This is accomplished by dissolving the ash in concentrated nitric acid, adding sulfuric acid, and evaporating the solution to dryness. By avoiding an excess of sulfuric acid it is possible to prevent the formation of calcium sulfate, thus making filtration unnecessary.

Method

The mice are killed by asphyxiation with illuminating gas, thoroughly washed with distilled water, and the alimentary tracts

removed and discarded. The livers are analyzed separately. The material is ashed in an electric oven at 105° and ashed in silica buckets, in a silica-lined and thermostatically controlled muffle furnace. The use of silica throughout insures against contamination from metals or from the walls of the furnace, while the accurately controlled temperature eliminates the danger of overheating. Tests show that a temperature of 650° is insufficient to ash organic matter completely but that a temperature greater than 700° is unnecessary. A higher temperature might cause the formation of salts which would be difficult to dissolve. After about 8 hours of heating at slightly less than 700°, the ashing is completed and the material, when cool, is dissolved in 10 cc. of concentrated nitric acid. 4 drops of concentrated sulfuric acid are added and the solution evaporated to dryness over an open flame to remove the chlorides. More sulfuric acid is unnecessary as a considerable amount will form more calcium sulfate than can be dissolved. The residue is then dissolved in 12 cc. of 15 per cent nitric acid and the solution made to volume. Since the manganese content of a mouse fed on a manganese-restricted diet is very small, the final volume should not be greater than 20 cc. For rats fed on a normal diet or for those which have had manganese added to the diet, the volume is made to 50 cc. The amount of nitric acid used in dissolving the residue should not be greater than 15 per cent of the final volume, because the author (2) has found that this is near the upper limit for developing the maximum intensity of permanganic acid color. To a measured amount of the solution in a graduated centrifuge tube are added 0.2 cc. of 0.25 per cent silver nitrate, as a catalyst, and 0.25 gm. of potassium persulfate. If the ash is thought to be low in manganese and the final volume is made to 20 cc. in order to concentrate the manganese as much as possible, 10 cc. of the undiluted solution should be used. If, however, the animal had been fed on a normal diet or if manganese had been added to the diet, then from 2 to 5 cc., when diluted to 10 cc. with distilled water, will contain enough manganese to give a convenient depth of color for reading. When the tube is heated in a water bath, the pure permanganic acid color, free from turbidity, appears within a few minutes and will persist for weeks without fading.

The color is compared with a standard similarly treated, in Nessler tubes of 10 cc. capacity, graduated in tenths of a cm., and 12 cm. in length. Most accurate readings can be made in these tubes when not more than 0.02 mg. of manganese in 10 cc. is measured. The oxidation may be brought about in the presence of nitric acid, sulfuric acid, or phosphoric acid. However, the

TABLE I

Results of Analyses and Recovery of Added Manganese in Dry Tissue of Mouse

Material	Whole mouse	Liver	Weight of dry sample used	Mn added	Mn found	Mn recovered
	gm.	gm.	gm.	mg.	mg.	per cent
Whole mouse minus liver (0.3 mg. Mn per day for 90 days)	6.5		0.26		0.0024	
			0.26		0.0027	
			0.26	0.0018	0.0043	97.2
Liver		0.45	0.45		0.0053	
Whole mouse minus liver (fed on dog biscuits)	5.8		1.16		0.0018	
			1.16		0.0016	
			1.16	0.0035	0.0053	102.8
Liver		0.33	0.33		0.0025	
Whole mouse minus liver (fed on dog biscuits)	6.7		1.34		0.0017	
			1.34		0.0019	
			1.34	0.0028	0.0045	96.4
Liver		0.42	0.42		0.0032	
Whole mouse minus liver (milk-fed)	7.4		7.40		0.0035	
Liver		0.43	0.43		0.0015	
Whole mouse minus liver (milk-fed)	6.3		6.30		0.0028	
Liver		0.39	0.39		0.0012	

writer has observed that the resulting color has a different tint with each acid. Therefore for accurate readings nitric acid should be used in the standard, since that acid is used in the sample.

[illegible] Table I and II [illegible] show the applicability of the procedure. Table I shows the results of the analyses of manganese in five mice. Three were fed on dog biscuits, and one of the three had 0.3 mg. of manganese added to

[illegible] water each day for 90 days. The other two received only milk supplemented with iron and copper.

Mice kept on a manganese-restricted diet in this laboratory are fed nothing but milk to which iron and copper are [illegible] therefore it was necessary to know the manganese content [illegible] food. Since both the ash of the mouse and that of milk contain very small amounts of manganese and large amounts of calcium and chlorides, the procedure described for the determination of manganese in the mouse can be applied directly to that of milk. The ash from 100 cc. of milk contains enough manganese to give

TABLE II

Results of Analyses and Recovery of Added Manganese in Three Samples of Skim Milk

Each determination was carried out on 100 cc. of milk.

Skim milk sample No.	Mn added	Mn found	Recovery of added Mn
	mg.	mg.	per cent
I		0.0023	
		0.0026	
	0.0008	0.0032	93.7
II		0.0033	
		0.0038	
	0.0012	0.0048	104.2
III		0.0038	
		0.0035	
	0.0015	0.0051	96.6

a color of sufficient intensity for satisfactory reading if the volume is made to 15 cc.

Table II shows the analyses of three separate samples of milk. The manganese for recovery was added before the material was ashed. In the whole animal this manganese was added to an aliquot part of the solution before the color was developed. The recovery of added manganese was satisfactory in all cases, and there was [illegible] calcium or chlorides. [illegible] analyzing tissues containing less calcium or chlorides than is found in the whole animal or in milk no change in the procedure is necessary.

SUMMARY

A procedure is presented for the determination of small amounts of manganese in ash which is high in calcium and chlorides. An essential feature in the method consists in removing chlorides with a minimum amount of sulfuric acid, thus preventing the formation of calcium sulfate. Filtration, therefore, becomes unnecessary and the danger of losing manganese in the process of filtering and washing is eliminated.

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IRON, COPPER AND MANGANESE CONTENT OF SOME COMMON VEGETABLE FOODS.

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[illegible] of the value of traces of copper as an adjunct [illegible] in the diet has been presented by workers [illegible] of Wisconsin (Waddell, Elvehjem, Steenbock and [illegible]). [illegible] of Bert Lunde, of Theis and Hoglund, and [illegible] seem to show that manganese may also [illegible]. While the accepted feeding of these [illegible] and on the clinical [illegible] is still some distance in the future, [illegible] as to the quantities of these elements contained in various [illegible] which are used as food is desirable. Peterson and Lindow[6] [illegible] to the fact that while knowledge of the carbohydrate, [illegible] protein contents of foodstuffs grown under different [illegible] is very extensive, research literature [illegible] of elements. These authors, together with Elvehjem[7], have performed valuable service in determining the iron, manganese, and copper content of a wide variety of vegetable foods. While [illegible] results are reported in practically all cases on the analysis of a [illegible] sample of each kind, they have also called attention to possible [illegible] in any given crop. Previous work in this laboratory by [illegible] Coupland von Kolnitz[8] has shown that large variations exist in the iodine content of vegetable foods, while Greaves and Hirst[9] have called attention to similar variations in the quantities of sodium, potassium, calcium, magnesium, sulfur, phosphorus, and iron present in cereal [illegible]. For purposes of practical value in nutrition work it is desirable [illegible] of variation and average values be determined.

In course for with the studies which have been made in this laboratory on the iodine content of foods, a large number of samples of vegetables grown in South Carolina were obtained through the cooperation of the several county agricultural and home demonstration agents of the [illegible]. Eighteen of the more commonly used vegetables were selected [illegible] the study. While it might be desirable to consider a larger number [illegible] of the same kind, it is believed that when five or more are [illegible] the results are fairly suitable for this general locality.

[illegible] dried by a current of air at room temperature, then

[illegible] of U. S. Department of Agriculture.

[illegible]

[illegible]

TABLE 1.

[illegible]

Variety	Dry matter (per cent)			Iron (mg. per kilogram of dry matter)			Manganese (mg. per kilogram of dry matter)			Copper (mg. per kilogram of dry matter)		
	Minimum	Maximum	Average	No. of samples	Minimum	Maximum	Average	No. of samples	Minimum	Maximum	Average	No. of samples	Minimum	Maximum	Average
Asparagus	11.0	11.6	6.6	16	310	125	207	8	80.0	27.7	44.1	9	21.0	11.6	15.3
[illegible]	12.0	8.7	9.3	4	210	152	111	6	95.1	33.1	55.4	6	12.3	5.6	9.1
[illegible]	11.1	7.3	9.0	3	335	130	72	5	[illegible]	[illegible]	[illegible]	3	[illegible]	[illegible]	[illegible]
[illegible]	10.1	5.7	8.5	15	370	149	201	13	55.0	25.3	39.7	14	13.3	5.5	9.6
[illegible]	19.6	6.2	7.7	8	305	74	182	7	97.7	50.7	59.7	8	8.0	4.5	6.3
[illegible]	10.8	8.5	10.0	8	330	111	204	8	90.9	19.1	42.9	8	11.4	7.5	10.7
[illegible]	[illegible]	13.0	17.3	5	795	335	577	3	53.1	[illegible]	21.1	2	21.8	[illegible]	22.5
[illegible]	7.5	5.7	8.6	...	...	...	...	3	52.2	19.1	31.1	3	15.1	20.4	22.7
[illegible]	10.5	4.9	6.5	9	4320	225	2110	8	102.2	62.9	120.4	6	16.9	9.1	10.9
[illegible]	16.7	9.9	12.9	5	140	72	101	5	62.5	33.5	45.3	8	13.4	5.9	9.4
[illegible]	15.3	6.3	11.2	6	305	75	153	5	63.2	57.7	62.3	9	23.5	5.0	11.5
[illegible]	20.7	16.8	19.6	19	185	65	119	20	11.3	5.6	9.1	43	19.6	3.5	7.4
[illegible]	12.9	5.4	9.1	13	1750	275	803	12	293.3	22.5	101.2	11	19.9	5.5	10.7
[illegible]	6.3	5.1	5.0	3	130	79	125	6	27.6	18.9	23.3	6	15.3	11.9	13.2
[illegible]	20.7	20.0	20.0	12	88	39	61	9	17.7	2.5	9.3	10	8.5	3.1	6.2
[illegible]	10.0	3.0	9.2	...	...	...	...	5	11.3	9.7	13.6	5	5.0	4.0	4.4
[illegible]	13.8	11.4	13.2	...	...	...	...	6	143.8	64.0	107.6	5	13.3	5.7	7.8
[illegible]	8.5	3.0	4.7	8	185	115	173	8	33.7	17.7	25.7	3	33.8	7.7	17.4

[illegible] 100°C., after which they were ground and bottled. Although [illegible] on the dry basis, the ground samples still contained from 6 to 8 per cent of moisture as determined by drying the powder at 100°C. For the determination of copper and manganese, respectively, 5 grams of dried vegetable were used, and 1 gram was used for the determination of iron. The methods followed, those of the Wisconsin workers, were subjected to a preliminary series of careful recovery experiments on known amounts of the metals sought and found to be as satisfactory as the authors suggested them to be. For purposes of comparison with the roots, the leaves of beets, carrots, and turnips were included. Carrot leaves may not be eaten, but beet leaves frequently are and turnip leaves are probably more popular than any other salad green in this locality. The results are summarized in Table 1.

It is apparent from the results given that among the several vegetable types there is a far wider degree of variation in iron and manganese than in copper. In iron the range is from an average of 2110 parts per million in beet tops to 31 in sweet potatoes, for manganese from 383 in beet leaves to 1.4 in sweet potatoes, while for copper it is only from 17.4 in tomatoes down to 1.1 in turnips. The variation among samples of the same product is also considerable, often in iron and manganese more than in copper, and greater in iron than in manganese. The significance of these wide variations is not apparent at present. Graves and Hirst were unable to find a correlation between the quantity of available moisture and the mineral content of grains, and it seems extremely doubtful that available moisture can be the controlling factor in these South Carolina vegetables.

In comparison with the values reported by the Wisconsin workers, the average values for iron and manganese reported in this paper are on the whole higher, in some cases very much higher. Out of thirteen varieties on which comparative values were obtained, ten South Carolina samples gave higher values for iron, while all of eleven varieties which could be compared showed higher values for manganese. On copper the differences are not so striking, all of the Wisconsin values falling within the range of the samples here reported. Of fifteen varieties which could be compared, averages on six are higher. The high concentration of copper in tomatoes is of interest in connection with stifling effects that have been produced in Florida by Allison, Bryan and Hunter[1] by fertilizing this crop with a small quantity of copper sulfate.

If these vegetables are divided into groups, some interesting relations develop. Iron, manganese, and copper in leaves and shoots average, respectively, 682, 168, and 11 parts per million; roots and tubers, in the same constituents average 132, 32.2 and 8.2 parts per million; likewise, fruits average 139, 22.8 and 12.5 parts per million, all on a dry basis.

[1] Bull. Agricultural Res. Inst., [illegible]

Leafy vegetables were found to be outstanding in their content of iron and manganese, and while the differences with regard to copper are not so striking, there is no doubt definitely higher in these [illegible] and fruits. This observation confirms that of the Wisconsin [illegible] and helps to establish the predominant value of the green leafy vegetables in the diet. [illegible] parallel [illegible] observations from this laboratory on the iodine content of vegetables.

Three root or tuber vegetables—beets, carrots, and turnips—were examined both as to roots and foliage. The comparative values are of interest and are shown in Table 2.

TABLE 2.

Iron, manganese, and copper content of roots and leaves of the same plant compared

(Parts per million.)

	IRON	MANGANESE	COPPER
Beets: roots	141	55.4	9.1
Beets: leaves	372	182.7	15.3
Carrots: roots	224	42.2	10.7
Carrots: leaves	517	121.1	[illegible]
Turnips: roots	...	13.6	[illegible]
Turnips: leaves	...	107.3	7.3

The differences shown in the results in Table 2 cannot be due to differences in water content, for if calculated back to the original basis [illegible] It is quite easy to think of the foliage as [illegible] the root in such crops as [illegible] principally storage tissue, and these differences may perhaps be indirect evidence of the essential function of these metals in the metabolism of the plant.

SUMMARY.

Data are presented for the iron, manganese, and copper content of a number of samples of eighteen kinds of vegetables which are used as food. The methods of analysis are those employed by workers at the University of Wisconsin. They were checked on known quantities of the metals sought and found satisfactory. The results are discussed in the light of analytical data and from the standpoint of the use of these vegetables in nutrition. When compared with roots and fruits, vegetables, the leafy vegetables were found to be outstanding in the amounts of iron, manganese, and copper.

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The Colorimetric Determination of Manganese in Biological Material.

BY MARION B. RICHARDS, D.Sc.

The possible importance of manganese as a factor in animal metabolism has been indicated by various authors.[1] With a view to studying more closely the function of this element in nutrition, it has been necessary to work out a technique for the accurate and rapid determination of small amounts of manganese in biological material, particularly in that of animal origin.

While various methods of determining manganese, as described in the literature, are fairly satisfactory under the conditions given, investigation has shown that in dealing with such minute amounts as are liable to occur in animal tissues, certain precautions are necessary which seem to have been overlooked by most workers.

The persulphate method of oxidation of the manganese to permanganate, in readiness for the colorimetric comparison, has been widely used since its introduction by Marshall[2] in 1901, and Wester[3] has worked out very carefully the conditions under which it can be used for the determination of very small amounts of manganese in such materials as the ash of plants. The periodate method of Willard and Greathouse[4] seems preferable to that of Marshall in that it requires no catalyst, and is claimed by its authors to be free from various faults of previous methods. It was originally applied to the determination of manganese in steels and iron ores, and the present paper seeks to show, as Wester has done for the persulphate method, the conditions under which it may be used for the determination of minute amounts of manganese in organic material. It will be seen that, due regard being paid to one or two details in the procedure, the periodate method is preferable to the persulphate method in determining small amounts of manganese, just as it is preferable in the estimation of larger amounts.

PROPOSED MODIFICATIONS.—It has been found that in the application of the periodate method to the determination of traces of manganese, two points in particular require attention:—(1) The acidity of the solution to be oxidised, and (2) the removal of chlorides.

1. ACIDITY OF THE SOLUTION DURING OXIDATION.—(*a*) *Danger of Excess Acidity.*—Willard and Greathouse, in describing their method, emphasise the danger of having insufficient acid present, and state that a very large concentration of acid does no harm. This is no doubt true for the amounts of manganese dealt with in their tests, but the lowest amount they used was 2·5 mgrm. of manganese, a quantity much beyond the amount present in a workable sample of animal material.

If the amount of manganese present is very small, e.g. 0·005 mgrm. or less, as quite frequently occurs in the analysis of animal organs, the danger lies in excess of acidity, rather than in its insufficiency. In such cases an acidity of 15 per cent. sulphuric acid (*i.e.* 15 c.c. of concentrated acid per 100 c.c. of solution) is the maximum permissible. An acidity of 20 per cent. is sufficient to prevent the full colour from developing, or to cause it to fade rather rapidly, the solution generally assuming a yellow tint. Moreover, it has been found that if the colour has been destroyed by excess of acidity, it cannot be fully restored by subsequent dilution of the solution.

In a recently published paper Skinner and Peterson[5] report that in a few instances during the oxidation with periodate the permanganate colour faded as the solution was reduced, a difficulty which they overcame by the addition of a few drops of syrupy phosphoric acid to the boiling solution. As their paper was published after the present tests of the method were satisfactorily concluded, I have not tested whether a colouration that has faded from excess of acidity can be restored by means of phosphoric acid, but it may be stated that with the acidity carefully restricted, no such fading has been experienced.

(*b*) *Rate of Colour Development at Different Acidities.*—Willard and Greathouse state that increase of acidity increases the rate of colour development, and while this is true for the concentrations of 15 per cent. sulphuric acid and over, with which they chiefly deal, it is not quite accurate where lower acidities are concerned. For all the amounts of manganese tested (0·0025–0·5 mgrm.) the rate of oxidation appears to be a minimum when the concentration of sulphuric acid is about 9–10 per cent., the colour appearing only very slowly at this concentration. Above this point the rate does increase with increasing acidity, but below it the rate increases as the acidity diminishes. Below 1 per cent. the colour develops very rapidly, but the solutions show the same yellowish tint and tendency to rapid fading as are found when the acidity is too great.

In view of these observations—the possibility of fading if the acidity is greater than 15 per cent. or less than 1 per cent., and the slowness of the reaction at 9–10 per cent., it is recommended that in dealing with animal tissues conditions should be regulated so that the final acidity is about 5–6 per cent.

In the analysis of plant material, which has in general a higher manganese content, it is very seldom *essential* that this low acidity should be maintained, but it is simple to adopt the same procedure for all classes of material, and to increase the acidity to 15 or 20 per cent. after addition of the periodate, if it is evident that considerable amounts of manganese are present.

It seems probable, in view of our results, that the "unexplained failures" sometimes experienced by Reimann and Minot[6] in using solutions of greater acidity than 2 per cent. nitric acid, have occurred in cases where the manganese present was very small in amount, and the variable acidity in the final solution arising from their acid-sulphate-fusion method of ashing, happened to overstep the limit suitable for traces of manganese.

II. THE REMOVAL OF CHLORIDES.—According to Willard and Greathouse, the presence of chloride does not interfere with the oxidation, as the chlorine may be driven off by adding excess of periodate. This may, however, involve boiling for chlorine for a considerable time before the colour comes up at all, and unless the chlorine is thoroughly removed, there is danger of the colour fading to some extent before the estimation is finished. If little manganese is present, this continued boiling is inadvisable, in view of the careful regulation of the acidity which has been found necessary. Hence it is recommended that all chlorides should be destroyed before the oxidation stage, by repeated evaporation with concentrated sulphuric acid—a point on which Wester also lays stress.[6]

DETAILS OF METHOD AS ADOPTED.—1. *Preparation of the Material for Oxidation.*—The process of wet ashing and the acid-sulphate-fusion method described by Reimann and Minot, are both rather cumbersome in view of the large number of routine analyses to be carried through, and the necessity for ease in manipulation at all stages of the determination. The method adopted therefore is that of ordinary ashing, with subsequent treatment of the ash with various acids.

Ash the material as completely as possible (in a silica basin) at a low-red heat, and evaporate to dryness with a little concentrated hydrochloric acid. Add a few c.c. of sulphuric acid (25 per cent. by volume), and 3-4 drops of concentrated nitric acid, and evaporate carefully to dryness on a water bath and sand bath, finishing off by gentle ignition over a Bunsen flame. Add 2-2·5 c.c. of sulphuric acid (25 per cent.) and a little water, and evaporate to white-fuming stage, thus removing all traces of chlorides.

After cooling, dilute and filter into a small flask (50 c.c.) for oxidation. Add to the solution one or two small pieces of pumice stone, previously purified by boiling with 5 per cent. sulphuric acid and a little periodate. Evaporate down to about 10 c.c., when the concentration of sulphuric acid will be 5-6 per cent. (allowing for the loss of acid at the fuming stage), and the solution is ready for oxidation.

II. *Oxidation.*—Add 0·3 grm. of sodium periodate (or potassium periodate), and insert a loosely-fitting pear-shaped glass stopper in the neck of the flask. Heat to boiling, and immerse in boiling water for 30 minutes. (Wooden test-tube holders serve to hold the flasks in position in the bath.)

If the whole solution is required for the colour-comparison, cool, and transfer for the determination to the colorimetric tube described below.

If the solution has sufficient colour to require dilution, either (*a*) dilute to the required volume with 5 per cent. sulphuric acid that has been boiled with a little periodate, or (*b*) add water till it has nearly the required dilution, and heat for 15 minutes longer in the bath. Transfer to a calibrated flask, make up to mark, and estimate the manganese by comparing with the standard solution.

III. *Colorimetric Tubes.*—The tubes used are 10 c.c. calibrated cylinders of [illegible] capacity of 12-13 c.c., and internal diameter 13-14 c.c., graduated

in tenths of a c.c., and made without the usual flange at the bottom, so that the tubes can be held closely together for the colour-comparison. These tubes are most suitable for very dilute solutions. With a standard which contains 0·001 mgrm. of manganese per c.c., 7-10 c.c. give a very convenient depth of tint for matching, but a detectable pink colour can be obtained with as little as 1 c.c., and differences of 0·2 c.c. give distinctly recognisable differences in tint.

If preferred, a colorimeter may be used when the solution is sufficiently strong, but in many cases the amount of manganese in organic materials is so small that it would require an excessive amount of material to give sufficient depth of colour for the colorimeter. Hence we prefer to work always with the above tubes, and usually with the above standard, bringing the colour of solution approximately to this strength by choosing suitable amounts of material for oxidation, and by suitable dilution.

[It may be noted that Wester in his critical study of the persulphate method, found it necessary to have special colorimetric tubes made with ground-glass caps, to protect the solutions from the action of reducing fumes. With the periodate method, we have found no necessity for such covers, under ordinary laboratory conditions.]

IV. *Standard Solutions.*—(*a*) *Stock Solution of Manganese Sulphate.*—Pure potassium permanganate (0·1584 grm.) is dissolved in about 100 c.c. of water, and reduced by sulphur dioxide. Instead of passing in sulphur dioxide, it is much simpler to generate the gas in the solution itself, by means of sulphuric acid and sodium sulphite or bisulphite. The solution is heated till the reaction takes place, and the excess of sulphur dioxide is then boiled off. This method of reduction does not seem to interfere in any way with the stability of the standard solutions. Dilute to 1 litre (1 c.c. of this solution=0·05 mgrm. of manganese).

(*b*) *Standard Solution for Comparison.*—Oxidise 20 c.c. of stock solution with periodate in the usual way, and make up to 1 litre with 5 per cent. sulphuric acid (previously boiled with periodate). (1 c.c. of this solution=0·001 mgrm. of manganese). This is the most useful concentration for the colorimetric tubes above described, but stronger solutions may be used if desired.

(*c*) *Stability of Standard Solutions.*—Willard and Greathouse state that a remarkable feature of the solutions oxidised by periodate is their great stability when a slight excess of the reagent is present. Such a solution, kept for 3 months in a stoppered flask, when compared with a similar solution freshly oxidised, showed no change whatever.

We have found that even the very dilute standard described above (0·001 mgrm. of manganese per c.c.) shows the same stability observed by Willard and Greathouse.

V. *Purity of Reagents.*—A great advantage of the procedure outlined is the [illegible] that the operations introduced during the ashing process are the strong

acids—hydrochloric, nitric, and sulphuric—and tests have shown that there is no difficulty in obtaining these entirely free from manganese. Nor have we ever found in our tests that any manganese is introduced as impurity from the silica basins, when subjected to the action of the acids as directed in the estimation. Kramer and Mhoot, on the other hand, find that the quartz beakers used in their acid-sulphate-fusion method frequently contain manganese, which is dissolved during the fusion process, resulting in contamination, which may amount occasionally to as much as 0·020 mgrm.

The only other chemical introduced in the present method is the periodate, which is almost invariably quite free from manganese. In only one lot have we found a slight manganese impurity, amounting to only 0·0005 mgrm. for 0·3 grm. periodate, the amount used in the determination.

Modifications of Usual Procedure.—The procedure described above is applicable to the great majority of vegetable and animal tissues, but in a few cases a slight modification of procedure is necessary:—(*a*) For substances containing much calcium, *e.g.* milk; (*b*) for substances containing much iron and very little manganese, *e.g.* blood.

(*a*) *Substances containing much Calcium.*—Unless most of the calcium is removed, it is difficult to avoid loss by spurting in evaporating to dryness with sulphuric acid. There is also danger of the minute amount of manganese present (*e.g.* in milk) being carried down with the calcium sulphate if the ash is treated with sulphuric acid in the usual way. The procedure adopted is as follows:—

(1) *Estimation of Manganese in Milk.*—One hundred c.c. of milk in a silica basin are evaporated down on the water-bath, protecting from possible contamination by particles of rust, which may contain considerable manganese. After asking completely, and taking down to dryness with hydrochloric acid, the ash is moistened with a little water, and broken up as finely as possible. Twenty-five c.c. of boiling sulphuric acid (33 per cent. by volume) are added, with careful stirring. After thorough cooling (overnight, if convenient), filter off into another silica basin, washing 6–8 times with 5 c.c. of 33 per cent. sulphuric acid. (N.B. The filter paper must have been previously tested for its resistance to this strength of acid. If the paper available is not strong enough for 33 per cent. acid, 20–25 per cent. may be used, as this will remove sufficient of the calcium sulphate to permit subsequent evaporation to dryness. Ten per cent. acid does not remove the calcium sulphate.) Evaporate the filtrate to dryness and proceed as usual, except that it may be necessary, when filtering at the final stage before oxidation, to re-filter through same before washing, in order to get an absolutely clear solution.

(*b*) *Substances containing much Iron and very little Manganese.*—If the amount of manganese present is minute, and the ratio of iron to manganese is high, *e.g.* in blood, proceed thus:—After evaporation to dryness with sulphuric acid, [illegible] over a Bunsen burner, instead of adding 2–2·5 c.c. of sulphuric acid [illegible] of strong hydrochloric acid and 0·5 c.c.

sulphuric acid. The phosphoric acid is used to reduce the yellow tint of the iron salt at the final matching stage. Further procedure is as usual. With no other animal tissue do we find this modification necessary. In the case of liver, the high amount of iron does not interfere, on account of the dilution necessitated by the high manganese content of liver.

Tests of Method. The method has been applied in a large number of analyses of plant and animal tissues, with very satisfactory results, judged by (1) concordance of parallel determinations on the same sample, and (2) recovery of added manganese from the samples analysed.

Some results for different classes of material are shown in Tables I and II.

Table I.

Concordance of Parallel Determinations.

Substance.		Manganese content of various samples. (Mgrms. of Mn per 100 grms. of dry matter.)				Average. (Mgrms. of Mn per 100 grms. of dry matter.)
Grass		61·3,	61·2,	61·7,	61·2	61·4
Tapioca		0·676,	0·673,	0·698,	0·681	0·682
Potatoes (peeled)	I.	0·707,	0·705,	0·711,	0·704	0·707
	II.	1·19,	1·17,	1·18,	1·18	1·18
	III.	0·467,	0·475,	0·473,	0·475	0·473
	IV.	0·677,	0·638,	0·655,	0·650	0·655
		(Mgrms. of Mn per 100 grms. of moist tissue.)				(Mgrms. of Mn per 100 grms. of moist tissue.)
Liver (various species)	I.	0·371,	0·368			0·370
	II.	0·229,	0·227			0·228
	III.	0·412,	0·420			0·416
	IV.	0·231,	0·237			0·234
	V.	0·248,	0·250			0·249
	VI.	0·137,	0·144,	0·137		0·139
Kidney	I.	0·152,	0·162			0·157
	II.	0·174,	0·174			0·174
Spleen (*b*)		0·018,	0·018			0·018
Milk (*a*)	I.	0·0039,	0·0048,	0·0042,	0·0038	0·0041
		0·0043,	0·0039,	0·0049,	0·0045	
		0·0034,	0·0039,	0·0049,	0·0049	
	II.	0·0037,	0·0039,	0·0040,	0·0037	0·0041
		0·0051,	0·0043			

(*b*) By the modification of method for substances containing much Fe, and little Mn.
(*a*) " " " " much Ca.

TABLE II.

RECOVERY OF ADDED MANGANESE.

Substance.	Amount analysed. Grms.	Mn added. Mgrms.	Total Mn found. Mgrms.	Manganese in substance taken. Mgrms.	Added Mn found. Mgrms.	Recovery. Per cent.
Grass	2·011	0·25	1·48	1·23	0·25	100
	2·010	0·25	1·46	1·23	0·23	92
	2·011	0·50	1·76	1·23	0·53	106
	2·011	0·50	1·74	1·23	0·51	102
	2·010	1·00	2·16	1·23	0·93	93
	2·013	1·00	2·20	1·23	0·97	97
Tapioca	10·003	0·025	0·095	0·068	0·027	108
	10·007	0·050	0·122	0·068	0·054	108
	c.c.					
Milk	100	0·0025	0·0067	0·0041	0·0026	104
	,,	0·0025	0·0067	,,	0·0026	104
	,,	0·0050	0·0097	,,	0·0056	112
	,,	0·0050	0·0095	,,	0·0054	108
	,,	0·010	0·0133	,,	0·0092	92
	,,	0·010	0·0136	,,	0·0095	95
	,,	0·020	0·0241	,,	0·0200	100
	,,	0·020	0·0237	,,	0·0196	98
	,,	0·040	0·0440	,,	[illegible]	[illegible]
	,,	0·040	0·0435	,,	[illegible]	[illegible]
	34	0·080	0·080	0·0014	0·0786	[illegible]
Blood	25	0·0025	0·0039	0·0012	0·0027	108
	,,	0·0050	0·0063	,,	0·0051	102
	,,	0·020	0·0194	,,	0·0182	91
	,,	0·040	0·0397	,,	[illegible]	[illegible]
	,,	0·080	0·0756	,,	0·0744	[illegible]

SUMMARY.—The periodate method of Willard and Greathouse for the colorimetric estimation of manganese can be applied to the estimation of very small amounts of manganese, such as occur in biological material, if due care be taken to drive off all chlorides before oxidation, and to see that the acidity of the solution does not exceed 15 c.c. of sulphuric acid per 100 c.c. of solution. It is recommended for reasons given, that the acidity be kept about 5 to 6 per cent., unless it is found that very considerable amounts of manganese are present. It has been shown that the method gives satisfactory results, both as regards agreement of parallel determinations, and as regards recovery of added manganese from the samples analysed.

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French Translation

Study of the Biological Modifications in Manganism

by J. Rodier (Rabat)

In the course of our inquiry on manganism in which we discovered 151 poisoned persons, we were interested in studying the biological modifications caused by the introduction of a poison into the organism. The objective was, on the one hand, to look for the obvious signs for a test of poisoning making it possible to discover illness before the appearance of nerve lesions in which the evolution is inexorable and irreversible and on the other hand, to determine exactly the modifications of biological constants produced by the poisoning, in a manner helpful for diagnosis and supportive to the experts. We therefore studied a certain number of subjects showing the customary disturbances owing to the period of the condition and in particular, disturbances in walking. The material problems owing to the remoteness and isolation of the mining centers, prevented us from carrying on the studies as long as we would have wished. Even if it was not possible to exactly specify a positive and irrefutable test for poisoning our studies nevertheless permitted us to come upon some findings which we considered useful to report here.

Exploration of the renal function:

Urinary albumin-- In the 151 cases examined, we only found one sole albuminuric with an elimination of 0.65 g/liter.

Urinary cytology-- This examination was carried out on the urine of 37 subjects. We did not note abnormal cytology both from the stand point of

organised and unorganised cells.

Azotemia-- The urea in the blood of 40 invalids was determined by the hypokromite method. If one considers that the amount of urea in normal subjects is between 0.20 g and 0.30 g p 1000, half of our patients had a normal amount with an average of 0.26 g/1000, the other half showed a average amount of 0.39 g/1000, some subjects even reached 0.50 g/1000. One might think, at first sight, that these figures are distinctly altered; nevertheless it is necessary to take account of the fact that the amount of urea in the blood has a tendency to be much higher than normal in North Africans and in Europeans living in Northern Africa, but for that one could speak of a pathological phenomenon.

Ambard's constant-- This test was carried out on 20 patients. If one takes into consideration that the normal constant is not pathological above 0.090; your results are expressed thus: 4 subjects (20 percent) showed a raised Ambard's constant with an average on 0.107 and an observed maximum of 0.13, the 16 others (80 percent) had a normal Ambard's constant, with an average of 0.06.

Phenolsulfonaphthalene test-- Following the technique employed, we were able to collect, 60 percent of the colorant injected intravenously, 70 min after the injection. The test was carried out with 20 patients. Eight of them (40 percent) showed a retention of colorant with an average amount of elimination around 38%. The 12 others (60 percent) had normal elimination with an average amount of 63.9 percent.

Exploration of hepatic function

Cholesterolemia--Total cholesterol-- Cholesterol was determined colorimetrically by using Liebermann's reaction. If one considers as normal

the cholesterol level located between 1.50 and 2 g/1000, our results which were carried out on 17 subjects are distributed as follows: 11 patients (65 percent) had a normal value with a general average of 1.70 g/1000, 6 patients (29 percent) had a decrease with 1.17 g/1000 as an average amount, only one subject showed an increase with a value of 2.72 g/1000.

Esterified cholesterol-- The examination was carried out on the 17 subjects already examined for total cholesterol. If one agrees that the normal value oscillates between 1 g and 1.30 g/1000, 15 subjects (88 percent) showed a normal amount with 1.08 g/1000 as the average value. Only two subjects (12 percent) had an esterified cholesterol lower than 0.65 g/1000 as an average.

Ratio $\frac{\text{Esterified cholesterol}}{\text{Total cholesterol}}$

If one considers as normal the values situated between 0.50 and 0.70, the 17 patients showed a normal ratio with a general average of 0.65.

Protenemia--total protein-- The technique used was that of Machebouef. By considering that the normal amount of serum protein varies between 70 and 85 g/1000, our results carried out on a sample of 19 were distributed as follows: 12 patients (64 percent) had normal amounts with an average value of 79 g/1000. The 7 others (36 percent) had reduced amounts with an average value of 62.7 g/1000.

Serine-- The serine level in healthy subjects varies between 40 and 50 g/1000. Our results with 10 patients were distributed as follows: 5(26 percent) showed a reduction with an average of 35 g/1000; the lowest value obtained was 30.8 g/1000; the rest of the patients (74 percent) had an average value of 48.5 g/1000.

Globulin-- The normal globulin value is located between 25 and 32 g/1000. 13 of our subjects (69 percent) belonged in this category with an average value of 31.5 g/1000. With the 6 others (31 percent) the amount was reduced with an average value of 20.15 g/1000

Ratio $\frac{\text{Serine}}{\text{globulin}}$

This ratio was calculated from the preceding results. If one considers as pathological, the values located below 1.20, 16 patients (85 percent) showed a normal ratio with an average value of 1.79. With 3 others (15 percent), the ratio was lowered with an average value of 0.99.

Flocculation reaction--thymol test-- This reaction was carried out according to the technique of Mac Lagan with a photometric reading. If one considers that the results expressed in Mac Lagan units are negative when they are below 3, doubtful when they are between 3 and 4, and positive when they are above 4, our results are distributed as follows: 12 subjects (60 percent) had a normal reaction with an average of 1.97 M.L.U.; 3 subjects (15 percent) a doubtful reaction with an average of 4 M.L.U. and 5 subjects (25 percent), a distinctly positive reaction with an average of 5 M.L.U.

Hanger test--the cephalin-- cholesterol test was only carried out on 11 subjects: 8 (73 percent) showed a normal reaction, 2 (18 percent) a doubtful reaction; only 1 a distinctly positive reaction.

Maillard coefficient: The Maillard coefficient was established with the urine of 18 patients following the determination of ammonia by the Ronchese procedure and the determination of urea by the hypobromite method. If one considers that the normal ratio is close to 7 in a

healthy person subjected to a mixed diet, our results appeared to cause an increase of the coefficient in 11 patients (61 percent) with an average amount of 10.9. The coefficient was normal in all the other patients (39 percent) with an average amount of 6.11.

Test for provoked galactosurea-- This test was carried out according to Fiessinger's technique. The measurements obtained are considered pathological when the amount of galactose eliminated exceeds 2g. Twenty patients were subjected to this test. In 18 subjects elimination was normal (90 percent) with an average amount of eliminated galactose of 0.74 g. In only 2 subjects, elimination was much higher with respectively 2.64 and 2.73 g.

Test with tetrabromophenolsulfonaphthalene-- According to the technique which we used, the normal quantity of dye recovered in the blood after 30 min. in a healthy subject, is on the order of 10 to 15 percent of the intravenously injected dose. This test was carried out on 20 patients. Eleven (55 percent) had a normal retention with an average of 12.5 percent, 2 (10 percent) showed a slight retention of the order of 22 percent, finally (35 percent) showed an unquestionable retention with an average established at 65 percent. In this last group, 3 total retainers were also observed.

Cholinesterase activity-- This measurement was carried out according to Vincent's method. By accepting that the normal value is located, in the healthy man, between 4 and 6, our results with 22 patients, is distributed as follows: 15 subjects (68 percent) had a normal value with an average activity of 4.68; seven others (32 percent) on the other hand, had a reduced activity with an average of 3, the lowest observed value was 1.5.

Various Examinations

Mineral elements-- Potassium

Potassium was determined in the plasma by flame photometry. If one takes into consideration that the average value is located between 0.16 and 0.20 g/1000, only 1 patient of the 60 studied, showed a slightly modified level of 0.257 g/100.

Sodium

This measurement was carried out by flame photometry of the plasma. If one takes into consideration that the normal level of sodium varies between 3.15 and 3.40 g/1000, the 60 subjects examined had a normal level.

Calcium

The Guillaumin technique was used. Unfortunately only 9 patients could be examined. Recalling that normal calcemia varies between 90 and 110 mg/1000, 7 patients (78 percent) had a normal amount, only 2 had lowered calcemia, the lowest result was 80.62 mg/1000.

Reduced glutathione

This measurement was carried out with 21 patients. If one takes into consideration that the normal level of reduced glutathione in a healthy subject is between 30 and 37 mg/100 ml of blood, our results appeared to be that 7 subjects (33.3 percent) showed normal levels of the average order of 34.72 mg. On the other hand, with 8 patients (38.2 percent), one observed a reduction with an average level of 15.5 mg/100 ml and with 6 others (28.5 percent), an increase with an average amount of 50.38 mg/100 ml.

Total glutathione

This measurement was carried out with 21 patients. If one takes into

consideration that the level of blood total glutathione in a normal subject is from 37 to 42 mg per 100 ml, our results appeared to show a normal level with 6 subjects (28.5 percent) with an average amount of 40.29 mg/100 ml, a reduced level with 3 patients (14.3 percent) with an average of 28.7 mg/100 ml and finally an increased level with an average value of 52.8 mg/100 ml in the 12 remaining (57.2 percent).

Alkaline reserve

This measurement was carried out by the Van Slyke technique. If one takes into consideration that the normal amount of CO_2 volume is 52 to 77, the 14 patients which we examined have a normal alkaline reserve.

Phosphatase activity-- Serum phosohate activity was measured according to the technique of King Armstrong.

Acid phosphatase. Our results were expressed in Guttman units. If one takes into consideration that this activity is normally weak on the order of 3 units, and that it is pathological above 6 units, the examination carried out on 20 patients is distributed as follows: 17 subjects (35 percent) had a normal level, 3 had a significant elevation which reached respectively 18, 27.5, 45 G.U.

Alkaline phosphatase

The results were expressed in Armstrong units. If one considers as normal the values located between 4 and 12 Armstrong units, the results of our determinations with 20 patients appeared to show an increase in, 2 subjects (10 percent) with respectively 15.5 and 17.5 Armstrong units, while all the others had normal activity.

Coproporphyrinurea-- In the course of our previous research, we thought about the existence of a considerable elimination of coproporphyrins in

the urine of manganics. The lack of appropriate medium materials prevented us from making the determination. As soon as it was possible, we took up this study again totally, by using the extraction method of Dobriner and Rhoads and by making the determination with fluorometry. With 62 patients, we did not record any measurements higher than normal (50γ/24h). A certain number of the same subjects (26) had a very weak level below 10γ/liter of urine.

Congo rouge-- This test was carried out with 18 patients following the technique of Adler and Reimann. According to this method less than 40 percent of dye was removed from the circulation of a normal subject in 1 hr. This was produced in 16 of our subjects (89 percent) who showed an average retention value of 26.8 percent. Only 2 subjects had an accelerated retention with respectively 55.3 percent and 57 percent.

Endocrine Examination

Elimination of 17-ketosteroids--

The 17 ketosteroids were determined photocolorimetrically by the technique of Cahen and Salter modified by Jayle on the urine of 74 patients. If one takes into consideration that the normal level of elimination in man is from 12 to 13 mg/24hr. Fourteen patients (19 percent) had a normal elimination varying between 11 and 13 mg with a general average level of 12.3 mg/24 hr. On the other hand, with 60 subjects (81 percent) one could observe a decreased elimination of 11 mg/24 hr; with certain patients likewise the elimination level scarcely reached 6.7 mg/24 hr. The general average of all the elimination levels was established at 8.69 mg/24 hr.

Basal metabolism-- This examination was carried out with 84 manganics by

the closed circuit method. Three determinations were carried out for each patient and the usual precautions for this examination were rigorously respected. By considering as normal variations of 15 percent to +15 percent, 45 subjects (54 percent) had an increased metabolism, this increase also reached 70 percent in some cases, the average was +31 percent. In 39 subjects (46 percent) the basal metabolism was normal with an average of +5.5 percent. None of our patients had an amount less than -13 percent.

We judged that it would be useful with 3 subjects considered to have a normal metabolism, to carry out the stress test of Durupt. This examination revealed to us that we happened in reality to be in the presence of subjects having an increase in basal metabolism which was hidden in the course of the first measurement, thus:

M. F.	At rest: +8 percent	After stress: +15 percent
Ben B	At rest: +2 "	After stress: +19 "
Ben H	At rest: +2 "	After stress: +35 "

It is possible to believe that if this counter-test had been systematically carried out, we would have been able to discover a certain number of increased metabolisms. If one proceeds with the establishment of the general average of all the basal metabolism measurements carried out, these established at +19.5 percent, make apparent therefore in an unquestionable manner, the elevation in this test during the course of manganese poisoning.

Hematological examination

Blood Corpuscle Count-- The counting of red blood corpuscles and white blood corpuscles was carried out on 18 patients by the classic cell

technique of Malassez.

Our results distributed themselves thus:

Red blood corpuscles per mm^3 9 subjects (19 percent) had a reduction of the amount of red blood corpuscles of 4,000,000 with an average of 3,340,000.

18 subjects (37 percent) had a red blood corpuscle measurement between 4,000,000 and 4,500,000 with an average of 4,320,000

21 subjects (44 percent) had a red blood corpuscle measurement above 4,500,000 with an average of 4,977,000

The general average of red blood corpuscles of all the examined patients was 4,458,000 per mm^3.

White blood corpuscles per mm^3, 9 subjects (19 percent) had less than 6000 white blood corpuscles with an average of 5,160

25 subjects (62 percent) had between 6,000 and 8,000 white blood corpuscles with an average of 7,290.

9 subjects (19 percent) had more than 8,000 white blood corpuscles with an average of 9,360.

The general average of white blood corpuscles of all the examined patients was 7,270 per mm^3.

It should be noted in connection with these examinations, that the normal level of red blood corpuscles in Moroccans, usually fluctuates between 4,000,000 and 4,500,000 per mm^3.

Hemoglobin-- Hemoglobin was measured by means of a Sahli hemoglobinmeter. The results of 43 examined patients, are distributed as follows:

% of hemoglobin	Number of cases	%of patients	Average hemoglobin level
from 90 to 100	19	44 percent	97 percent
80 to 90	11	25.5 "	84 "
70 to 80	11	25.5 "	71 "
below to 70	2	5 "	60 "

Leukocyte formula-- This examination was carried out on 112 patients. It became quickly apparent that a certain number of those poisoned showed an inversion of the leukocyte formula with an increase in the number of lymphocytes. We therefore decided to classify our results by taking into account variations in the lymphocyte level. We considered as a leukocyte formula which is distictly modified, that in which the lymphocyte level for 100 white cells was above 45; as the formula in which the modification was slight, that in which the lymphocyte level varies between 35 and 45 per 100; and as the normal formula that in which the lymphocyte level was less than 35 per 100. The results classified thus are distributed as follows: distinctly altered leukocyte formula, 46 cases (41 percent) with a lymphocytic average of 57.2 per 100; leukocytic formula in which the modification was slight, 33 cases (29.5 percent) with a lymphocytic average of 33.9 per 100; normal leukocytic formula 33 cases (29.5 percent) with a lymphocytic average of 27.3 per 100.

The average leukocytic formula for each of the 3 selected categories is respectively:

	P. N. %	P. M. %	P. H. %	L. %	M. %
A Moyenne des formules considérées comme normales	66,4	2,7	0,3	27,3	3,3
B Moyenne des formules dont la modification est légère	51,8	2,9	0,2	39,9	5,2
C Moyenne des formules nettement modifiées	31,8	3	0,4	57,2	4,6
D Moyenne générale de toutes les formules	51	2,9	0,2	41,5	4,4

A - Average of formulas considered to be normal
B - Average of formulas in which there is slight modification
C - Average of formulas distinctly modified
D - General average of all the formulas

Examination of the cerebrospinal fluid

Albumin-- The albumin determination was carried out by photometric measurement after precipitation with trichloroacetic acid; 22 patients were examined. The quantity of albumin normally present in the cerebrospinal fluid is 0.15 to 0.20 g per 1000. 18 patients (82 percent) had a normal level, with 0.20 g per 1000 as an average amount. The remaining 4 (18 percent) had an increase in which the average amount was established at 0.42 g per 1000.

Glucose-- The determination was carried out on 21 samples by the modified method of Fontes-Thivolle. Recalling that the normal amount of glucose in a healthy subject oscillates between 0.50 and 0.80 g per 1000, our results are distributed as follows: 19 patients (90 percent) had normal amounts with an average of 0.67 g per 1000, only 2 subjects had a reduced level respectively at 0.20 and 0.33 g per 1000.

Chloride-- The measurement was carried out with 21 patients by the method of Charpentier-Volhard. Recalling that cerebrospinal fluid in a healthy subject contains an average 7.30 g per 1000 of chloride expressed as NaCl,

18 patients (86 percent) had a normal level with an average of 7.28 g per 1000, 3 showed a slight modification, the level was a little lowered with an average of 6.5 g per 1000.

Cytology-- This examination was carried out on 17 patients. None of the subjects examined had a leukocytic measurement above 3 per mm^3. The general average was located at 0.97 per mm^3.

Reaction with colloidal benzoin (Dr. Ninard)-- This examination carried out on the cerebrospinal fluid of 20 patients was normal in 17 subjects (85 percent), only 3 had a sufficiently weakly deviating reaction, indicating a weak meningeal reaction.

Conclusions

If the biological study that we have carried out on subjects poisoned by manganese, did not make apparent large modifications among the elements studied, it is nevertheless evident that one should take certain important variations into consideration.

From the renal standpoint, it does not appear that manganese poisoning is accompanied by serious disturbances. Nevertheless, the phenolsulfonaphthalene test indicated a retention of dye in 40 percent of the patients.

From the standpoint of hepatic examination, the tests carried out, were in general little altered, except for the one concerning the P.S.P. test and the Maillard coefficient which were respectively altered in 45 percent and 61 percent of the patients.

The endocrine examination indicated the most distinct modifications. The elimination of 17-ketosteroids was decreased in 80 percent of the subjects, and the general average of elimination for all the patients was

8.69 mg/24 hr. The basal metabolism was increased, sometimes very extremely, in 53 percent of the patients, since the average of all the basal metabolism studies was established at + 19.5 per 100.

From the hematological point of view, the examinations revealed an unquestionable inversion of the leukocytic formula in the direction of a lymphocytosis in 41 percent of the patients while 29.5 percent had a slight modification. Finally, the blood corpuscle count indicated a slight polycythemia in 44 percent of the manganics.

In conclusion, it appears desirable that in manganics, as much from the viewpoint of discovery as of examination the blood cell count, the establishment of the leukocytic formula, the measurement of basal metabolism and the study of the amount of urinary ketosteroids eliminated, should be carried out.

[illegible] p. 100). Chez les 6 autres (81 p. 100) le taux [illegible] s'est élevé avec un chiffre moyen de [illegible] p. 100).

Rapport [illegible] / [illegible]. — Ce rapport a été calculé d'après les résultats précédents. [illegible] pathologique les valeurs [illegible] de 1,20. 10 malades (83 p. 100) présentent un rapport normal avec une valeur moyenne de 1,7 [illegible] chez les 3 autres (15 p. 100), le rapport était diminué avec une valeur moyenne de 0,9.

— RÉACTION DU MONOCYTAIRE. — *Test de Rimed.* — Cette réaction a été pratiquée selon la technique de Mac Lagan avec lecture photométrique. Si on considère que les résultats exprimés en unités Mac Lagan sont négatifs quand ils sont inférieurs à 3, douteux quand ils sont compris entre 3 et 4, et positifs quand ils sont supérieurs à 4, nos résultats se répartissent ainsi : 12 sujets (60 p. 100) avaient une réaction [illegible] avec une moyenne de [illegible] 8 sujets (40 p. 100) une réaction nettement positive avec une moyenne de 5,72 U. M. L.

Test de Hanger. — Le test de sédimentation a été pratiqué sur 11 malades : 8 (73 p. 100) ont présenté une réaction normale : 2 (18 p. 100), une réaction douteuse ; 1 seul a eu une réaction nettement positive.

CHOLESTÉROLÉMIE TOTALE. — Le cholestérol de Millbert a été dosé sur les urines [illegible] malades après dosage de l'azote urinaire sur le procédé Kjeldahl et le dosage [illegible] par la méthode de Pfaff. Si l'on considère que le rapport normal est compris entre 7 chez un individu sain, comme [illegible] un régime salé, nos résultats font apparaître une augmentation du cholestérol chez 11 malades (61 p. 100) avec un chiffre moyen de [illegible]. Le cholestérol est normal chez les 7 autres sujets (39 p. 100) avec un chiffre moyen de 6,11.

ÉPREUVE DU LAVAGE GASTRIQUE PHOSPHATÉ. — Cette épreuve a été pratiquée selon la technique de Hasslinger. Les chiffres obtenus sont considérés comme pathologiques [illegible] la quantité de chlorure éliminée [illegible] 2 g. 20 malades ont été soumis à cette épreuve. L'élimination fut normale chez 16 sujets (80 p. 100) avec un taux moyen de chlorures éliminé de 0,71 g. Chez 2 sujets seulement, l'élimination a été plus importante avec respectivement 2,01 et 2,03 g.

ÉPREUVE A LA TÉTRABROMOPHÉNOLSULFONEPHTALÉINE. — Suivant la technique que nous avons utilisée, le chiffre normal de colorant retrouvé dans le sang après 30 mn chez un sujet sain est de l'ordre de 12 à 15 p. 100 de la dose injectée par voie intraveineuse. Cette épreuve a été pratiquée sur 20 malades. 11 (55 p. 100) ont eu une rétention normale avec une moyenne de 12,5 p. 100, 2 (10 p. 100) ont montré une rétention légère de l'ordre de 22 p. 100, 7 enfin (35 p. 100) ont présenté une rétention indéniable avec une moyenne s'établissant à 35 p. 100 ; dans ce dernier groupe, on a même noté 3 rétentions totales.

ÉLIMINATION CHLORURÉMIQUE. — Cette mesure a été étudiée selon la méthode de Vincent. En admettant que les taux normaux se situent chez l'homme sain entre 4 et 6, nos résultats sur 22 malades se répartissent ainsi : 15 sujets (68 p. 100) [illegible] un chiffre normal avec une moyenne [illegible] de 4,68 ; 7 autres (32 p. 100), par contre, [illegible] avec une moyenne de 5, le chiffre le plus élevé étant de 1,5.

Explorations diverses.

[illegible] *Potassium.* — La potassium a été dosé dans le sérum par [illegible] 20 malades. Il ressort que le taux moyen est situé entre 0,16 et [illegible] malades, [illegible] qui ont été étudiés, présentait un taux inférieur à la normale [illegible] 17 g. p. 100).

[illegible] dans le sérum par photométrie de flamme. Si [illegible] normal des chlorures varie de 3,17 à 3,70 g p. 1 000, les 60 sujets [illegible] un taux normal.

Cal[illegible]. — La [illegible] que nous avons utilisée fut celle de Kaufmann. Malheureusement, [illegible] le calcémie normale varie entre [illegible] 110 mg [illegible] 70 p. 100 avaient un chiffre normal, 2 seulement [illegible] 1 le [illegible] de 50 à 2 mg p. 100.

[illegible] — Ce [illegible] pratiqué [illegible] malades. Si l'on sait que le [illegible] normal du phosphore [illegible] chez un [illegible] est compris entre [illegible] 27 [illegible] 7 sujets (37,3 p. 100) [illegible] 12 mg. Par contre, chez [illegible] 2 p. 100) [illegible] 12,2 p. 100) [illegible] taux moyen de 15,5 mg p. 100. Chez les 6 autres (26,7 p. 100) une [illegible] avec un chiffre moyen de 20,[illegible] mg p. 100.

[illegible] — Ce [illegible] a été [illegible] sur 21 malades. Si l'on considère que le taux [illegible] normal est de [illegible] 42 mg [illegible] 78, nos résultats font apparaître des cas [illegible] chez 8 sujets (38 p. 100) [illegible] 14,3 p. 100) avec une moyenne de [illegible] 7 mg p. 100 [illegible] une hausse [illegible] avec une valeur moyenne de 32,8 mg [illegible] chez les 12 derniers (57,2 p. 100).

[illegible] — Cette [illegible] a été effectuée selon la technique de Van Slyke. [illegible] que le [illegible] normal de [illegible] en CO² est de 52 à 77, les 14 [illegible] que nous avons [illegible] avaient une réserve alcaline normale.

[illegible] — L'activité phosphatasique du sérum a été mesurée selon la technique de King Armstrong.

Phosphatase acide. — Nos résultats sont exprimés en unités Guttmann. Si l'on considère que cette activité est normalement faible, de l'ordre de 3 unités, et qu'elle est [illegible] au maximum à 6 unités, les examens pratiqués sur 20 malades se répartissent [illegible] : 17 sujets (85 p. 100) avaient un taux normal, 3 avaient une élévation importante qui atteignait respectivement 18, 27,5, [illegible] U. G.

Phosphatase alcaline. — Les résultats sont exprimés en unités Armstrong. Si l'on considère comme normaux les chiffres compris entre [illegible] 12 unités Armstrong, les résultats [illegible] sur 20 malades [illegible] apparaître une [illegible] chez 2 sujets (10 p. 100) [illegible] 15 unités [illegible], tandis que tous les autres [illegible] avaient été normaux.

CONCLUSIONS. — Au cours de nos recherches ultérieures, nous avons pensé à l'intérêt [illegible] dans les urines des malades [illegible] nous avons pu permettre le [illegible] nous avons [illegible] reprendre cette étude en [illegible] en pratiquant le dosage

Étude des modifications biologiques dans le mucoviscidose.

Par M.

J. RODIER

(Rabat).

[illegible]

Exploration de la fonction rénale.

Albumine urinaire. — Chez les 151 malades examinés, nous n'avons trouvé qu'un seul albuminurique avec une élimination de 0,15 g/l.

Leucocyturie urinaire. — Cet examen a été pratiqué chez les urines de 37 sujets. Nous n'avons pas relevé de cytologie anormale, tant au point de vue éléments organisés que non organisés.

Azotémie. — L'urée dans le sang a été dosée chez 91 malades par la méthode à l'hypobromite. Si l'on considère que le taux d'urée chez le sujet normal se situe entre 0,26 g et 0,30 g p. 1 000, la moitié de nos malades avait un taux normal avec une moyenne de 0,26 g p. 1 000. L'autre moitié présentait un taux moyen de 0,39 g p. 1 000, certains sujets atteignant un taux de 0,5 g p. 1 000. On peut, d'ailleurs, penser, à première vue, que ces chiffres sont nettement modifiés, d'autant plus que, tenant compte du fait que le taux d'urée dans le sang artériel [illegible] à être plus élevé que la normale chez le

[illegible] Nord, sans pour cela [illegible] pathologique.

Azotémie d'Ambard. — L'examen a été pratiqué sur 20 malades. Si l'on considère que la constante de [illegible] est [illegible] au dessus de 0,063, nos résultats [illegible] constante d'Ambard [illegible] 0,063, les 19 autres [illegible] avec et une [illegible] d'Ambard normale, avec une moyenne de [illegible]

Bleu de méthylène intraveineux. — Suivant la technique utilisée, [illegible] 60 p. 100 du colorant injecté par voie intraveineuse. L'examen a été [illegible] sur 20 malades. Parmi eux (10 p. 100) présentant une rétention du [illegible] avait un chiffre moyen d'élimination se situant [illegible] avec un taux moyen de [illegible] normale avec un taux [illegible]

Exploration de la fonction hépatique.

[illegible] a été dosé [illegible] comme [illegible] (0,5) et 2 g p. 1 000 [illegible] nos résultats qui ont [illegible] 11 malades [illegible] avaient une valeur [illegible] 1,75 g p. 1 000. 5 malades (20 p. 100) avaient [illegible] seulement [illegible] 2,72 g p. 1 000.

Cholestérol. — L'examen a porté sur les 17 sujets déjà examinés pour le [illegible]. Si l'on admet que le chiffre normal oscille entre 1 g et 1,50 g p. 1 000, [illegible] 1,29 g p. 1 000 comme chiffre [illegible] 5 p. 1 000 seulement avaient un cholestérol estérifié abaissé à [illegible] g p. 1 000 en moyenne.

Rapport $\frac{\text{Cholestérol total}}{\text{Cholestérol total}}$. — Si l'on considère comme normales les valeurs [illegible] entre 0,70 et 0,79, les 17 malades présentant un rapport normal avec une protéine sérique de 0,65.

Protéinémie. — *Protéines totales.* — La technique utilisée a été celle de Mache-[illegible]. En considérant que le taux normal des protéines sériques varie entre 70 et 8[illegible] g p. 1 000, nos résultats portant sur 19 prélèvements se répartissent ainsi : [illegible] malades (61 p. 100) avaient des chiffres normaux avec une valeur moyenne de 7[illegible] g p. 1 000. Les 7 autres (39 p. 100) avaient des chiffres diminués avec une [illegible] moyenne de 62,7 g p. 1 000.

[illegible] — Chez les [illegible] sujets [illegible] les taux de sérine varient entre 40 et 50 g p. 1 000. [illegible] sur les malades se répartissent ainsi : 5 (26 p. 100) présentaient une [illegible] avec une moyenne de 35 g p. 1 000, le chiffre le plus bas obtenu étant [illegible] 3 g p. 1 000. Les autres malades (74 p. 100) avaient un taux moyen de [illegible] g p. 1 000.

[illegible] — La [illegible] globulines [illegible] entre 25 et 32 g p. 1 000. [illegible] sujets (6 p. 10 [illegible]) [illegible] avec un taux moyen de

par [illegible] Sur 62 malades, nous n'avons relevé aucun chiffre supérieur à la normale (0,24 bg). Chez le nombre de sujets atteints (20) avaient un taux très faible, 0,01 bg à 0,04 d'urine.

[illegible] la technique [illegible] au colorant sont [illegible] chez le sujet normal en 1 h. C'est ce qui s'est produit pour 16 de nos sujets (53 p. 100) qui ont présenté un chiffre de rétention moyen de 36,8 p. 100. 2 sujets seulement avaient une rétention modérée avec respectivement 55,3 p. 100 et 57 p. 100.

Exploration endocrinienne.

[illegible] 17 surrénales. — Les 17 surrénales ont été dosées photocolorimétriquement sur la technique de Gahon et Sailer modifiée par Jayle sur [illegible] de 71 malades. Si l'on considère que les taux normaux d'élimination chez l'homme sont de 12 à 13 mg 24 h., 11 malades (19 p. 100) avaient une élimination [illegible] entre 11 et 13 mg avec un taux moyen urinaire de 12,2 mg 24 h. [illegible] sujets (81 p. 100) un taux de cette élimination inférieure à 12 mg 24 h. Chez certains malades, le taux d'élimination n'atteignait que 7 mg 24 h. La moyenne générale de tous les taux d'élimination s'établit à [illegible] mg 24 h.

[illegible] DE BASE. — Cet examen a été pratiqué sur 81 manganiques par [illegible] en circuit fermé. 3 déterminations ont été exécutées pour chaque malade [illegible] les préparations habituelles pour cet examen ont été rigoureusement respectées. On considérait comme normales des variations de — 15 p. 100 à — 15 p. 100. 45 sujets (54 p. 100) avaient un métabolisme augmenté, cette augmentation atteignant même une quotité de 70 p. 100, elle était en moyenne de [illegible] p. 100. Chez 30 sujets (37 p. 100) le métabolisme basal était normal avec une moyenne de + 5,5 p. 100. Aucun de nos malades n'eut un chiffre inférieur à — 13 p. 100.

Nous avons [illegible], chez 3 sujets considérés comme ayant un chiffre de métabolisme normal, de pratiquer le test de l'effort de Dorept. Cet examen nous a révélé que nous nous trouvions en réalité en présence de sujets ayant une augmentation du métabolisme de base qui avait été masquée au cours des premières mesures, ainsi :

M. E.	Au repos : + 3 p. 100	Après effort : + 15 p. 100
[illegible]	Au repos : + 2 —	Après effort : + 19 —
[illegible]	Au repos : + 2 —	Après effort : + 35 —

Il est très vraisemblable de penser que si cette contre-épreuve avait pu être pratiquée régulièrement, nous aurions pu en révéler un certain nombre de métabolismes augmentés. Si on procède à rétablissement de la moyenne générale de tous les chiffres de métabolisme de base [illegible], celui-ci s'établit à + 19,5 p. 100, fait [illegible] qui [illegible] la valeur de l'élévation de cette épreuve au cours [illegible] manganiques.

Examens hématologiques.

NUMÉRATION [illegible] — [illegible] globules rouges et globules blancs [illegible] classique [illegible] de Malassez. [illegible]

GLOBULES ROUGES PAR MM³	GLOBULES BLANCS PAR MM³
[illegible] chiffre de globules rouges [illegible] 4 millions avec [illegible] globules.	Parmi 19 p. 100 [illegible] moins de 6 000 globules blancs [illegible] moyenne de 5 160.
[illegible]	[illegible] 62 p. 100 [illegible] entre 6 000 et 8 000 globules blancs avec une moyenne 7 [illegible].
[illegible]	[illegible] de 8 000 globules blancs [illegible] moyenne de 9 [illegible].
La moyenne [illegible] globules rouges [illegible] 4 128 [illegible] par mm³.	La moyenne [illegible] en globules blancs de [illegible] par mm³.

Il [illegible] normal [illegible] globules rouges sous le [illegible] par mm³.

[illegible] hémoglobine [illegible] hémoglobinomètre de Sahli. Sur 43 malades examinés, les résultats [illegible] ainsi :

[illegible]	NOMBRE DE CAS	[illegible]	TAUX MOYEN d'hémoglobine
De 90 à 100	19	[illegible] p. 100	97 p. 100
[illegible] à [illegible]	11	[illegible] —	84 —
70 à 80	11	[illegible] —	71 —
Inf. à 70	2	[illegible] —	60 —

FORMULE LEUCOCYTAIRE. — Cet examen a été effectué sur 112 malades. Il nous est apparu [illegible] qu'un certain nombre d'malades présentaient une [illegible] de la formule leucocytaire avec une augmentation du nombre de lymphocytes. Nous avons alors [illegible] de classer nos résultats en tenant compte des variations du taux de lymphocytes. Nous avons considéré comme formule leucocytaire nette [illegible] celle dont le taux de lymphocytes pour 100 éléments blancs était supérieur à 45, comme [illegible] dont la modification était légère, celle dont le taux de lymphocytes variait entre 35 et 45 p. 100, comme formule normale celle dont le taux de lymphocytes était inférieur à 35 p. 100. Les résultats ainsi [illegible] les formules leucocytaires [illegible], 48 cas (43 p. 100) [illegible] lymphocytaire de 57,2 p. 100. Les formules leucocytaires [illegible] légère, 31 cas (27 p. 100) avec une moyenne lymphocytaire de 39,9 p. 100. Formule leucocytaire normale, 33 cas (29,5 p. 100), avec une moyenne lymphocytaire de 27,3 p. 100.

Le formule [illegible] pour chacune des 3 catégories [illegible] pathologiques.

	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Magnan. Les formules considérées comme normales	66.1	2.7	0.3	27.3	3.3
[illegible] modifiées	51.8	2.9	0.2	39.9	3.2
[illegible] modifiées	51.8	3	0.1	37.2	4.8
Moyenne générale de toutes les formules	51	2.9	0.2	31.5	4.1

Exploration du liquide céphalorachidien.

Albumine. — Le dosage de l'albumine a été pratiqué par mesure nephélométrique après précipitation par l'acide trichloracétique. 22 malades ont été examinés. L'albumine existait normalement à la dose de 0.15 à 0.20 g p. 1 000 dans le liquide [illegible] 10 malades, 12 malades (54 p. 100) accusaient un taux normal, avec 0.20 g p. 1 000 comme chiffre moyen. Les 4 dernières (18 p. 100) avaient une augmentation dont le chiffre moyen s'établissait à 0.37 g p. 1 000.

Glucose. — Le dosage a été pratiqué sur 21 prélèvements par la méthode de Bertrand modifiée. Il retient que les taux normaux de glucose chez le sujet sain oscillent entre [illegible] g p. 1 000 [illegible] 13 malades (70 p. 100) présentaient des taux normaux avec un moyenne 0.67 g p. 1 000, 2 sujets seulement avaient un taux élevé respectivement à 0.20 et à 0.27 g p. 1 000.

Chlorures. — Ce dosage a été pratiqué sur 21 malades par la méthode de Charpentier-Volhard. On retient que le liquide céphalorachidien d'un sujet sain [illegible] moyenne 7.[illegible] g p. 1 000 de chlorures exprimés en ClNa, 18 malades (90 p. 100) avaient un taux normal avec en moyenne 7.23 g p. 1 000, 3 présentaient une légère modification, le taux étant un peu élevé avec une moyenne de 6,5 g p. 1 000.

Cytologie. — Cet examen a été pratiqué sur 17 malades. Aucun des sujets examinés n'avait un chiffre de leucocytes supérieur à 3 par mm³. La moyenne générale ne s'élevait à 0.97 par mm³.

Réaction au benjoin colloïdal (docteur Ninard). — Cet examen effectué sur le liquide céphalorachidien de 20 malades a été normal chez 17 sujets (85 p. 100), 3 seulement avaient une réaction assez faiblement élevée, indiquant une faible [illegible] méningée.

Conclusions.

[illegible] Méthode biologique que nous avons pratiquée sur les sujets intoxiqués par le manganèse ne fait pas apparaître de modifications massives dans les éléments étudiés, il ressort toutefois que l'on doit tenir compte de certaines variations importantes.

Au point de vue rénal, il [illegible] manganique s'accompagne de troubles importants. [illegible] à la phénolsulfonephtaléine indique [illegible] rétention du colorant chez 40 p. 100 des malades.

[illegible] pratique, les [illegible] pratiqués sont, dans l'ensemble, [illegible] normaux, sauf en ce qui [illegible] l'épreuve de l'U. S. P. et le coefficient de Maillard [illegible] 61 p. 100 des malades.

[illegible] les plus nettes. L'[illegible] des sujets, et le [illegible] les malades [illegible] 24 h. Le métabolisme [illegible] chez 5.1 p. [illegible] des malades [illegible] 115 calories par [illegible] s'établit à + 19,5 p. 100. [illegible] sujet de cette hémolyse [illegible] les examens [illegible] une inversion [illegible] de la formule leucocytaire dans [illegible] d'une lymphocytose chez 11 p. 100 des malades, [illegible] que 29.5 [illegible] modification [illegible]. Enfin, les numérations globulaires [illegible] chez [illegible] manganiques.

En conclusion, nous avons [illegible] chez le manganique, tant au point de vue [illegible]. Il est [illegible] à la numération globulaire [illegible] de la formule leucocytaire, à un [illegible] un métabolisme de base et à [illegible] de l'élimination [illegible] des 17-cétostéroïdes urinaires.

(Travail du Service de Toxicologie de l'Institut d'Hygiène du Maroc.)

THE INDUCTION BY Mn^{++} OF HERITABLE RESPIRATORY DEFICIENCY IN NON-DIVIDING POPULATIONS OF *SACCHAROMYCES*

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SUMMARY

1. Mn^{++} can induce heritable respiratory deficiency in *Saccharomyces* without the aid of cell division.

2. The mutagenicity and toxicity of Mn^{++} for non-dividing cells is determined by the cellular free amino acid contents and the relative Mg^{++} concentration of the medium in which the cells are exposed.

3. By proper adjustment of Mg^{++} and Mn^{++} concentrations it is possible to reduce the mutagenic from the toxic properties of Mn^{++}.

4. It is suggested that Mn^{++} induces inactivation of respiratory deficiency in *Saccharomyces* by direct hit of normal protein syntheses resulting in decay of one or more essential physiological systems.

INTRODUCTION

A variety of chemical and physical agents have been reported capable of inducing respiratory-deficiency in yeast[1-5]. All reports agree in asserting that treated cells must be actively budding in order for the inducing event to occur. Most recently, Nakai *et al.*[6] noted the production of respiratory-deficient mutants in populations of *Saccharomyces* growing actively in the presence of high concentrations of Mn^{++}. During unsuccessful attempts to effect somatic segregation in *Saccharomyces* by treatment with manganous salts, I have made the unique observation that Mn^{++} induces the transformation to respiratory deficiency in non-budding cells as well. The present communication defines the extent to which the mutagenic efficiency of Mn^{++} for non-dividing cells depends upon the levels of the free amino acid pools of the cells and the Mg^{++} concentration of the medium in which they are exposed.

METHODS

The test organism employed was a respiratory-sufficient, adenine-requiring stock of *Saccharomyces* which produces red pigment when growing aerobically on limiting concentrations of adenine. Respiratory-deficient variants of specimen cells appear as white colonies in a population of about 0.[illegible]

Cells were cultivated in shake cultures at 28° upon the [illegible] mg medium: Glucose, 40 g; asparagine, 2 g; ammonium lactate, 2 g; KH_2PO_4, 1.5 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2$, 0.33 g; $FeSO_4 \cdot 7H_2O$, 3 mg; $ZnSO_4 \cdot 7H_2O$, 3 mg; $CuSO_4 \cdot 5H_2O$, [illegible] mg; $MnSO_4 \cdot 4H_2O$, [illegible] mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.15 mg; $Na_2B_4O_7 \cdot 10H_2O$, 0.9 mg; biotin, 10 mg; thiamine hydrochloride, 1 mg; pyridoxine hydrochloride, 0.[illegible] mg; nicotinic acid, 0.[illegible] mg; calcium pantothenate, 0.[illegible] mg; folic acid, 0.[illegible] mg; distilled water, 1000 ml.

To deplete cells of their free amino acids, log-phase cells were harvested, transferred to [illegible] and incubated for 5 or 12 h. For some experiments the free amino acid pools of cells depleted for 12 h were reconstituted by exposure to incubation in growth medium enriched with 0.5% casein hydrolysate. The magnitudes of the pools were estimated from their glutamic acid contents according to procedures which have been described elsewhere[7]. The relative protein-synthesizing capacities of unadapted, depleted, and reconstituted cells were assessed by measurement of the rate of a lysine-sensitive formation [illegible] anaerobic conditions using conventional manometric techniques.

For the induction experiments, cells were harvested from growth or depletion media, washed thrice [illegible] M KH_2PO_4, and inoculated to a density of about $1 \cdot 10^8$ cells ml^{-1} into flasks containing 50 ml of nitrogen-free medium, to which was added Mn^{++} as $MnSO_4 \cdot 7H_2O$. The flasks were incubated at 28° with shaking. To confirm the [illegible] of cell division under these conditions, microscopic examinations were made of aliquots of the cultures [illegible] $1 \cdot 10^8$ cell/ml; it was observed that budding cells which [illegible] had ceased to complete their division cycle during the period of incubation. Under these conditions, growth inhibition while depleted cells were fixed buds and did not initiate budding. In order to evaluate cell survival and the appearance of [illegible] after 24- and 72-h incubation, 1-ml samples were removed from each flask and plated after appropriate dilution. The plating medium was prepared from the growth medium described above by reducing the adenine and [illegible] concentrations to [illegible] and 20 mg respectively, and adding 20 g agar/l. The respiratory capacities of the cells plated were determined from the color characteristics of the colonies they produced after growth incubation at 28°; respiratory-competent individuals produced red colonies, respiratory-deficient ones produced white colonies, and cells of unstable genetic potential gave rise to either orange or pink and white sectored colonies.

RESULTS AND DISCUSSION

The results summarized in Tables I and II show that, in general, the ability of Mn^{++} both to induce respiratory variants and to inactivate cells is inversely proportional to the concentration of Mg^{++} in the medium and directly proportional to the free amino acid pool of the cells. In particular instances, though, the mutagenic and toxic properties of Mn^{++} can be separated. For example, when cells which had depleted of amino acids were incubated for 24 h in the presence of $4 \cdot 10^{-3}$ M and [illegible] M Mn^{++}, there was approximately 70-fold increase in the frequency of respiratory-deficient mutants with no decrease in cell survival.

Lethal biological responses were not detected unless the molar concentration of Mn^{++} [illegible]

[illegible] were at least four times greater than those of Mg^{++} (Table I). Undoubtedly a number of physiological processes including Mg^{++} exchange might be disturbed by the presence of excess ions Mn^{++}. However, KORNBERG *et al.*[7] have shown that the uptake of Mn^{++} and Mg^{++} by the yeast cell is regulated by the same protein carrier system and that the preferential affinity of the system for Mg^{++} is so great that no Mn^{++} uptake can occur when the two ions are present in equimolar concentrations.

TABLE I

[illegible] OF CELL SURVIVAL AND OF RESPIRATORY VARIANTS APPEARING IN AMINO-ACID-DEPLETED AND -REPLENISHED POPULATIONS OF *Saccharomyces* EXPOSED TO $4 \cdot 10^{-3}$ *M* Mn^{++} AND VARIOUS CONCENTRATIONS OF Mg^{++}

Mg^{++} M	Alternatives of the cellular free amino acid pool	Days 1-2 exposure to $4 \cdot 10^{-3}$ M Mn^{++}: 24 h			72 h		
		% Survival	% Stable variants	% Total* variants	% Survival	% Stable variants	% Total* variants
$2 \cdot 10^{-3}$	undepleted	210	0.3	0.3	199	0.8	0.8
	depleted 5 h	100	0.4	0.2	95	0.3	0.3
	depleted 12 h	94	0.3	0.3	99	0.3	0.4
	replenished	101	0.2	0.4	98	0.4	0.9
$1 \cdot 10^{-3}$	undepleted	119	0.4	0.4	71	1.0	2.0
	depleted 5 h	97	0.3	0.3	88	0.3	0.4
	depleted 12 h	95	0.3	0.3	89	0.4	0.4
	replenished	100	0.5	0.6	48	0.6	1.0
$5 \cdot 10^{-4}$	undepleted	103	0.9	20.0	19	15.9	37.5
	depleted 5 h	88	0.3	0.6	62	1.4	2.6
	depleted 12 h	92	0.3	0.5	70	0.7	1.7
	replenished	90	2.8	3.6	12	0.8	0.5
$2.5 \cdot 10^{-4}$	undepleted	34	12.0	34.3	9.5	41.4	62.8
	depleted 5 h	79	1.8	3.5	29	38.1	17.0
	depleted 12 h	77	0.6	1.7	67	1.0	3.0
	replenished	59	5.2	10.1	18	30.0	43.0
$1 \cdot 10^{-4}$	undepleted	16	41.7	59.3	9		
	depleted 5 h	59	11.1	23.0	22	49.2	79.4
	depleted 12 h	67	1.6	5.3	39	3.2	12.3
	replenished	12	29.5	39.8	8	56.2	60.0

* Pooled values for stable and unstable respiratory variants.

TABLE II

THE EFFECTS OF AMINO ACID DEPLETION OR REPLENISHMENT UPON CELLULAR FREE GLUTAMIC ACID CONTENTS AND PROTEIN-SYNTHESIZING CAPACITIES

Alternatives of the cellular free amino acid pool	Free glutamic acid contents in μmoles/g. d.w. (± S.E.)*	Relative rates of adaptive enzyme formation
Undepleted	2[illegible].6 ± 0.07	100
Depleted 5 h	1.2 ± 0.45	55
Depleted 12 h	3.4 ± 0.30	8
Replenished	2[illegible].3 ± 0.88	85

* Triplicate determinations.

[illegible]ly, then, of Mn^{++} to determine the entry of Mn^{++} into the cell is certainly a crucial specific factor underlying the present observations.

Several factors have possible bearing upon the direct correspondence between the free amino acid contents of cells and their susceptibilities to Mn^{++}. It is possible that free amino acids might facilitate the intracellular concentration of Mn^{++} by virtue of their chelating affinities. However, the fact that cells showed a continuous increase in response during their entire 72-h period of exposure to Mn^{++} implies the primary operation of a more dynamic process. Alternatively, the possibility exists that depletion of cells free amino acids might also decrease the activity of the ion transport system for Mn^{++}, and that the relation between cellular free amino acid contents and sensitivities to Mn^{++} is only an adventitious one. HALVORSON AND SPIEGELMAN[10] have shown that amino acid depletion does alter certain forms of proteins of the yeast cell. They also demonstrated that these proteins are not restored upon short-term replenishment of the free amino acid pool. The results presented in Table I, however, reveal that such replenishment brings about an appreciable increase in cellular responsiveness to Mn^{++} and indicate, therefore, the cardinal involvement of the free amino acids of the cells *per se*. Perhaps the most satisfactory explanation of the observed effects resides in the close correlation between the free amino acid contents of cells and their capacities for protein synthesis (Table II); that is, the induction of respiratory deficient variants, as well as cellular inactivation, result from decay of one or more physiological systems due to abnormal protein synthesis (or contingent RNA synthesis) occurring under the influence of Mn^{++}.

The yeast cell is peculiar in that its period of maximal cytoplasmic growth normally coincides with cell division. The previous studies indicating the respiration deficiency can be induced only in actively budding cells have stressed the importance of the division process in the induction, but have failed to consider adequately the possible significance of concurrent cytoplasmic growth. Consequently, proposals have been advanced to the effect that respiratory variants arise at mitosis as a result of a mechanical defect in the distribution of the heritable cytoplasmic determinants of respiration[11-13]. The present demonstration that respiratory variants can be induced when cytoplasmic growth occurs divorced from cell division suggests that a proper definition of the general induction by which respiration-deficient yeasts are induced must await more critical appraisals of the relative importance of cell growth and cell division in the induction process.

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Special Article

[illegible]

THE TRACE ELEMENTS IN NUTRITION

MAURICE E. SHILS, Sc.D.

[illegible]

These special articles on foods and nutrition have been prepared under the auspices of the Council on Foods and Nutrition. [illegible] the opinion of the Council. These articles [illegible] as a Trend Book on Nutrition [illegible].

The many mineral elements which exist in animal tissues occur in widely varying amounts. They range [illegible] be expressed in kilograms, down to those which are most inclusive in milligrams and even micrograms, and which have been termed "trace elements." The dividing line between trace and non-trace elements is purely arbitrary and a matter of choice. Some nutritionists[1] include in the former category any element occurring in the tissues or nutritionally necessary in amounts equal to and less than iron, but others[2] consider only those elements below iron.

Presaging the point of view that traces of minerals might exert profound and specialized physiologic effects were the discoveries that iodine occurs in the thyroid,[3] copper in octopus blood[4] and in the hemocyanin of crustacea,[5] zinc in the hemocyanin of oysters,[6] vanadium in the blood pigment of the sea squirt[7] and

[illegible]

manganese in the blood of the radfish: Fundulus squamosus.[illegible] Nevertheless, with the exceptions of iron and iodine, little physiologic significance was attached to the others of the trace elements until comparatively recently. Beginning with the investigations on the role of iodine, especially those of Bertrand in France, the importance of the trace elements has come to be realized [illegible] through the study of experimental animals [illegible] discussed in previous papers. Knowledge of the [illegible] of these trace elements and of those discussed [illegible] indicates that the role of "trace" [illegible] is [illegible] the activities of hormones and [illegible] deficiency results in [illegible] to date at least twenty trace elements other than iron, copper and iodine have been reported to occur, [illegible] in [illegible] zinc, bromine, [illegible], boron and aluminum.

EXCEPTIONS

Conclusive evidence for the essential nature of manganese for animals was first demonstrated in 1931.[illegible] Although earlier investigators[illegible] claimed that they had shown this fact, their results were not conclusive.

Orent and McCollum[illegible] found that rats reared from weaning on a diet adequate except for manganese developed sterility and inadequate lactation after three days. Females on the same diet delivered litters, which survived but were feeble; in addition they failed to suckle normal stock young. Hemoglobin regeneration, estrus and growth were not affected by the deficiency.

When mice were reared on a manganese low diet consisting of whole milk supplemented with iron and copper, decreased growth and an abnormal estrus cycle resulted.[illegible]

Daniel and Everson[illegible] fed a manganese deficient mineralized milk diet to rats and examined the [illegible] that the females produced nonviable young. The [illegible] their findings differed from those of Orent and McCollum, but[illegible] in that the deficient females could [illegible] foster young.

Recently we[illegible] found that manganese is essential for the normal growth of the rat. The [illegible] the female reproduction [illegible] the deficient females while being their own young could suckle normal foster young. The new young which did survive to weaning uniformly show [illegible] in coordination, most pronounced in the hind [illegible] with poor growth and [illegible] to the reproductive. Beyer and coworkers[illegible] have likewise found that manganese is essential for [illegible] the symptoms observed in mice are [illegible] and in addition, a marked delay in the opening of [illegible] vaginal orifice.

The differing findings of the Hopkins and [illegible] may be due to the presence or absence of [illegible] in the magnesium content of the diets used. Purified diets have not yet been found [illegible] whole milk in manganese content. In [illegible] different symptoms occur at different levels of [illegible] It is [illegible] that nutritional dietary interrelationships [illegible] have caused the differences.

The species differences in deficiency symptoms and requirement, of which we are well aware in the case of the vitamins, extends to the trace elements. [illegible] investigations of manganese deficiency in [illegible] resulted in knowledge of practical [illegible] requirements of this species are much less [illegible] of not as well studied and the [illegible] are different. Manganese may be a [illegible] prevent the development of an [illegible] so-called perosis. The symptoms [illegible] the tibiometatarsal joint, twisting and [illegible] of the distal end of the tibia and of the proximal end of the metatarsus and slipping of the epiphysis [illegible] under from its condyles, resulting in severe [illegible] The deficient chicks have shortened leg bones, [illegible] vertical curvature.

It has long been known that excess calcium and phosphorus in the diet intensify perosis and that injected manganese is more efficiently utilized than that given orally. In vitro and in vivo experiments have helped explain these observations by showing that manganese

[illegible]

precipitated from solution by insoluble calcium phosphate or by ferric hydroxide and strontium carbonate.

The chick embryo, like that of the rat, requires manganese for normal development. Lyons and Todhunter observed a very low hatchability of eggs of deficient hens; the embryos which developed exhibited severe chondrodystrophic and other lesions which in chief included bowed leg and wing lesions resembling [illegible]

The fact that only slight differences in manganese content are found between the decidedly different yolks[illegible] and egg shells[illegible] of normal and deficient hens chickens is indication that probably manganese plays some indirect or catalytic role in calcium and [illegible] metabolism. [illegible] as a factor in reproductive [illegible] in rats[illegible] and lameness in pigs[illegible] has been pointed out.

The specificity of manganese as the inorganic factor [illegible] the observed deficiencies of choline[illegible] and of biotin[illegible] reach in protein in food is an indication that [illegible] other factors necessary to protein [illegible] metabolism resulting in [illegible]

[illegible] suggested[illegible] that the [illegible] epiphysis[illegible] in children[illegible] resemble those of patients [illegible] and histologic comparisons of these bone [illegible] studies would be of interest.

A relationship between manganese and thiamine has been postulated.[illegible] Large amounts of thiamine or manganese given to rats caused reproduction and lactation failure, which could be prevented by increasing the intake of the other of the two substances.[illegible] In a thiamine [illegible] diet receiving excess manganese [illegible] more quickly depleted than those not receiving [illegible] The implications of the work merit its being repeated.

That the need for manganese is widespread is indicated by the fact that, in addition to the animal species already discussed, it has been found essential for plants,[illegible] including fungi[illegible] and several bacteria.[illegible]

There is no definite information about the human requirements for manganese nor is there evidence of deficiency ever occurring in man. Iverson and [illegible], on the basis of balance studies, suggest [illegible] the diet of preschool age children should [illegible] between 0.01 and 0.20 mg. of Mn per gram per kilo [illegible] weight; retention in children 8 to 12 [illegible] 0.12 mg. [illegible] [illegible] is found in the diet [illegible] substantially equivalent amounts are excreted. [illegible] manganese content of various foods has been determined, and it is apparent that plant foodstuffs are the [illegible] source in the diet.[illegible] In contrast to [illegible] animals, with liver containing [illegible] manganese. [illegible] [illegible] of pregnancy.[illegible]

After oral, subcutaneous or intraperitoneal administration, manganese is excreted almost entirely in the feces, only small amounts being excreted in the urine.[illegible]

After the necessity for copper as a concomitant for iron in hemoglobin formation had been established, a controversy arose as to whether certain other elements [illegible] in the synthesis [illegible] but, despite the evidence to the contrary is quite conclusive.[illegible]

Acute and chronic manganese poisoning in man and experimental animals has been reviewed.[illegible]

Manganese and Enzyme Activity.—Sumner[illegible] in 1887 first implicated manganese in urease[illegible] [illegible] we now know[illegible] as the activator of the oxidase [illegible]

[illegible]

this element has been found to activate a number of enzymes.

Of particular interest is the observation of Wilson and his co-workers[8] that manganese-deficient chicks with perosis have a lower blood and bone phosphatase activity than normal birds and that the decrease in phosphatase activity precedes the appearance of perotic symptoms. This decreased activity may be due to an decreased manganese concentration, but also to a general decrease in the amount of enzyme present.[8] [illegible] phosphatase in liver, kidney, pancreas and bone,[8] and, although other divalent ions (magnesium, cobalt and iron) also activate phosphatases, they are not as effective as manganese.

[illegible] treating effect of manganese on [illegible] Gallbacher and Pincus[9] to point out that [illegible] manganese [illegible] gives [illegible] manganese [illegible] although cobalt and nickel can produce [illegible] The evidence for the [illegible] manganese has been [illegible] by the determination [illegible]

Among the other enzymes whose activity increases with the addition of manganous ions are (a) phosphoglucomutase[11] (which causes a transfer of phosphate from carbon atom 1 of glucose-1-phosphate to carbon atom 6 in carbohydrates, nucleotides, [illegible] and peptides), (b) diphosphatase,[12] (c) [illegible],[13] (d) [illegible] dehydrogenase[14] (which catalyzes the reaction of isocitric acid to α-ketoglutaric acid and carbon dioxide),[15] which contain diphosphothiamine. In yeast carboxylase, magnesium seems to be the naturally occurring activating ion, but in vitro manganese can quantitatively replace it. (e) The adenosinetriphosphatase activity of the muscle protein, myosin,[16] has been found to be activated strongly by calcium and manganese.[16b] All the enzymes listed are activated in vitro by one or more divalent ions in addition to manganese. The determination of the nature of the activating metallic ion or ions and the mechanism of the activation may be of value.

Richards[50] has found the presence of manganese necessary for the in vitro and in vivo synthesis of [illegible] ascorbic acid. [illegible] Furthermore, it has been found that in manganese deficiency in the rat there is no lowering of the ascorbic acid content of various tissues;[50] Richards' findings have not been substantiated.[51]

COBALT

[illegible] in both plant and animal tissues in very small [illegible] Distribution in animal tissues has been [illegible] by [illegible] and confirmatory evidence which [illegible] highest concentrations in glandular organs, especially the pancreas, liver, spleen and kidneys. [illegible] injected cobalt, unlike manganese, is excreted in [illegible] the [illegible]

[illegible] on finding that the pancreas [illegible] high in cobalt and nickel, suggested that the [illegible] might be connected with the synthesis of insulin. He claimed a prolongation of insulin hypoglycemia [illegible] injection of cobalt; nickel had less effect. [illegible] of cobalt has not been confirmed.[52] Nickel [illegible] recently been found to delay insulin [illegible] However, a number of metal salts have been found to influence the onset and extent of insulin hypoglycemia, notably zinc, so that such action is not [illegible]

A peculiar property of cobalt [illegible] ionic form is its ability to produce [illegible] when ingested or injected.[53] This effect [illegible] has been produced in rats, mice, guinea pigs, [illegible] dogs, pigs, chickens and frogs. The range of [illegible] concentrations in which cobalt acts is [illegible] 0.05 mg. in the entire body of [illegible] to produce polycythemia.[53] It is [illegible]

[illegible]

with an increased blood volume resulting from the increase in red blood cells and with little alteration in the total and differential leukocyte counts.[71] The action of cobalt appears to be on the erythropoietic centers, since hyperplasia[72] and stimulation of the erythrogenic precursors in the bone marrow[73] and [illegible] increase in reticulocytes[74] have been observed following its administration. The spleen plays no [illegible] in the phenomenon. The polycythemia [illegible] for months [illegible] rats.

[illegible] that produced by [illegible].[76] Liver, choline and certain vasodilator drugs have also been found effective in reducing the level of cobalt polycythemia [illegible]. When fed to rats, liver did not reduce cobalt polycythemia [illegible], on the contrary, increased it.[77]

[illegible] the sulfur containing amino acids, methionine, cysteine and cystine, particularly the latter, prevent the toxic effects induced by cobalt and nickel administration.

[illegible] have found that the polycythemia of adult dogs on a mineralized milk diet supplemented with cobalt is only temporary; in young dogs cobalt has more toxic effect with little influence [illegible] hemopoietic response to iron and copper resulted in [illegible] anemic and fed cobalt prior to the administration of the iron and copper. Hemopoietic activity was dramatically resumed on the feeding of whole dry liver or liver extract. The factor or factors in liver [illegible] this response have not been determined. These investigators suggest that, under the conditions of the experiment, cobalt feeding creates an unnaturally large demand for certain hemopoietic precursors. The possibility that liver contains something more than iron and [illegible] which is intimately concerned with the stimulation of hemopoiesis recalls the finding of Filmer and Underwood[81] that the curative properties of whole liver [illegible] treatment of enzootic marasmus (a cobalt deficiency disease in sheep and cattle) could not be accounted for on the basis of the cobalt content; they suggested at that time that liver provides some necessary organic factor for the elaboration of which cobalt is required.

Copper deficient rats fail to develop polycythemia when fed cobalt.[78] Schultze[82] has shown that cytochrome oxidase activity is diminished in copper deficient rats and fails to respond to cobalt alone, whereas the feeding of cobalt to animals receiving [illegible] in the bone marrow.

The knowledge that cobalt is a biologic essential is the result of research carried out in Australia and New Zealand. The story of this work has been reviewed in some detail recently;[83] consequently, only a brief summary is needed here.

[illegible] by progressive emaciation and anemia. Western Australia has had its "Denmark disease" or enzootic marasmus, South Australia its "coast disease," New Zealand its "bush sickness," Scotland its "pine disease," Kenya its "nakuruitis," and Florida its "salt sickness."

Deficiency of iron had been regarded as the cause of all these diseases for some years, but in 1933 this view was questioned because of the [illegible] Underwood [illegible] in 1931 discovered that the livers and spleens of affected animals contained excessive amounts of iron, hardly a condition associated with deficient iron intake. The iron deficiency theory was completely discredited by Filmer and Underwood[84] who demonstrated that by using an iron free extract of limonite, a hydrated ferric oxide used extensively in treatment, they could cure enzootic marasmus, implicating some contaminant of the iron compound as the curative factor. In 1935 Marston[85] and Lines[86] and almost simultaneously Underwood and Filmer[88] found that cobalt was the curative agent in "coast disease" and enzootic marasmus. Lines[86] and Marston[85] had been led to try cobalt through the Waltners'[69] work on cobalt polycythemia. In 1936 Askew and Dixon[89] in New Zealand found cobalt effective in the treatment of bush sickness. Cattle and sheep suffering from Florida salt sickness,[90] Kenya nakuruitis[91] and Scotch pine disease [illegible] since been treated successfully with small amounts of cobalt. Certain areas of western Canada and of Michigan are now reported to have [illegible]

[illegible footnotes 71–81]

82. Schultze, M. O.: The Relation of Copper to [illegible] and Hemoglobin [illegible] Activity of the Bone Marrow of Rats, J. [illegible] 1941.

83. Underwood, E. J.: The Significance of the "Trace Elements" in Nutrition, Nutrition Abstr. & Rev. [illegible]

84. Filmer, J. F., and Underwood, E. J.: [illegible]

85. Marston, H. R.: [illegible]

86. Lines, E. W.: The Effect of the Ingestion of Minute Quantities of Cobalt by Sheep [illegible] "Coast Disease" [illegible] J. Council Sc. & Ind. Res. [illegible]

87. [illegible]

88. Underwood, E. J., and Filmer, J. F.: [illegible]

89. Askew, H. O., and Dixon, J. K.: [illegible]

90. [illegible]

91. [illegible] Kenya, 1936, 1937; [illegible]

92. [illegible]

[illegible] deficiency.[illegible] The deficiency may be complicated by a lack of other elements. Salt sickness may be associated with an iron and copper deficiency, and there have been sporadic outbreaks in areas with cobalt [illegible] of an ataxia in young lambs resulting from central nervous system degeneration which can be prevented by administration of copper.[illegible]

[illegible] sheep and cattle develop severe deficiency symptoms. Cobalt deficiency has as yet not been produced in rats [illegible] with diets containing a low (0.0[illegible]) [illegible] element [illegible] essential to this species but in all [illegible] mammals.

[illegible] comparative [illegible] of iron, copper and cobalt than to iron and copper alone.[illegible] The [illegible] when dog [illegible] were used [illegible] copper [illegible] anemia produced by [illegible] about half [illegible] by hemorrhage, but, in about an equal number of cases treated with iron and copper in which [illegible] was [illegible] [illegible] of [illegible] combated [illegible].

[illegible] of the cobalt in the treatment of human [illegible] has been reported for children[illegible] with some favorable results and for adults[illegible] with negative results. The adult cases were very few in number. A [illegible] has reported that in children, in contrast to experimental animals, cobalt administration results in an increase in the erythrocyte count but in no increase in hemoglobin.

Cobalt shares with manganese the ability to activate [illegible] a number of enzymes.[illegible] It has not been [illegible] as the physiologic ion in any enzyme system.

ZINC

Although several of the earlier investigators[illegible] claimed to have shown the importance of zinc in nutrition, unequivocal proof for its needs by [illegible] the work of Todd, Elvehjem and Hart in 1934.[illegible] Using a diet containing only 1.6 parts per million of zinc, they found that the growth of the deficient rats was much inferior to the controls and that an alopecia developed over various parts of the body. With still more restrictive diets the Wisconsin investigators[illegible] have produced greater differences in growth and have again observed changes in the fur. The need for zinc in rats has been confirmed,[illegible] and it has been shown that [illegible] [illegible] Microscopic study of rats [illegible] developing [illegible] parakeratosis [illegible] cells; the skin showed hyperkeratinization, [illegible] of the epidermis and loss of hair follicles with [illegible] of the sebaceous glands. Several animals [illegible] conical keratinization similar to that described in [illegible] chickens at the [illegible].[illegible]

[illegible] role of the [illegible] in insulin. Scott and Fisher[illegible] have [illegible] occurrence in insulin; this close association has been confirmed.[illegible] Zinc[illegible] (as well as some other elements) [illegible] the activity of insulin [illegible] while [illegible] is [illegible]. Scott and Fisher[illegible] [illegible] the average zinc content of the pancreas of diabetic patients was only half that of the normal [illegible], the insulin content was only one fourth. However, a recent report[illegible] indicates that [illegible] and the non-diabetic is the same.

Carbonic anhydrase, the enzyme which catalyzes the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$,[illegible] appears to be a zinc protein compound,[illegible] although reports [illegible] to the zinc content.[illegible] It has been found in the [illegible] gastric mucosa,[illegible] pancreas[illegible] and renal [illegible] apparently all of the zinc in the erythrocytes is in this

[illegible] Sieve, E. E.; Elvehjem, C. A., and Hart, E. B.: [illegible] Relationships of Zinc in Nutrition to the Rat. J. Biol. Chem. [illegible] 350 (Sept.) 1935. Hove, E.; Elvehjem, C. A., and Hart, E. B.: [illegible] Physiology of Zinc in the Nutrition of the Rat. [illegible] Zinc Deficiency in Rats. Am. J. Physiol. [illegible] (Dec.) 1935.

[illegible] Hart, E. B., and McCollum, E. V.: Effects of Acute Zinc Deficiency in the Rat. Proc. Soc. Exper. Biol. & Med. [illegible]

[illegible] Day, H. G.: The Effects of Zinc Deficiency in the [illegible] Diet. [illegible] 189 (March) 1937.

[illegible] Day, H. G., Jr., and Day, H. G.: Histological [illegible] Effects of Low Fat & Diet Especially Low in Zinc. J. Nutrition [illegible] 1938.

[illegible] Pearson, O. A., and Wilbach, R. R.: [illegible] Rat in Riboflavin Deficiency with a Note on Cobalt [illegible] Vitamin A deficiency. J. Exper. Med. [illegible]

[illegible] Scott, D. A.: Crystalline Insulin. Biochem. J. [illegible]

[illegible] Scott, D. A., and Fisher, A. M.: Crystalline Insulin. [illegible]

[illegible] Lu, L. L.; Perry, J. D.; Livingood, J. J., and Blanchard, [illegible] The Solubility and Dialyzable Properties of Insulin and Its Zinc Salt. Relationship Zinc. Science [illegible] (Oct.) [illegible]

[illegible] Boothby, [illegible] and Jandorgard, L. [illegible]: Divided Doses of Insulin. [illegible] (May) [illegible] [illegible] and Boothby, Charles L.: Some Effects of Zinc [illegible] Metabolism of Insulin in Rats. Am. J. Physiol. [illegible] Scott and Fisher.

[illegible] Scott, D. A., and Fisher, A. M.: The Insulin and the Zinc Content of Normal and Diabetic Pancreas. J. Clin. Invest. [illegible] (Aug.) 1938.

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[illegible] Rudolph, J. J. W.: Recent Work on the Carbon Dioxide Transport. [illegible] Physiol. Rev. [illegible] (April) 1938.

[illegible] Keilin, D., and Mann, T.: Carbonic Anhydrase. Nature [illegible] (Oct.) 1938.

[illegible] Hart, E. B.; Elvehjem, C. A., and Phillips, E. B.: The [illegible] J. Biol. Chem. [illegible] (Sept.) 1938. [illegible] J. Biol. Chem. [illegible] (Sept.) 1938. [illegible] J. Biol. Chem. [illegible] (Aug.) 1938. [illegible] Soc. [illegible]

[illegible] Dyckerhoff, H. W.: Gastric Carbonic Anhydrase. J. Biol. Chem. [illegible] (Nov.) 1938.

[illegible] Le répartition de l'adrénaline [illegible] Archives [illegible] Physiol. [illegible] Lucas, H. J., and Bell, [illegible] Archives [illegible] Exhibition in the Sciences and Conservation [illegible] Rochester [illegible] 1939. [illegible] (Oct.) 1938.

[illegible] Dyckerhoff, H. W., and Wilbrandt, A. [illegible]

[illegible] which, incidentally, is inhibited by sulfanilamide.[118] The enzyme mixture may also contain zinc.[119]

In view of these findings, it is interesting to note the observations on the physiology of zinc deficient animals. However there have been found no disorder of carbohydrate metabolism,[illegible] no significant decrease in the [illegible] anhydrase activity[120] and no decrease in the concentration of urease.[121] On the other hand, the [illegible] showed [illegible] decreases in pancreatic lipase activity (monopentic) and intestinal phosphatase activity.[122] Catalase activity [illegible] and kidney [illegible] decreased in zinc deficiency.[123] [illegible] concentration in mammalian [illegible] is taken [illegible] of [illegible] of [illegible] of [illegible] greater concentrations, milk, for example, containing 3 to 4 mg. per liter. The average daily diet contains 12 [illegible] mg. of zinc, [illegible] 8 mg. [illegible] per day. For the present there is no information on the zinc requirement of man. Several balance studies, [illegible] children of preschool age[124] and the other on children 8 to 12 years old,[125] have shown zinc retention, [illegible] possible requirements. [illegible] weight for the preschool age child.

FLUORINE

The consideration of fluorine in any discussion of trace elements is pertinent for at least two reasons: first, because of the undeniable effects associated with prolonged chronic fluorine intoxication, and, second, because of the recent interest in the effect of fluorine on the incidence and severity of dental caries.

Fluorine associated with various minerals is widely distributed in nature, especially in areas rich in phosphates, aluminum and volcanic ash. It is present [illegible] in very small amounts in plant and animal tissues. McClure[126] has collected data on the fluorine content of meats and vegetables.

After passing through fluorine rich mineral deposits [illegible] contaminants with the element, and consequently this water during the period of tooth formation, particularly of the permanent teeth, results in a disease known as chronic endemic dental fluorosis, commonly called mottled enamel. Recognition that the disease is water borne came[129] in 1916, but it was not until 1931 that three independent investigations[130] implicated fluoride as the causative agent. Epidemiologic studies and animal experimentation fully support this view. Surveys of populations[131] indicate an orderly uniformity in the group response to the fluoride concentration of the communal water supply with respect both to the incidence and to the percentage distribution of the severity of the mottled enamel [illegible] as shown in the table. According [illegible] expressing one part per million [illegible] considered of public health significance.[132]

The widespread occurrence of mottled enamel in the United States is indicated in figure 1. Its distribution is worldwide.

As fluorine acts during the period of calcification [illegible] the affected child [illegible] instead of having the [illegible] lustrous, translucent appearance, the teeth have dull chalky white patches distributed over the surface and in [illegible] the whole tooth surface may [illegible] a dead white unglazed appearance. In addition there

Relation of Fluoride Concentration to Incidence of Mottled Enamel in Children

[illegible]

City and State	Fluoride Content in Parts per Million of Drinking Water	Mottled Enamel in 11 Year Old Children	
		Number of Children Examined	Percentage Affected
Junction City, Kan.	0.7	95	1.1
Eau Claire, Ill.	1.5	176	[illegible]
Waterloo, Iowa	1.5	47	8.1
Clovis, N. M.	2.2	45	7.6
Panhandle, Texas	2.9	37	8.0
Amarillo, Texas	3.9	35	8.5
Cameron, N. C.	4.9	39	8.3
Lubbock, Texas	4.1	43	9.3

may be discrete or confluent pitting of the enamel. The affected teeth often take on a characteristic brown stain, the frequency of occurrence increasing with age. Mottled enamel is a permanent disfigurement (figs. 2, 3, 4 and 5). The microscopic appearance is that of a hypoplasia of the enamel and dentin. There is a failure in the development of the cementing interprismatic substance of the enamel[133] with incomplete calcification of the enamel rods[134] and of the dentin.[135] Pitting is the result of the breaking off of the end of the enamel prisms.

The solution to the problem lies in the reduction of the fluoride intake by a change in the community source

[illegible]

130. McKay, F. S., in collaboration with Black, G. V.: [illegible] Mottled Teeth, Dent. Cosmos 58:543, [illegible] (June) 1916.

131. Churchill, H. V.: Occurrence of Fluorides in Some Waters of the United States, Indust. & Eng. Chem. 23: [illegible] Smith, Theodore; Lantz, E. M., and Smith, H. V.: [illegible] Mottled Enamel Based on Survey of Human Teeth, Tech. Bull. [illegible] Agr. Exper. Sta., June 1931, [illegible] Velu, H.: [illegible] chronique, Compt. rend. Soc. de Biol. [illegible]

132. Dean, H. T., and Elvove, E.: Further Studies on the Minimal Cause of Chronic Endemic Dental Fluorosis, Public Health Rep. [illegible] Sept. 1941.

133. Hoag, V.: [illegible] in McKay, F. S., [illegible] Mottled Enamel: Investigations of the Dental Profession of the Laboratory of Dentistry, Dent. Cosmos [illegible] (July) 1916.

134. Wilson, J. L.: Mottled Teeth: Data on Structural and Pathological Conditions of Teeth, Dental Rev. [illegible] (Sept.) 1925.

135. Fosdick, L. S.: A Contribution to the Histology of Mottled Teeth, J. Am. Dent. Assoc. 12: 1952 (Dec.) 1925; Ainsworth, N. J.: Mottled Teeth, Dent. Rec. 45: [illegible] (Sept.) 1925.

simple,[illegible] by dilution of high fluoride waters or by treatment of the water to remove the fluoride.[illegible] Various aspects of endemic dental fluorosis have been reviewed in a number of recent papers.[illegible]

Chronic fluorine poisoning may occur through exposure to dusts or fumes of fluoride containing minerals in industrial processes. Rohdich[illegible] in his monograph reviews the [illegible] discusses the [illegible]

[illegible] and osteosclerosis was the outstanding feature. In the bone system was affected eventually, but the cerebral cortex and pelvis were affected first [illegible] Greenwood[illegible] in his comprehensive [illegible]

[illegible] of fluoride containing insecticides used in spraying fruits and vegetables. This has necessitated a federal food tolerance on such spray residues of 1/100 grain of fluoride per pound of food, which is equivalent to about 2.9 parts per million.

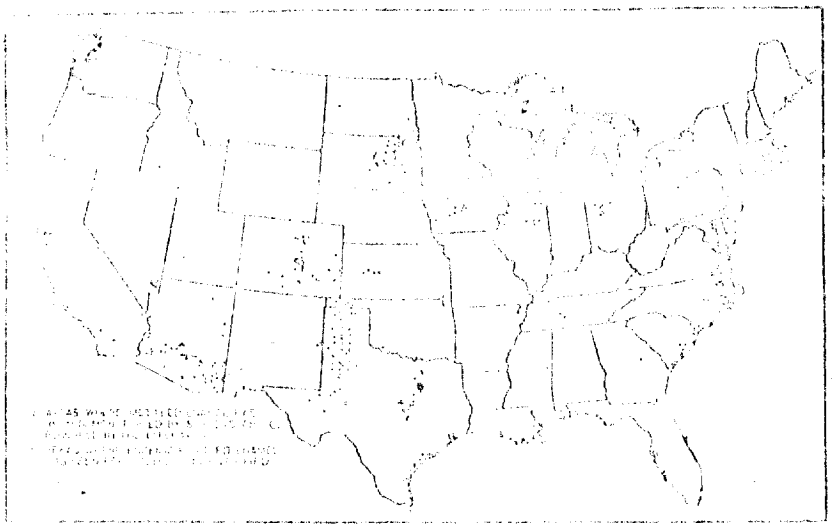

Fig. 1. Distribution of mottled enamel in the United States. In August 1933 there were about [illegible] and several [illegible] in the United States where not [illegible] (Dean and Arnold[illegible]).

McCollum and his coworkers[illegible] and Schulz and Lingg[illegible] first showed the detrimental effects of the inclusion of fluoride in the diet of experimental animals. Their observations[illegible] on the bleeding and fragility of the bones and teeth and of the overgrowth of the maxillary incisors following excessive erosion of the opposing mandibular incisors[illegible] have been confirmed by numerous investigators who have added to our knowledge. The use of natural phosphates or superphosphates with high fluoride content as mineral supplements to farm animals has frequently resulted in chronic fluoride poisoning. Stunting of growth, [illegible] lameness due to bone abnormality [illegible] changes similar to the [illegible] have been described.[illegible]

Additional evidence for the action of fluoride as a modifier of calcium and phosphorus metabolism is the observation[illegible] that fluoride decreases the severity of rickets in rats, prolongs the life of rachitic [illegible] and restores normal calcification when vitamin D is [illegible] administered together with vitamin D [illegible] given to the normal mother causes delay and [illegible] disorganization of calcification of the bones of suckling rats.[illegible] Greenwood[illegible] cites [illegible] pathologic bone changes [illegible] people living in endemic mottled areas for long periods of time. However, in a recent [illegible] study[illegible] of persons [illegible] plies contained 1.5 to 3 parts per million of fluoride and where dental fluorosis was widespread, no [illegible] strable skeletal sclerosis occurred even though the water was taken for long periods. Smith[illegible] was unable to find any bony changes in children with mottled enamel. [illegible] gested that teeth are much more sensitive to fluoride than the bone skeleton.

Fluoride is known as an enzyme poison, specifically inhibiting the formation of phosphoglycerate from phosphoglycerate. The [illegible] the literature on its effect on [illegible] phatases in vivo are conflicting.[illegible] In vitro experiments indicate an inhibition of phosphatases[illegible] and esterases.[illegible]

[illegible]

[illegible] Mc Clure, Shamonds, Becker and Barnes,[illegible] Science [illegible]

[illegible] Pease, A. W.: Chronic Fluorine Poisoning in Dairy Cattle, Nutrition Abst. & Rev. 8: 1, 1939.

[illegible] Hunt and Murray, M. M.: Observations on Chronic Fluorine Poisoning in Animals, Proc. Roy. Soc. Med. 32: 1, 1939.

[illegible] Mc Clure, K., and Hunt, S. B.: Effect of Fluorine on Activity of Vitamin D in Rachitic Rats, J. Nutrition 7: 1, 1934.

[illegible] Hunt, B. B., and Kramer, M.: Effect of Fluorine on Healing of Rickets, Bull. Proc. Soc. Exp. Biol. & Med. 31: 1, 1933.

[illegible] Greenwood, G. T.: Glycogen and Calcification, J. Physiol. [illegible]

[illegible] Dobbs, P. C.; Farrell, O. L.; Peggy, George and Clark, [illegible] Science on Chronic Fluoride Poisoning, J. A. M. A. [illegible]

[illegible] Smith, Macaulay, G.: Fluorine Toxicosis: A Public Health Problem, J. Pub. Health 27: 1, 1937.

[illegible] Hoffman, G. [illegible] Bland, T. W., [illegible] Mc Clure, [illegible]

The synergistic action of thyroid and fluoride has been noted. Fluoride when given in conjunction with thyroid accentuates the effect on the basal metabolic rate produced by the thyroid, whereas the fluoride alone has no effect.[illegible] Fluoride enhances the toxicity of thyroid in chicks and vice versa.[illegible] Thyroid and thyrotropic hormone likewise enhance the bleaching of rat incisors by fluoride.[illegible]

[illegible] the content of cow's milk [illegible] by increasing [illegible] ever, it is possible that women exposed to fluorides may be able to transmit enough fluoride in their milk to affect the development of the teeth.[illegible] From these as what meager evidence[illegible] it would appear that [illegible] relatively little, when compared on a basis as [illegible] to the fluoride dosage [illegible] papers on the arising from the use of dicalcium phosphate as a dietary supplement during pregnancy and for [illegible] and children.

[illegible] has become of even greater practical importance since the discovery of Armstrong[illegible] and Armstrong and Brekhus[illegible] that the amount of [illegible] contained in a fluoride diet [illegible] amount [illegible] has been found to differ between normal [illegible] teeth, and the suggestion was made that the increased fluorine content might play a role in the resistance to caries.

[illegible] papers have since appeared of a clinical, [illegible] and experimental nature with supporting evidence for a relationship between fluorine and the incidence of dental caries. It is interesting to note that before the finding of Armstrong and Brekhus there were many observations in the literature[illegible] pointing [illegible] increased severity of caries in areas of endemic dental fluorosis.

[illegible]

Dean and his co-workers[illegible] have published a valuable series of papers concerned with human epidemiologic studies. They indicate an inverse relationship between the fluoride concentration of the water supply and the incidence of dental caries in children. [illegible] this in communities with a water supply containing fluorides in concentration but slightly above the [illegible] and threshold of endemic dental fluorosis [illegible] where the incidence [illegible] dental caries [illegible] in communities using fluoride [illegible] Further work[illegible] had shown the inverse relationship between endemic dental fluorosis and dental caries. [illegible]

[illegible] Dean and Arnold and Dean, H. T., McKay, F. S., and Elvove, Elmer, Pub. Health Rep. 53: 1443, 1938, through the courtesy of Dr. Dean of U. S. Public Health Service.

Fig. 5. Mottled enamel of moderate degree. Note the [illegible] pitting and lack of luster particularly [illegible] the molar and premolar 4.

[illegible] the latter results indicate that the limited immunity to dental caries is not dependent on the presence of macroscopic mottled enamel. The results at Bauxite, Ark.,[illegible] indicate that exposure to high fluoride water for only the first several years of life resulted in increased resistance to caries for at least several years after a change to a low fluoride water supply. [illegible]

[illegible]

[illegible] studies the salivary Bacillus acidophilus counts reflected the dental caries rate. Evidence that the sodium needs further study is given in an recent report of a study of school children exposed for [illegible] years to a community water which was increased in fluoride content from [illegible] to 0.7 parts per million. The dental caries experience was studied by [illegible] in comparison with [illegible]

[illegible] inhibit incipient dental caries [illegible] when given during tooth development [illegible] or after eruption of the teeth.[illegible] The mechanism by which

[illegible]

[illegible]

FIG. 5.—Normal enamel.

fluoride acts is not completely established, but it appears to be by one or both of the following: (a) by the fluoride entering the tooth structure and giving it earlier resistant properties or (b) by inhibiting bacterial growth on food particles and on the teeth. There is evidence for both effects.

[illegible]

Fluorides in minute amounts limit acid production by mouth bacteria, as do fluorosed and fluoride-treated enamel and dentin; in larger amounts growth is inhibited.[illegible] In vitro and in vivo acquisition of fluoride by teeth has been studied;[illegible] it has been found that enamel and dentin can acquire fluoride and that in acid samples of low reduced solubility,[illegible] although [illegible] the amounts of fluoride [illegible] [illegible] Nygard and McClure[illegible] [illegible] cutaneous injection of the fluoride as contrasted to oral administration produced no significant reduction in induced dental caries of rats, although the fluoride content of the teeth increased. They believe that [illegible] fluoride acts by inactivating [illegible] [illegible] the pH [illegible]

Fluorine's consumption effect on dental caries in minute amounts unfortunately means that for the present, at least, it is not an inhibitor of [illegible] [illegible] whether topical application of fluoride or its intake by persons whose permanent teeth have already erupted are effective and [illegible] inhibiting dental caries. One recent preliminary report on topical application to a small [illegible]

There is no evidence from the investigations [illegible] fluorine low diets[illegible] that this element is [illegible] However, more refined nutritional experiments [illegible] elucidation of its role in tooth and bone [illegible] show that it is necessary.

SELENIUM

Selenium poisoning has gained prominence [illegible] lem in livestock nutrition and as a possible [illegible] heard to man since 1933, when selenium was found in samples of wheat which had previously been shown to be toxic to livestock and to laboratory animals.[illegible] The ingestion of selenium-bearing vegetation has been thought to be concerned with the pathological conditions of farm animals known as "alkali disease" [illegible] "blind staggers."[illegible] Plants growing in saline [illegible] areas often contain high concentrations of the element in some organic form and presumably in the [illegible]

[illegible]

[illegible] tion.[175] The affected localities are limited in size [illegible] widely scattered throughout the Great Plains of [illegible] western United States.[176]

Selenium poisoning results in stunting of growth, [illegible] tion, loss of hair, decreased reproductive power, [illegible] ly and cirrhosis of the liver, gastric disorders and [illegible].[177] Cattle, [illegible] and horses develop [illegible] of hoofs, abnormalities of the horns and [illegible] in the [illegible] [illegible] of the embryos into constituents.[178] Experiments with various species have demonstrated[179] that daily inges[illegible] of selenium in amounts as low as [illegible] [illegible] of weight causes minor symptoms and that [illegible] in excess of [illegible] per kilogram daily [illegible] [illegible] the inorganic selenium for the [illegible]

It is noteworthy that within certain limits the toxicity of plant and inorganic selenium is determined by the [illegible] selenium ratio in the diet rather than by the selenium intake.[180] Selenium compounds inhibit cellular [illegible] probably by poisoning enzymes, such as [illegible] which are dependent on sulfhydryl groups for activity.[181]

[illegible] living in areas where [illegible] [illegible] nonseleniferous areas.[182] Study is [illegible] selenium [illegible] its widespread occurrence in animals as well as [illegible] foodstuffs from seleniferous localities. There [illegible] definite clinical evidence of human selenium intoxication in these areas, but there is suggestive evidence that man is not immune.[183] The seriousness of selenium [illegible] in the [illegible] populations is undoubtedly reduced by the fact that much of the flour and vegetables con[illegible] come from nonseleniferous regions.[184]

BORON

Although boron is known to be essential for plants, there is as yet no experimental evidence that it is necessary for animals. It appears to exist normally in small amounts in animals,[185] in milk,[186] and in eggs.[187] Numerous investigators have observed the rapid urinary excretion of the element.[188] Two recent studies[189] have shown that rats can thrive on diets extremely low in boron (less than 0.3 microgram per rat daily).

ALUMINUM

The use of aluminum in cooking utensils [illegible] has created some [illegible] [illegible] in its role in normal [illegible] [illegible] indicates that ingested aluminum is absorbed in but small amounts[190] and that the amounts occurring in the usual human dietary (about 12 to 15 mg. daily[191]) are not harmful.[192] This element is widely distributed in nature and has been found [illegible] very small amounts in plant and animal [illegible] milk [illegible] of aluminum as a dietary constituent. Rats [illegible] for six weeks on a diet containing as little as 1 microgram of aluminum daily appeared normal;[193] nevertheless, when the aluminum intake was [illegible] there was definite absorption and storage. On the basis of balance studies with young children, Sepulveda[194] suggests that this element is not essential.

CONCLUSION

Six trace elements, namely iron, copper, iodine, [illegible] and zinc, have been definitely proved to be essential to animal life. Our knowledge of the human requirement for manganese, cobalt and zinc is so meager that the possibility of deficiency of any one of them occurring cannot be dismissed, although, because of their broad distribution in nature and probably small requirement, the likelihood of any acute or widespread deficiency appears remote. The essential trace elements and the other trace elements which occur in living matter but whose importance is unknown stand as a challenge to nutritionists and physiologists. The indispensable "traces," just as the vitamins, appear to be keys to fundamental physiologic processes the mechanisms of which are either only partially understood or in most cases completely unknown.

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THE IRON AND MANGANESE CONTENT OF FEEDING STUFFS.*

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That the inorganic elements play an important rôle in plant and animal metabolism has long been recognized. However, it is only in comparatively recent years that the importance of some of the less obvious of these elements has been realized and attempts made to determine their function in the animal organism (1–5). For example, the wide-spread occurrence of manganese in the tissues of animals (6–9) is an indication that it probably serves a useful purpose there. Direct evidence of the nutritional value of manganese has been furnished through various feeding experiments. Richet, Gardner, and Goodbody (10) have demonstrated that manganese, if administered in small amounts, produces a favorable effect upon the growth of dogs. More recently McCarrison (11) reported that manganese, when ingested by young rats to the extent of 1 part in 617,700 parts of ration, was beneficial in promoting growth. However, when the manganese was increased to 1 part in 12,000, a retarding influence upon the rate of growth became evident after 32 days.

In view of the demonstrated function of manganese in animal metabolism, a knowledge of the content of this element in the various feeding stuffs is highly desirable. A few papers (12–15), have already been published on this subject, but many of the materials commonly consumed by farm animals are not included in these papers.

Unlike manganese, iron has been recognized for many years as important in nutrition. Consequently, the lack of analytical

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data with regard to the iron content of various feeding stuffs is rather surprising. Henry and Morrison (16) in their tables of the mineral matter in representative feeding stuffs give the iron content for only one-half of the materials listed. Additional analyses have been reported (12–14, 17) but the number of samples was rather limited.

In this paper will be presented the manganese content of 54 feeding stuffs and the iron content of 51 of the same samples.

EXPERIMENTAL.

Preparation and Ashing of Samples.—The feeding stuffs were either obtained from the local market or were taken directly from the field. After being dried, all coarse materials were reduced to a finely divided state, care being taken to avoid the introduction of iron or manganese. The samples were ashed in a muffle furnace at a cherry-red heat until all carbon had been oxidized. Samples which were extremely hard to ash were first burned at a somewhat lower temperature and the partially oxidized mass was then extracted with water and filtered. After the residue was dried it was ignited to a carbon-free ash.

Methods of Analysis.

Iron.—The iron analyses were made according to the ferric thiocyanate method given in Standard Methods for the Examination of Water and Sewage by the American Public Health Association (18). Precautions with respect to dishes and reagents described in a previous publication (19) were observed.

In order to secure representative samples from the various feeds 2 to 4 gm. portions were usually ashed. The ash was dissolved in HCl (1:4) made to a definite volume and aliquots assumed to contain from 0.1 to 0.3 mg. of iron were taken for analysis. In the analysis of some of the samples it was thought that it might be desirable to remove the silica by evaporating the solution to dryness, but no color could be obtained with a sample which had received such treatment. No attempt was therefore made to remove the silica.

The high concentration of phosphates in the cereals frequently prevented the full development of the thiocyanate color. This

difficulty was overcome by oxidizing the HCl solution of the ash with 30 per cent H_2O_2, a reagent which had been found successful in such cases by Dr. C. A. Elvehjem of this laboratory.

All analyses were run in triplicate and to one of them was added 0.1 mg. of iron for recovery. If a satisfactory recovery of the added iron was obtained it was felt that the iron content

TABLE I.

Detailed Data Illustrating Procedure in Determination of Iron in Representative Samples of Feeding Stuffs.

Material.	Weight of sample	Weight of sample in aliquot.	Fe in standard.	Readings.		Fe in sample.	Recovery of added Fe.	Fe
				Standard.	Aliquot.			
	gm.	*gm.*	*mg.*	*mm.*	*mm.*	*mg.*	*per cent*	*per cent*
Alfalfa hay.	2.	0.8	0.1	25	17.8	0.351		0.0176
	2.	0.8	0.1	25	18.0	0.348		0.0174
	2*	0.8	0.2	20	22.3	0.449	99	
Corn, yellow.	2	2.0	0.1	25	27.7	0.090		0.0045
	2	2.0	0.1	25	28.1	0.089		0.0045
	2*	2.0	0.2	20	20.5	0.195	105	
Corn stover.	4	1.6	0.2	20	26.8	0.373		0.0093
	4	1.6	0.2	20	26.6	0.376		0.0094
	4*	1.6	0.2	20	21.0	0.476	102	
Rape.	2	0.8	0.2	20	20.4	0.490		0.0245
	2	0.8	0.2	20	20.6	0.485		0.0243
	2*	0.8	0.2	20	17.0	0.588	101	
Tankage.	1	0.2	0.1	25	26.1	0.483		0.0483
	1	0.2	0.1	25	25.9	0.479		0.0479
	1*	0.2	0.1	25	19.2	0.571	90	

* A quantity of the standard iron solution containing 0.1 mg. of Fe was added to this sample before ashing.

of the sample was being accurately determined. There was no appreciable difference whether the iron was added prior to ashing or just before oxidizing the solution with permanganate.

The detailed data for five of the 51 samples are given in Table I. These five samples are representative of the different classes of feeding stuffs and include materials which range from low to high

in iron content. The recovery of added iron is from 90 to 106 per cent and shows the reliability of the method. The same procedure was followed in the analysis of all the other samples. If a satisfactory recovery was not obtained the first time, the analysis was repeated with particular attention to the ashing and to the oxidation of the hydrochloric acid extract.

Manganese.—This element was determined by the procedure given in Methods of Analysis of the Association of Official Agricultural Chemists (20). Certain modifications in procedure and precautions with respect to reagents and dishes have been described in a previous publication (21).

Samples high in calcium frequently showed a separation of $CaSO_4$ while the solution was being evaporated for the removal of chlorine. In such cases it was necessary to shake the beaker continuously to prevent spattering and loss of manganese. A satisfactory reading of the colorimeter in the presence of this precipitate was impossible and therefore it was removed by filtering through a layer of manganese-free asbestos in a Gooch crucible. The residue was washed with small portions of water until the volume of the filtrate was approximately 30 cc.

In a few instances during oxidation with KIO_4 the permanganate color faded as the volume was reduced. This difficulty was overcome by the addition of a few drops of sirupy phosphoric acid to the boiling solution.

As in the analysis of iron, triplicate determinations, one of which was a recovery, were made upon each feed. The percentage recovery was slightly lower when the manganese was added before the sample was ashed. The lowest recoveries were obtained with those samples which yielded large amounts of ash. It is probable that the loss was due to occlusion of the added manganese in the silica and other insoluble compounds. Samples of such size were taken that the color could be compared with that of a standard containing from 0.1 to 0.25 mg. of manganese. When concentrations greater than these are present, the KIO_4 may fail to develop completely the permanganate color.

Table II illustrates the procedure in detail for the determination of manganese in five feeding stuffs. The difficulties encountered in the analyses of these feeds are representative of those met with in the analyses of all the materials. The recovery was better than

90 per cent for each of these samples. In only eight analyses was the recovery less than 90 per cent.

DISCUSSION.

The amounts of iron and manganese per kilo of dry matter for all the samples are given in Table III.

TABLE II.

Detailed Data Illustrating Procedure in Determination of Manganese in Representative Samples of Feeding Stuffs.

Material.	Weight of sample.	Mn in standard.	Readings.		Mn in sample.	Recovery of added Mn.	Mn
			Standard.	Sample.			
	gm.	*mg.*	*mm.*	*mm.*	*mg.*	*per cent*	*per cent*
Alfalfa hay.	2.5	0.148	20	16.5	0.179		0.0072
	2.5	0.148	20	16.8	0.176		0.0071
	2.5*	0.247	15	13.7	0.271	94	
Corn, yellow.	15	0.148	20	37.0	0.080		0.00053
	15	0.148	20	36.0	0.082		0.00054
	15*	0.148	20	16.4	0.181	100	
Cottonseed meal.	15	0.247	15	12.2	0.304		0.0020
	15	0.247	15	12.6	0.294		0.0020
	15*	0.247	15	9.4	0.394	96	
Oat straw.	2	0.148	20	17.0	0.175		0.0087
	2	0.148	20	17.0	0.175		0.0087
	2*	0.247	15	13.7	0.271	96	
Sugar beet roots.	1	0.148	20	14.8	0.200		0.0200
	1	0.148	20	14.0	0.211		0.0211
	1*	0.247	15	12.5	0.296	92	

* 0.099 mg. of Mn was added to this sample.

Iron.—The iron content in the 51 feeds ranged from 8.9 mg. per kilo for polished rice to 750 mg. for coconut oil meal. 80 per cent of the samples contained between 40 and 500 mg. per kilo. The average for all the samples was 203 mg. per kilo.

The variations observed in a single feed were enormous. For example, five samples of alfalfa varied from 126.5 to 681.8 mg. per

TABLE III.

Iron and Manganese Content of Feeding Stuffs.

Material.	Fe in dry matter (100°).	Mn in dry matter (100°).
	mg. per kg.	*mg. per kg.*
Alfalfa hay	174.7	71.2
" " first cutting (Wisconsin)	242.8	46.0
" " second " "	126.5	47.9
" " first " (Colorado), Sample 1	681.8	27.0
" " " " " " 2	497.5	44.0
Barley	65.1	19.0
Blood meal	512.5	8.9
Bluegrass	425.5	59.0
Brewers' dried grain	170.9	38.3
Buckwheat feed	69.8	89.3
" hulls	99.3	97.2
Coconut oil meal	750.0	87.0
Corn, yellow	44.8	5.4
" gluten meal	183.9	5.9
" oil cake meal	182.9	15.0
" silage	352.9	34.8
" stover	93.7	53.7
Cottonseed meal	53.9	19.9
Distillers' dried grain	360.3	24.7
Earner	51.7	35.2
Gluten feed	483.0	26.9
Groats	36.8	37.4
Hominy feed	94.4	16.4
Ivory nut meal	465.0	14.7
Kafir grain, white	39.1	15.9
Linseed meal	105.8	49.4
Meat meal	241.5	18.7
" and bone meal	438.5	10.0
Millet, Japanese	58.7	35.6
Oats	42.3	31.8
Oat straw	61.3	87.1
Rape	243.9	51.1
Rice, brewers'	11.0	16.8
" polished	8.9	12.0
Rye, Sample 1	57.4	50.1
" " 2		32.3
Sorghum fodder	54.3	41.1
Soy bean seed	59.5	29.5
" " hay	304.0	84.1

TABLE III—*Concluded.*

Material.	Fe in dry matter (100°).	Mn in dry matter (100°).
	mg. per kg.	*mg. per kg.*
Sugar beet roots (field)	53.5	205.7
" " tops (field)	178.7	712.4
" " " (greenhouse)	478.5	102.9
Sweet clover hay	212.8	46.1
Tankage	480.8	14.3
Timothy hay	30.2	32.0
Vetch hay	354.5	106.5
Wheat	46.3	54.5
" bran, Sample 1	162.8	140.4
" " " 2		140.4
" gluten	71.0	27.4
" middlings, flour	120.3	129.6
" " standard	192.4	155.8
" straw, Sample 1	71.0	24.1
" " " 2		30.8

kilo. The first cutting of alfalfa contained 242.8 mg. per kilo whereas the second cutting from the same plot of land contained only 126.5 mg. Apparently the available iron supply was being rapidly diminished by the growing plant.

The average iron content arranged in descending order and expressed as mg. per kilo of dry matter for some of the principal classes of feeding stuffs are: 4 animal products, 418.4; 4 green forage crops, 331.7; 8 legume hays, 324.3; 19 by-product concentrates, 189.6; 3 cured forage feeds from grasses and cereals, 59.4; 9 grains and seeds, 51.6; 2 straws, 43.7. The group containing the grains and seeds showed the least variation, the limits for this group being 39.1 and 65.1 mg. per kilo. The percentage of iron was greater in the by-product concentrates than in the materials from which they are manufactured.

Manganese.—The amount of manganese as given in Table III varied from 5.4 mg. per kilo for yellow corn to 712.4 mg. for sugar beet tops. Of the entire number of samples 90 per cent contained from 10 to 150 mg. per kilo. The average for all the samples was 63 mg. per kilo.

The manganese in different samples of the same feed varied widely. Sugar beet tops grown in the field contained 712.4 mg. per

kilo as compared to 102.9 mg. per kilo in a sample grown in a greenhouse. The manganese in five samples of alfalfa hay grown on different soils varied from 27 to 71.2 mg. per kilo. In contrast to the variations in iron, the manganese figures for the first and second cuttings were much alike, 46 and 47.9 mg. per kilo respectively. One sample of rye contained 32.3 mg. per kilo, another contained 50.1 mg. but two samples of wheat bran were found to contain the same amount of manganese.

From the standpoint of manganese, the group containing green forage crops far surpasses any of the other groups of feeding materials. The average for this group was 231.4 mg. per kilo, whereas the next highest group showed an average manganese content of only 59.1 mg. The grains and seeds were not found to be as rich in manganese as the by-products obtained from them. These facts indicate a concentration of the element in the seed coat.

Comparison of Distribution of Iron and Manganese.

In previous publications (21, 22) it was reported that the iron content of human foods was from 2 to 25 times as great as the manganese. In the animal feeding stuffs listed in Table III, the average iron content is about 3 times that of the manganese. In the animal products, such as tankage, the iron is more than 30 times as abundant as the manganese. In the grains and seeds nine of the 51 samples contained more manganese than iron. A change in iron content is not related to a change in manganese. Of the five samples of alfalfa the one containing the greatest amount of iron contained the least amount of manganese.

SUMMARY.

The manganese content of 54 samples of a wide variety of feeding stuffs and the iron content of 51 of the same samples are given.

The figures (dry basis) range from 5.4 to 712.4 mg. per kilo for manganese and from 8.9 to 750 mg. for iron. Great variations for both elements were observed in different samples of the same feeding stuff.

The average iron content was 3 times the average manganese content. In only nine samples did the manganese exceed the iron.

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THE DETERMINATION OF MANGANESE IN ANIMAL MATERIALS.*

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In a study of the manganese metabolism of rats it became necessary to analyze many animals for their manganese content. Serious interference, due to the precipitation of calcium sulfate, was encountered when the official periodate method was applied to these samples. The same difficulty had been experienced previously in the analysis of feedingstuffs (1), but in that work the calcium sulfate, formed during evaporation, was removed by filtration. This procedure did not work well when applied to the analysis of whole animals. There is so much calcium in the bones that a satisfactory concentration of the sample could not be accomplished without removing the calcium sulfate several times during the evaporation.

An attempt was made to remove all of the calcium sulfate at one time by adding ethyl alcohol to the solution until the mixture contained 70 per cent by volume of alcohol. While the calcium sulfate was completely precipitated by this procedure, distillation of the filtrate failed to get rid of all the alcohol, and, as a result, the development of the permanganate color proved very troublesome; three oxidations with periodate were sometimes necessary before the readings against the standard reached a maximum. There was also a tendency to form a brownish surface film, which made comparison with the standard difficult.

While the work was proceeding the paper of Davidson and Capen (2) appeared giving a modification of the official method in which hydrochloric acid is replaced by nitric, sulfuric, or phos-

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phoric acid, thus shortening the method and giving as good or better results. Upon trying phosphoric acid in the analysis of our animal materials, it was found that the interference of calcium salts was almost entirely overcome. The new method gave uniformly higher results and more complete recoveries of added manganese than the official method combined with the removal of calcium sulfate by alcohol.

The percentage of manganese in animal materials is so extremely small that more than 50 gm. of dry material must be taken in some instances to furnish 0.05 mg. of manganese. It is necessary to have at least this quantity of manganese in 25 cc. of solution if the color is to be read in a colorimeter, even of the long, prism type. Such a weight of material is unwieldy and requires many hours to ash. In our metabolism studies it was highly desirable to be able to obtain analyses on a 10 to 20 gm. sample. This quantity of material is sufficient if the solutions are compared in 50 cc. Nessler tubes. Eimer and Amend tubes No. 20466 with polished colorless glass discs which are fused on to the glass tubes are particularly satisfactory. If the column of solution is about 200 mm. in depth, 0.01 mg. of manganese in 50 cc. gives a readable color. The procedure finally adopted is as follows:

Method.—The porcelain dishes in which the material is to be ashed must be extracted with dilute HCl (1:4) prior to use. Ash the material in a muffle furnace at a cherry-red heat. To the contents of the dish add 5 cc. of sirupy phosphoric acid and sufficient distilled water (30 to 50 cc.) to permit thorough extraction of the ash, and heat on the water bath for about 30 minutes. Allow the dish to cool and then filter the extract through quantitative filter paper or manganese-free asbestos. Transfer the filtrate to a 250 cc. beaker, add approximately 0.3 gm. of KIO_4, and boil gently until the full development of the permanganate color takes place. Cool to about 40°, transfer to a Nessler tube, dilute to the mark, and mix by pouring back into the original beaker and again into the Nessler tube. Compare at once with a similarly treated standard of approximately the same strength. Two standards should be prepared and the one nearest the unknown should be used for comparison.

Fold a piece of white paper so that it forms an inverted V and reflects the maximum amount of light vertically and place on a

larger sheet of white paper. Pour the lighter colored solution into one Nessler tube and while holding this and the empty tube over the reflector pour the stronger solution into the second tube until the colors of the two tubes match. With a rule graduated in mm. measure the column of liquid in each tube. Pour out some of the stronger solution and again match the two solutions. Readings should check within 2 or 3 mm. This simple method of matching

TABLE I.

Detailed Data Illustrating Procedure in the Determination of Mn in Tissues of the Rat.

Rat tissue.	Weight of sample dried at 100°.	Mn in added std.	Readings.		Mn in sample.	Recovery of added Mn.	Mn
			Standard.	Sample.			
	gm.	*mg.*	*mm.*	*mm.*	*mg.*	*per cent*	*mg. per kg.*
Bone	15	0.03	191	171	0.0335		2.233
"	15	0.03	190	173	0.0330		2.200
" + 0.03 mg. of Mn	15	0.03	187	182*	0.0616	94.5	
Hide	20	0.02	155	197	0.0157		0.785
"	20	0.02	167	187	0.0173		0.865
" + 0.03 mg. of Mn	20	0.03	179	118	0.0455	95.7	
Muscle	15	0.01	173	197	0.0088		0.587
"	15	0.01	176	200	0.0088		0.587
" + 0.03 mg. of Mn	15	0.03	195	151	0.0387	99.7	
Whole animal	13.75	0.05	186	205	0.0453		3.295
" " + 0.01 mg. of Mn	13.75	0.05	229	203	0.0548	95.0	

* The sample was made to 100 cc. instead of 50 cc.

the colors avoids the necessity of preparing a large number of standards.

In case a cloudiness appears before the reading can be taken it may be removed by concentrating the solution to about 20 cc., transferring it to a Nessler tube, and allowing the precipitate to settle out as the solution cools. The clear solution may then be decanted off, reoxidized, and compared without any interference from turbidity.

The detailed data for three tissues of the rat and for one whole

animal are given in Table I. No difficulties have been encountered in the analysis of other animal materials that have not been experienced in the analysis of these four. In fact, the analysis of bone represents the most difficult and tedious determination to which the method has been applied. Although it was necessary to remove the precipitate several times before oxidation could be accomplished without loss from spattering, a 94.5 per cent recovery of the added manganese was obtained. In this determination, as in all of our tissue analyses, the manganese for recovery was added prior to ashing. When the manganese content of the whole individual is desired this procedure cannot be employed

TABLE II.

Applicability of the Method to Some Typical Animal Materials.

Material.	Weight of sample.	Mn in sample.	Recovery of added Mn.
	gm.	*mg.*	*per cent*
Beefsteak, T-bone	20	0.0130	93.0
Cheese, American	34.2	0.0265	91.5
Cod, fresh	15	0.0095	104.5
Eggs	15	0.0165	103.5
Lamb chops	15	0.0185	91.5
Lobster	10	0.0252	91.8
Milk	412	0.0135	89.3
Pork chops	5	0.0062	95.8
Shrimp	15	0.0105	99.5
Turkey, dark meat	20	0.0315	88.5

because of the difficulty in obtaining representative samples of the dried body. In Table I the recovery on the whole animal was obtained by adding the manganese to an aliquot of the acid extract just prior to oxidation. A comparison of the percentages in the seventh column shows that the recovery of manganese is about the same regardless of whether it is added before or after the ashing.

That the method is applicable to a wide variety of animal materials is shown in Table II by the data obtained in the analysis of ten representative foodstuffs. Although the actual amount of manganese in these samples ranges from 0.0062 to 0.0315 mg., recoveries ranging from 88.5 to 104.5 per cent were obtained.

SUMMARY.

The periodate method has been adapted to the determination of the small quantities of manganese found in animal tissue without the necessity of taking a large and unwieldy weight of sample. As little as 0.01 mg. of manganese has been satisfactorily determined. Good recoveries of added manganese were obtained.

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French Translation

Content of Manganese in Rabbit Hair in The Course of Oral Chronic Administration of Manganese Sulfate

by G. M. UMARJI, K. G. ANANTANARAYANAN and R. A. BELLARE

Manganese in the form of the dioxide or pyrolusite is a mineral with a large field of application. Thanks to the fundamental work of Bertrand, its indispensable role as an oligo-element for life in plants as well as animals is recognized. But workers working with this mineral, especially those exposed to dust inhalation or to manganese fumes are often attacked by "manganese sickness" or "manganism" (psychic problems, asthenia, anorexia, unclear speech, muscular weakness, paraplegia etc.). Considering these serious repercussions, leading inexorably to complete incapacitation and practically insensitive to treatment, chronic manganese poisoning is considered one of the most serious industrial poisonings. First described in 1837 by Couper (1) the illness has recently been studied by a number of researchers notably by Boyer (2) Rodier (3) Ritter and Marti-Freed (4) and Thibault (5) in Morocco; by Penalver (6) in Cuba, Makarchenko (7) in the Soviet Union, Sykora (8) in Czechoslovakia, Szobor (9) in Hungary, Nujogi (10) Barval, Wadia et al. (11) Chatterjee et al. (12) in India, Lutier (13) in France etc. One must mention as well the experimental toxicological study of manganese carried out by Lemos (14).

Rodier (3) who carried out systematic studies on the biological changes in manganism, observed distinct endocrine changes (reduction of 17-Ketosteroids by 80%, the general average for all those sick was 8.69 mg/24 hr

as against the normal content of 12-14 mg/24 hr and an increase in basal metabolism of about 19.5 percent) and from the hematological standpoint an indisputable inversion of the leucocytic formula for manganese of 44 percent. These results, presented in another communication (15) concerning the Mn content of the hair of exposed miners, appear impressive to us. The average content in healthy miners is 365 mg/kg, in those suspect it is 232 mg/kg and only 105 mg/kg in those subjects who reach chronic poisoning. The average Mn content in normal hair includes between 1.6 and 4.5 mg/kg [Copeman (16), Anke and Schneider (17), Besson and Brecej (18), Umarji and Bellare (19)]. Thus the manganese content of the healthy miners' hair is considerably greater (100 times or more) than that of normal subjects but with the onset of manganism it undergoes a significant drop.

The pulmonary route of absorption alone is considered as dangerous (Rodier, Penalver, Thibault, Szobor), but Kawamura et al. (20) have reported 16 cases of manganism in Japan in subjects who drank water contaminated with Mn coming from electric batteries. This led us to study the effects of orally administering a manganese salt to laboratory animals, examining their behavior, their growth and above all the Mn content of their fur.

Materials and Methods --

Six young rabbits (white), weighing between 2,000 and 2,300 g were grouped, two by two in 3 groups. The first group of two rabbits (Nos. 1 & 2) received daily 75 mg of manganese sulfate dissolved in drinking water. The second group (Nos. 3 & 4) received 7 mg daily and the third group (Nos. 5 & 6) acted as controls.

At the beginning of the experiment , the fur on the back of each rabbit was removed in order to determine the Mn content. During the 3 or 4 months of the chronic administration of Mn, the newly regenerated fur was removed and carefully cleaned of all external contamination. The manganese was determined by the method of Wiese and Johnson (21) as previously described by us (19).

Results --

The average content of Mn in the fur of all of the rabbits before the start of the experiment was 1.07 mg/kg, the same order (1.11-1.36 mg/kg) as that found by Bertrand and Mediggreceaunu (22), though Bresson and Brecej (18) found between 0.28 and 0.56 mg/kg. The results of the experiment are shown in the Figure.

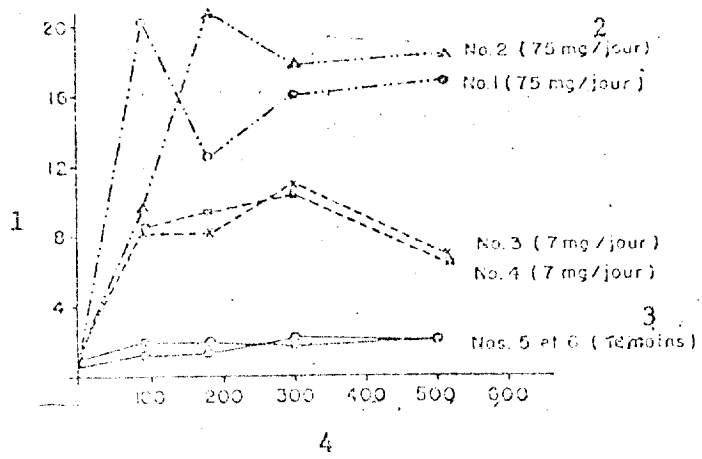

Figure. Manganese content of the fur of a rabbit receiving oral manganese sulfate

1 Manganese content of fur in p.p.m.
2 Mg/day
3 (controls)
4 Time of administration in days

In the controls the value gradually increased to 2 mg/kg after 510 days. In the group receiving 7 mg/day, the content was 8.4 mg/kg after 90 days, 10.6 mg/kg after 300 days, but beyond (510 days) a drop towards 6.5 mg/kg is observed. The group receiving 75 mg per day showed a contents of 15.5 mg/kg after 90 days, 16.8 mg/kg after 180 days and 17.75 mg/kg after 510 days.

In the case of two rabbits receiving 75 mg/day, the first showed a contents of 20.4 mg/kg already after 90 days but at the 180th day, the value underwent a sharp drop to 12.6 mg/kg; paralleling this, a weight loss and a paralysis of the hind paws was observed in which the extremities became insensitive to a needle prick. The paralysis lasted around two months and then gradually disappeared, despite the continuous administration of manganese sulfate (75 mg/day), while the anesthesia lasted for a long time. This amelioration corresponded to an increase in the manganese content of the fur to 16 mg/kg. The manganese content of the fur of the second rabbit increased less rapidly; 10.6 mg/kg after 90 days and 21.0 mg/kg after 180 days. Towards 300 days, it likewise underwent a slight drop (17.9 mg/kg) while one observed a weight loss, a slight transitory paralysis and an insensitivity to pricking in the extremities of the hind paws, for several months.

The weight gain of the controls during 300 days of the experiment was on the average 700 mg, that of the rabbits receiving 7 mg/day was 525 g. Rabbit No. 1 receiving 75 mg/day only gained 175 g while No. 2 showed an increase of 450 g.

Conclusion --

The chronic peroral administration of manganese sulfate to rabbits increases the manganese content of the fur according to the dose. The increase is greater than ten times with a dose of 75 mg/day. One observes with this manganese dose anorexia, weight loss, anesthesia, and also a paralysis of the extremities of the hind paws. With the setting in of these symptoms, one observes a drop in the Mn content of the fur interesting to place in parallel with Rodier's observations in manganese miners in Morocco. But the paralysis of the rabbits under our experimental conditions was not irreversible as is the case with true manganism in man.

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Toxicol. et expérimentale.

Toxicité du manganèse chez le lapin au cours de l'administration chronique de sulfate de manganèse par voie orale.

par G. M. Umarji, K. G. Ananthanarayanan et R. A. Bellare.

Le manganèse, en forme de biosyde de pyrolusite, est un minéral [illegible] au cycle d'usage d'application. Grâce aux travaux fondamentaux [illegible] sur le rôle indispensable comme oligo-élément à la vie, tant [illegible] et végétale, M. [illegible] et avec ce [illegible] à l'intoxication de [illegible] en de [illegible] de manganèse, et avec [illegible] par le [illegible] du manganèse [illegible] (troubles psychiques, asthénie, anorexie, [illegible], lourdeur musculaire, paraplégie etc.). Vu ses graves [illegible] considérant inexcusablement avec une incertitude totale [illegible] toxicologique. Dans l'intoxication chronique par [illegible] apparaît comme une des plus importantes. Intoxication [illegible] et [illegible] de 11,7 pg. chez [illegible] (1), le manganèse [illegible] par de nombreux chercheurs, notamment par Boyer (2), Oudin (3), Hiller et Shetlaw (4) et Thibault (5) en France; par Jenniker (6) à Cuba, Nikonchenko (7) en Union Soviétique, Sykora (8) en Tchécoslovaquie, Szonyi (9) en Hongrie, Nyssen (10), Rossi, Modin et coll. (11), Chatterjee et coll. (12) aux Indes, [illegible] en France et [illegible] l'intoxication [illegible] par Lemos et [illegible] qui protestent, l'intoxication [illegible] chez les [illegible] dans le manganèse, chez des ouvriers [illegible] la fréquence des 17 cétostéroïdes chez 32 n. 100, la moyenne générale de tous les malades étant 5,69 mg. 24 h contre le taux normal

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de 12,41 mg/24 h ; augmentation du métabolisme de base d'environ 13,5 p. 100 et, du point de vue hématologique, une injection intra-veineuse de la formule [illegible] p. 198 des manganiques [illegible] présentés dans une autre communication (15) concernant [illegible] nous semblaient [illegible] chez les mineurs [illegible] aspects et [illegible] chez les [illegible] toxicoallergique. La concentration moyenne en Mn dans les cheveux humains est comprise entre 1,6 et 1,5 mg/kg [illegible] (16), [illegible] et Schedler (17), [illegible] et Breed (18), Umarji et [illegible] (19). [illegible], la teneur en Mn des cheveux des mineurs sains, [illegible] idéal, est [illegible] (deux fois et plus) à celle des sujets [illegible] scientifiques.

La voie pulmonaire d'absorption seule est considérée comme [illegible] (Bertier, Fontaine, Thibault, [illegible]), mais Kawamura et [illegible] un syndrome de manganèse au Japon chez des sujets qui avaient bu de l'eau contaminée avec du provenant de piles électriques. Ceci nous a conduits à étudier les seuils de l'administration [illegible] de manganèse par voie orale chez des animaux de laboratoire en [illegible] leur croissance, leur [illegible] et surtout la teneur en Mn dans leurs poils.

Matériel et Méthodes. — Six jeunes lapins (blancs), pesant entre [illegible] et 2 kg, [illegible] et groupés, deux par deux, en trois groupes. Le [illegible] lapins (N° 1 et 2) recevait chaque jour 75 mg [illegible] Mn [illegible] dans de l'eau de boisson. Le deuxième groupe [illegible] et le troisième groupe (N° 5 et 6) [illegible] témoin.

[illegible] de l'expérience, les poils sur le dos de chaque lapin étaient prélevés en vue du dosage du Mn. Tous les 3 ou 4 jours, [illegible] les poils nouvellement [illegible] étaient prélevés et soigneusement débarrassés de toute contamination extérieure. Le manganèse a été dosé par une modification [illegible] et Johnson (20), comme décrit précédemment par nous (16).

Résultats. — La teneur moyenne des poils en Mn de l'ensemble des lapins avant le début de l'expérience était de 1,97 mg/kg, du même ordre que celle (1,5-1,56 mg/kg) trouvée par Bertrand et Medigreceanu (22) auxquels les [illegible] et Breed (18) en trouvent entre 0,28 et 0,56 mg/kg. Les résultats de l'expérience sont présentés dans la figure. Chez les

(15) J. Bedier, *J. B. I. Indust. Méd.*, 1955, t. 12, p. 21.
(16) R. G. Cuperus, *J. Forensic M. L.*, 1956, t. 2, p. 118.
(17) H. [illegible] et H. J. S. [illegible], *Z. d. Z. Verhütung u. Stoffwechselkrankheiten*, [illegible], t. 2, p. [illegible].
(18) [illegible] et [illegible], *Bull. Soc. Médecine des Sciences*, 1961, t. [illegible], p. 150.
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(20) R. Kawamura, T. Ikeda, S. Ichizumi, R. Yamada, S. Tsubaki, [illegible] Rodriguez, [illegible] S. Kano, *Sekingaku Zasshi*, 1959, t. 537, p. 1 (Biochem. Abstr. 1961, t. 46, p. [illegible]).
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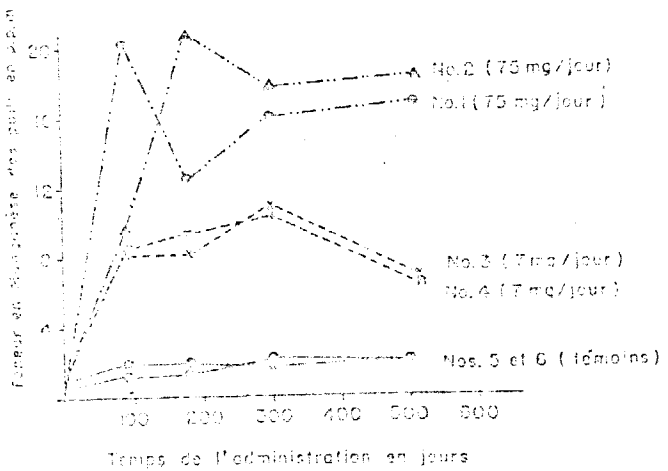
(22) C. [illegible] et M. Medigreceanu, [illegible] *Inst. Pasteur*, 1954, t. [illegible], p. [illegible]

[illegible], la valeur a graduellement monté jusqu'à 2 mg/kg après [illegible] jours. Chez le groupe recevant 7 mg/jour, la teneur est de 8,4 mg/kg après 90 jours, 10,6 mg/kg, après 300 jours, mais, au delà (540 jours) [illegible] une chute vers 6,5 mg/kg. Le groupe recevant 75 mg par [illegible] de 15,5 mg/kg après 90 jours, 16,8 mg/kg après 180 jours et 17,75 mg/kg après 540 jours.

Dans le cas des deux lapins recevant 75 mg/jour, le n° 1 présentait la teneur de 20,1 mg/kg déjà après 90 jours, mais au 180e jour, la valeur a subi une chute brusque à 12,6 mg/kg ; parallèlement, on a observé une perte de poids et une paralysie des pattes arrière, dont les extrémités étaient devenues insensibles à la piqûre par aiguille. La paralysie a duré environ deux mois et a évolué graduellement disparu, malgré l'administration continuelle de sulfate de Mn (75 mg/



Teneur en manganèse des poils chez le lapin recevant du sulfate de Mn par voie orale.

jours, tandis que l'anesthésie a duré plus longtemps. Cette amélioration correspondait avec une augmentation de la teneur en Mn des poils à [illegible] mg/kg. La teneur en Mn des poils du lapin n° 2 s'est accrue moins rapidement 19,6 mg/kg après 90 jours et 21,9 mg/kg après 180 jours. [illegible] 360 jours, elle subit également une chute légère (17,9 mg/kg), [illegible] tandis que s'observent une perte de poids, une légère paralysie [illegible] et une insensibilité à la piqûre des extrémités de pattes [illegible] pendant quelques mois.

[illegible] de poids des témoins durant 360 jours de l'expérience était [illegible] de 700 g, celui du lapin recevant 7 mg/jour de 525 g. Le [illegible] n° 1, recevant 75 mg/jour, a gagné seulement 175 g, alors que [illegible] n° 2 a montré une augmentation de 450 g.

Conclusion. — L'administration chronique de sulfate de manganèse par voie orale chez le lapin, accroît la teneur en Mn des poils selon la dose. L'accroissement est supérieur à dix fois avec la dose de 75 mg/

[illegible]. On observe, avec cette dose de Mn, de l'anorexie, de la perte de poids, de l'anosmie et même une paralysie des extrémités des pattes [illegible]. Avec l'atténuation de ces symptômes, on note une élévation [illegible] dans la teneur en Mn des poils, intéressante à noter en parallèle [illegible] Mn [illegible] dans [illegible], d'où [illegible] le cas le vrai manganisme [illegible].

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EFFECT OF MANGANESE COMPOUNDS ON THE FAT CONTENT IN THE LIVERS OF ANIMALS POISONED BY CARBON TETRACHLORIDE

O. I. Voynar and V. N. Galakhova

In an earlier study [1] we found that subcutaneous injections of manganous chloride promote the deposition of glycogen and reduces the content of total lipids and lipid phosphorus in the livers of white rats.

Injections of carbon tetrachloride are known to increase the content of triglycerides and phospholipids in the liver while suppressing the outflow of triglycerides from the liver to the plasma [2]. Accordingly, we thought it would be interesting to study the effect of manganese on the amount of fat and phospholipids in the livers of animals poisoned with carbon tetrachloride.

PROCEDURE

Experiments were performed on 4 groups of white rats. The first was the control (normal animals). In the other groups, fatty infiltration of the liver was induced by subcutaneous injections of carbon tetrachloride 4 or 5 days in succession (0.4 ml/100 g of body weight). A second group received only carbon tetrachloride and they were killed by decapitation on the 5th or 6th day from the start of the experiment.

A third group was injected subcutaneously with manganous chloride (0.7 mg/kg) for 15 days, after which the animals received a wound in the liver and were killed with an injection of carbon tetrachloride on the 5th or 6th day from the start of the experiment.

A fourth group of rats received carbon tetrachloride from the beginning of the experiment. The animals were divided into 2 subgroups. One received manganous chloride at the above dose for 15 days after they were poisoned with carbon tetrachloride. The other subgroup did not receive the substance. The animals of this group were killed on the 20th or 21st day from the start of the experiment.

A total of 45 animals were used in the experiment. All the animals received the usual mixed food. We determined the amount of total fat, lipid phosphorus, phospholipids, and moisture content in their livers.

Soxhlet's method was used to determine the total fat. Lipid phosphorus was determined in the sediment photocolorimetrically after evaporation of the fat solvent and combustion.

The resulting data were analyzed by the method of variational statistics. The standard deviation (m), significance (t), and probability of error (p) were determined.

RESULTS

The results of determining the total lipids, lipid phosphorus, and phospholipids in the livers of different groups of animals are given in the table. It is evident from the data that there was a sharp increase in the amount of fat, phosphorus of the lipid fraction, and phospholipids in the livers of rats poisoned with carbon tetrachloride (group 2) comparable to that in the livers of the control.

Content of Total Lipids and Lipid Phosphorus in the Livers of the Control Rats and Rats Poisoned with CCl_4 and the Quantitative Changes in These Substances After Subcutaneous Injection of Manganous Chloride

(Mean Data)

1 Визначувані показники	2 Групи тварин				
	3 контрольна (I)	4 отруєні CCl_4 (II)	5 попередньо одержували $MnCl_2$, отруєні CCl_4 (III)	6 отруєні CCl_4 (IV)	
				7 одержували $MnCl_2$	8 не одержували $MnCl_2$
9 Загальна кількість ліпідів на сиру вагу в %	4,8±0,17	13,0±0,99 $p<0,001$	11,6±0,98 $p<0,01$	4,1±0,6 $p<0,001$	7,0±1,16 $p<0,001$
10 Ліпідний фосфор, мг%	75,0±10,4	212±21,2 $p<0,001$	203±14,1 $p<0,5$	24,0±3,0 $p<0,001$	100±7,9 $p<0,001$
11 Вологість	70,4±0,76	67,4±1,2 $p<0,001$	67,4±1,2	71,8±1,0 $p<0,001$	71,0±1,1 $p<0,001$

1 - Indices determined
2 - Groups of animals
3 - control (1)
4 - poisoned with CCl_4 (II)
5 - previous given $MnCl_2$, poisoned with CCl_4 (III)
6 - poisoned with CCl_4 (IV)
7 - given $MnCl_2$
8 - not given $MnCl_2$
9 - Total lipids in wet weight, %
10 - Lipid phosphorus, mg%
11 - Moisture content

The rats given manganous chloride before being poisoned with carbon tetrachloride (group 3) likewise exhibited some increase in the amount of fat and phospholipids. The data show that previous administration of manganous chloride could not completely prevent fatty infiltration of the liver, but it helped to reduce the amount of fat deposited in the organ.

The effect of manganese after the animals were poisoned with carbon tetrachloride (group 4) was quite clear. In the livers of the poisoned rats and of those given manganous chloride, the amount of fat and phospholipids decreased almost 2-fold and 6-fold, respectively, compared to that in the poisoned animals not given manganese. A comparison of these data with the data obtained in the control reveals that in the rats given manganese before they were poisoned with carbon tetrachloride, the fat content even decreased a little below the normal level while the amount of phospholipids decreased by a factor of 3. At the same time in the poisoned rats not given manganese, the fat content remained almost one and one-half times higher; the amount of phospholipids also remained high. It is evident from our findings that manganese compounds increase the capacity of the liver to eliminate fat.

CONCLUSIONS

1. The content of total lipids, lipid phosphorus, and phospholipids increases sharply in the livers of white rats poisoned with carbon tetrachloride.

2. The animals given manganous chloride beforehand and then poisoned with carbon tetrachloride had less fat in the liver than did the rats who did not receive manganese before they were poisoned.

3. The administration of manganous chloride to rats after they were poisoned with carbon tetrachloride showed that manganese helps deposited fat to disappear quickly and markedly reduces the amount of both fat and phospholipids in the liver.

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УДК 577.170.49:599.71

ВПЛИВ СПОЛУК МАРГАНЦЮ НА ВМІСТ ЖИРУ В ПЕЧІНЦІ ТВАРИН, ОТРУЄНИХ ЧОТИРИХЛОРИСТИМ ВУГЛЕЦЕМ

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(Надійшла до редакції 16.XII 1961 р.)

В нашій попередній роботі [1] було встановлено, що підшкірні ін'єкції хлористого марганцю сприяють відкладанню глікогену і знижують вміст загальних ліпідів і ліпідного фосфору в печінці білих щурів.

Відомо, що введення чотирихлористого вуглецю призводить до підвищення вмісту тригліцеридів і фосфоліпідів у печінці і що поряд з цим чотирихлористий вуглець виявляє гальмуючий вплив на вихід тригліцеридів з печінки в плазму [2]. В зв'язку з цим становило інтерес вивчити вплив марганцю на зміну вмісту жиру і фосфоліпідів у печінці тварин при отруєнні їх чотирихлористим вуглецем.

Методика

Досліди проведено на чотирьох групах білих щурів. Перша з них була контрольною (нормальні тварини), у щурів решти груп викликали жирову інфільтрацію печінки шляхом введення під шкіру чотирихлористого вуглецю протягом 4—5 днів підряд (0.4 *мл* на 100 *г* ваги тіла). Тваринам другої групи вводили лише чотирихлористий вуглець і на п'ятий-шостий день від початку досліду їх забивали шляхом декапітації.

Тваринам третьої групи протягом 15 днів підшкірно вводили хлористий марганець (0.7 *мг/кг*), після чого у тварин викликали ураження печінки і на п'ятий-шостий день від початку введення чотирихлористого вуглецю їх умертвляли.

Щурам четвертої групи на початку досліду вводили чотирихлористий вуглець. Тварин ділили на дві підгрупи: одні після отруєння чотирихлористим вуглецем протягом 15 днів одержували хлористий марганець у вищезазначеній дозі, інші цієї речовини не одержували. Тварин четвертої групи умертвляли на 20—21-й день від початку досліду.

Всього під дослідом перебувало 45 тварин. Всі тварини одержували звичайний змішаний корм. В їхній печінці ми визначали кількість загального жиру, ліпідного фосфору, фосфоліпідів та вологість.

Визначення загального жиру проводили за методом Сокслета. Фосфор ліпідів визначали в осаді фотоколориметричним методом після випаровування жиророзчинника і спалювання.

Одержані дані обробляли методом варіаційної статистики. Встановлювали середню квадратичну помилку результату (m), достовірність (t), імовірність помилки (p).

Результати дослідів

Результати визначення загальних ліпідів, ліпідного фосфору і фосфоліпідів у печінці різних груп тварин наведені в таблиці. З наведених у ній даних видно, що в печінці щурів, отруєних чотирихлористим вуглецем (друга група тварин), різко підвищується вміст жиру, фосфору ліпідної фракції і фосфоліпідів порівняно до їх вмісту в печінці контрольних тварин.

Вміст загальних ліпідів і ліпідного фосфору в печінці контрольних і отруєних CCl_4 щурів та кількісні зміни цих речовин при підшкірному введенні хлористого марганцю (середні дані)

Визначувані показники	Групи тварин				
	контрольна (I)	отруєні CCl_4 (II)	попередньо одержували $MnCl_2$, отруєні CCl_4 (III)	отруєні CCl_4 (IV)	
				одержували $MnCl_2$	не одержували $MnCl_2$
Загальна кількість ліпідів на сиру вагу в %	4,8±0,17	13,6±0,99 $p<0,001$	11,6±0,98 $p<0,01$	4,1±0,6 $p<0,001$	7,0±1,16 $p<0,001$
Ліпідний фосфор, мг%	75,0±10,4	212±21,2 $p<0,001$	203±11,1 $p<0,5$	91,0±3,0 $p<0,001$	103±7,9 $p<0,001$
Вологість	70,4±0,76	67,4±1,2 $p<0,001$	67,4±1,2	71,8±1,0 $p<0,001$	71,0±1,1 $p<0,001$

У щурів, яким вводили хлористий марганець до отруєння чотирихлористим вуглецем (третя група тварин), також спостерігається деяке підвищення вмісту жиру і фосфоліпідів. Ці дані вказують на те, що за допомогою попереднього введення хлористого марганцю повністю запобігти розвитку ожиріння печінки не вдається, проте марганець, введений до отруєння, певною мірою сприяє зменшенню відкладання жиру в печінці.

Досить виразною виявилась дія марганцю при введенні його тваринам після отруєння чотирихлористим вуглецем (четверта група тварин). В печінці отруєних щурів і тих, що одержували хлористий марганець, кількість жиру знижувалась майже вдвічі, кількість фосфоліпідів — у чотири рази порівняно до отруєних тварин, які не одержували марганцю. Зіставляючи ці дані і дані, одержані на контрольній групі тварин, можна бачити, що у щурів, які одержували марганець до отруєння чотирихлористим вуглецем, вміст жиру проти норми навіть дещо знижується, а кількість фосфоліпідів зменшується в три рази. Поряд з цим у отруєних щурів, які не одержували марганцю, кількість жиру залишається підвищеною майже в півтора раза, підвищеним залишається і вміст фосфоліпідів. З одержаних даних випливає, що сполуки марганцю підвищують здатність печінки звільнятися від жиру.

Висновки

1. У печінці білих щурів, отруєних чотирихлористим вуглецем, різко збільшується вміст загальних ліпідів, ліпідного фосфору та фосфоліпідів.

2. У тварин, які попередньо одержували хлористий марганець і потім були отруєні чотирихлористим вуглецем, знайдено меншу кількість жиру в печінці, ніж у тварин, які до отруєння марганцю не одержували.

3. Введення хлористого марганцю тваринам після отруєння їх чотирихлористим вуглецем показало, що марганець сприяє швидшому зникненню відкладеного жиру, значно знижує його вміст і зменшує кількість фосфоліпідів у печінці.

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ВЛИЯНИЕ СОЕДИНЕНИЙ МАРГАНЦА НА СОДЕРЖАНИЕ ЖИРА В ПЕЧЕНИ ЖИВОТНЫХ, ОТРАВЛЕННЫХ ЧЕТЫРЕХХЛОРИСТЫМ УГЛЕРОДОМ

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Резюме

Исследовалось влияние марганца на изменение содержания общих липидов и фосфолипидов в печени животных при отравлении их четыреххлористым углеродом.

В печени белых крыс, получавших четыреххлористый углерод, отмечается резкое увеличение содержания общих липидов и липидного фосфора. У животных предварительно получавших хлористый марганец и затем отравленных четыреххлористым углеродом, найдено меньшее количество жира в печени, чем у животных, не получавших марганца до отравления. Введение хлористого марганца животным после отравления их четыреххлористым углеродом показало, что марганец способствует более быстрому исчезновению отложений жира, значительно снижает его содержание и уменьшает количество фосфолипидов в печени.

EFFECT OF MANGANESE COMPOUNDS ON THE FAT CONTENT IN THE LIVER OF ANIMALS INTOXICATED WITH CARBON TETRACHLORIDE

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Summary

The authors investigated the effect of manganese on the change in the content of total and phospholipids in the liver of animals on intoxication with carbon tetrachloride.

An increase in the quantity of total lipids and lipid phosphorus is observed in the liver of albino rats receiving carbon tetrachloride. In animals receiving manganese chloride before being poisoned with carbon tetrachloride a smaller quantity of fat was found in the liver than in rats that did not receive manganese before intoxication. Administration of manganese chloride to animals after intoxication with carbon tetrachloride showed that manganese causes a more rapid vanishing of adipose layers, considerably reduces the fat content and that of phospholipids in the liver.

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J Biol Chem

A NEW METHOD FOR THE MICRODETERMINATION OF MANGANESE IN BIOLOGICAL MATERIALS*

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(Received for publication, August 15, 1939)

In our studies on perosis in chicks, it was found desirable to be able to determine small amounts of manganese of the order of 0.1 to 10 micrograms. Such a method was necessary for the determination of manganese in bones, blood, and similar materials. The formaldoxime method of Sideris (1) was found unsuitable, as the determination is carried out in alkaline solution. Under these conditions phosphates precipitate, causing a masking and adsorption of the color. The standard method in which the manganese is oxidized to permanganate and the color thus developed compared with a standard was not sufficiently sensitive. The smallest amount detectable with the use of an Evelyn photoelectric colorimeter and a 5200 Å. filter was 25 micrograms in 25 cc.

In 1913 Dietz (2) and later Feigl (3) and Olszewski (4) described a very sensitive qualitative test for manganese. In alkaline solution manganese dioxide and the permanganate ion oxidized benzidine to give a blue color. With this test it was possible to detect 1 part of manganese in 250,000,000. Stratton and coworkers (5) in 1932 and Clark in 1933 (6) suggested the use of benzidine for the quantitative determination of permanganate. However, the blue color obtained was found to fade in 2 minutes, thus seriously limiting the method.

Fundamentally, the method depends on the oxidation of benzidine. In dilute solutions of oxidizing agents the meriquinoidal

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type of compound (7) is formed, which is green in acid and blue in [illegible] solution (8). When a neutral solution of dilute permanganate was added to a solution of purified benzidine in 5 per cent acetic acid, a deep blue color developed. Attempts to evaluate the color by means of the Duboscq photoelectric colorimeter and a [illegible] (9) were not with fullness, as it was found that the color developed started to fade immediately on formation. Thus accurate estimations of the small amounts of manganese present were impossible.

TABLE I

Stability of Color for Various Amounts of Manganese

Time	Manganese determination		
	1 milligram	0.5 milligrams	0.25 milligrams
min.			
1	73^1	49^1	16^3
2	73^2	$[illegible]^1$	11
3	74^1	33^1	12^2
4	74^1	33	12^1
5	74^1	32^1	12
6	74^1	32^1	12
7	74^1	32^1	12
8	74^1	32^1	12
9	74^1	32^1	12
10	75	32^1	12
11	75	32^1	12^1
12	76	33	12^1
13		33	12^2
14		33^1	12^2
15			12^2

However, when solutions of benzidine were added to permanganate solutions made strongly acid with nitric acid, a yellow-green color instead of the blue was obtained. In tests of this color with the photoelectric colorimeter, with a filter A, filter, it was found to be a great deal more stable. Table I shows the values obtained and the color developed with various amounts of [illegible] and the color developed with [illegible] amounts of manganese. From these data it is seen that the maximum color [illegible] for 5 [illegible] of manganese [illegible] for 5

It was decided to carry out the determination in nitric acid solution. Sulfuric acid could not be used, as sulfate forms a precipitate with barium, and hydrochloric acid was also undesirable, as it reduces permanganate ion and yields free chlorine in the presence of oxidizing agents.

As other oxidizing agents give the same color with benzidine (2), this reaction is not specific for permanganate. In the determination manganese ion must be oxidized to permanganate ion and the reagent chosen for this oxidation must be one which will not leave any excess oxidizing agent to react in contact with the benzidine. For this purpose sodium bismuthate was selected, as it readily decomposes in acid solution, leaving insoluble bismuth oxide, which can be easily filtered off.

The following metals, namely, cobalt, nickel, thallium, and silver, each in an amount of 5 milligrams, have been found by Feigl (3) to give the same blue color with benzidine. When salts of these metals were used in our regular procedure, no color was obtained. Ferric iron (3), and copper were also found not to interfere with the determination.

Moreover, chlorides (and other halides) do interfere by the formation of free chlorine (5) on treatment with the oxidizing agent and hence it is necessary to remove them from the sample. Silver nitrate was used, but here again trouble was encountered. The excess silver ion reacts with sodium bismuthate to form an unstable compound, silver bismuthate which is soluble in dilute nitric acid solution (6). This allowed some oxidizing agent to pass through the filter and react with the benzidine, thus yielding high results. We have also observed a similar effect with cobalt. However, with concentrations of cobalt up to the order of 20 times that which would ordinarily be found in biological material no interference was encountered. It is necessary, therefore, to remove the chloride by adding three or four drops of nitric acid and evaporating to dryness on the hot plate, care being used to prevent spattering. Two to three evaporations are sufficient to free the sample from chlorides.

The nitric acid used must be free of oxides of nitrogen, as these [illegible] the reagent, [illegible] blue. These oxides are [illegible] by treatment with nitric acid [illegible] with some persistent

Another difficulty encountered was the fading of the permanganate after filtering off the sodium bismuthate and before adding the benzidine. It was found simpler to filter directly into the colorimeter tubes containing the benzidine solution, and thus no loss occurred owing to fading of permanganate.

Procedure

Reagents—

Redistilled water throughout.

Benzidine (p-diaminobiphenyl). A 1 per cent solution in 5 per cent acetic acid, stored in a dark bottle. The benzidine was purified (10) as follows: "20 gm. of benzidine were dissolved in 150 cc. of 70 per cent alcohol (7 volumes of 95 per cent alcohol plus 3 volumes of water) at about 70°, allowed to crystallize in the refrigerator, filtered with suction, and washed with a little cold 50 per cent alcohol. The crystals were redissolved in about 135 cc. of 70 per cent alcohol." 1 gm. of carbon was added and after further heating, filtered hot on a hot water funnel through paper previously washed with some hot alcohol. "Finally the flask and filter were washed with about 30 cc. of warm 50 per cent alcohol. The filtrate was cooled in the refrigerator, and the benzidine filtered with suction, and dried in a dark place." The air-dried substance contained water and traces of carbon which were removed by recrystallization from dry benzene. The product was stored in a bottle of brown glass.

Nitric acid (concentrated) free from all traces of oxides of nitrogen.

Sodium bismuthate, $NaBiO_3$.

Shredded asbestos, purified by boiling with HNO_3 and washing thoroughly with redistilled water.

Standard manganese solution containing 1 microgram per cc., made by proper dilution of a solution containing 0.1 mg. per cc.

Method

A sample containing approximately 1 to 10 micrograms of manganese is weighed out and ashed in a well extracted porcelain dish at cherry-red heat. The ash is taken up in 3 to 5 cc. of concentrated HNO_3 and 10 cc. of water. The mixture is heated to boiling to permit [illegible] of the ash and transferred [illegible] to a 100 cc. beaker. It is then evaporated to dryness on a hot-plate, again taken up in 3 to 5 cc. of concentrated HNO_3 and 10 cc. of water, and again evaporated to dryness. This procedure is carried out a third time. The sample, which should now be free of chlorides, is taken up in 3 cc. of concentrated HNO_3 and 10 cc. of water and warmed slightly to bring into solution. Allow to cool and add approximately 0.2 gm. of sodium bismuthate; heat and allow to boil for 2 to 3 minutes. Cool to below [illegible] (11), add 0.2 to 0.5 gm. of sodium bismuthate, mix thoroughly, and let stand a few minutes to allow the excess sodium bismuthate to decompose. The excess sodium bismuthate is filtered off through a Gooch crucible. The Gooch crucible and suction flask are so arranged that the solution filters directly into the colorimeter tube containing 2 drops of the benzidine solution in 3 cc. of redistilled water. The solution is made up to a volume of 25 cc. (the colorimeter tube having been previously calibrated to this volume) and thoroughly mixed. A yellow-green color develops which is read at the end of 5 minutes on the Evelyn photoelectric colorimeter with a 4200 Å. filter. Blanks prepared by using the same amounts of nitric acid and sodium bismuthate should be water-white.

It has been found possible to perform six to eight filtrations through the same filter and then read the colors developed on the colorimeter. In this way thirty to forty determinations can be read in 1 hour.

Results

Known solutions of manganese were analyzed; the amounts of manganese found are given in Table II. The best results are obtained when the sample contains from 1 to 10 micrograms of manganese.

Several samples of food mixtures were analyzed by this procedure and that described by Skinner and Peterson (12). The results obtained are as follows:

Sample No.	Method	No. of determinations	Mn found per gm.
			micrograms
R[illegible]	Periodate	2	3.85
R[illegible]	Benzidine	6	3.35 (3.2–3.6)
R[illegible]	Periodate	2	[illegible]
R[illegible]	Benzidine	3	3.02

TABLE II

Analysis of Known Solutions for Manganese Content

Mn taken	Mn found
micrograms	*micrograms*
0.1	0.13
0.25	0.21
0.5	0.61
1.0	1.2
2.0	2.0
4.0	4.1
5.0	5.1
7.0	7.0
10.0	9.65
12.0	12.3
15.0	15.4

TABLE III

Recoveries Obtained with Added Manganese

Sample	Weight of sample	Mn in sample	Mn added	Mn recovered	Added Mn recovered
	gm.	*micrograms*	*micrograms*	*micrograms*	*per cent*
604	5	30.7	20	20.1	101
604	5	38.0	20	19.5	97.5
610	5	33.8	20	20.3	102
610	1	7.11	3	3.2	106
$CaCO_3$	1	112.0	50	49	98
$Ca_3(PO_4)_2$	0.5	23.5	5	5	100
NaCl	5	8.9	5	4.6	92
Calcium lactate	0.5	10.7	5	4.65	93

TABLE IV

Analysis of Bone for Manganese Content

Ration	Weight of bone	Mn found	Mn per gm. bone
	gm.	*micrograms*	*micrograms*
Low Mn	2.40	5.62	2.34
" "	1.65	3.15	1.92
" "	0.54	0.76	1.42
" "	1.50	2.94	1.96
" "	1.42	3.34	2.35
" "	1.52	3.63	2.39
Mn added	0.97	3.63	3.74
" "	1.88	10.70	5.70
" "	1.68	6.79	4.04
" "	1.42	8.94	6.31
" "	1.86	8.45	4.54
" "	2.07	7.46	3.60

Known amounts of manganese were added to the various samples [illegible] obtained are shown in Table III.

In [illegible] samples of chickens were analyzed for their manganese content. One group received a low manganese ration; the other the same ration plus 50 parts per million of added manganese. The results obtained are shown in Table IV.

Preliminary determinations were made on the blood of chickens receiving the same rations as above, the results of which are as follows:

Ration	Weight of blood	Mn found	Mn per 100 gm. blood
	gm.	*micrograms*	*micrograms*
Low Mn	44	3.4	8.00
Added Mn	37	5.95	16.00

In all our work the Evelyn photoelectric colorimeter was used. Since some laboratories may not be equipped with this instrument, the applicability of the method was studied with the Dubosq colorimeter. It was found possible to determine between 2 and 15 micrograms of manganese with this instrument. This procedure is neither as sensitive nor as rapid as with the use of the Evelyn photoelectric colorimeter.

SUMMARY

A method for the determination of 0.1 to 10 micrograms of manganese with the Evelyn photoelectric colorimeter has been described. Recoveries ranging from 92 to 106 per cent have been obtained.

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TABLE III.—ANALYSIS OF BADDELEYITE.

[illegible]	SiO_2 found, %	[illegible]	[illegible] found, %	[illegible] found, % (colorimetric).	Al_2O_3 found, %	Fe_2O_3 found, %	H_2O found, % (colorimetric).	Total found, %
[illegible]	15.85	[illegible]	71.[illegible]6	0.78	5.[illegible]	0.63	2.[illegible]	99.54
[illegible]	15.73	2.[illegible]	71.18	0.66	5.76	0.92	2.[illegible]	99.61
[illegible]	15.46	2.16	71.23	0.75	5.82	1.12	2.64	99.98
[illegible]	15.72	2.44	71.35	0.75	5.46	0.75	2.94	99.41

Summary.

The above results show that the cupferron reagent may be used in acid solution for the quantitative precipitation of iron, titanium, and zirconium from solutions containing these elements mixed with aluminum and manganese. The first three of these elements may be separated by standard methods, as by the procedures outlined above. The aluminum and manganese may be determined by standard methods after the removal of the iron by cupferron.

Analyses were made both of mixed solutions of pure salts and of minerals, with accurate results.

The addition is due R. B. Moore, of the United States Bureau of Mines, for suggesting the analysis of zirconium minerals as an inviting field for investigation.

[illegible], Ind.

[FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF MICHIGAN.]

DETERMINATION OF MANGANESE BY OXIDATION.[1]

BY HOWARD H. WILLARD AND LUCIEN H. GREATHOUSE.

Received July 27, 1917.

Historical.

The determination of manganese by comparing the color intensities of its permanganate solution has been a practical method for the rapid [illegible] for about forty-five years. W. Crum[2] first [illegible] the oxidation of manganous salts by means of lead dioxide. In dilute nitric solution, [illegible] the first [illegible] for a sample to method. [illegible] proposed [illegible] following [illegible] the same [illegible] manganese [illegible] 4, 8, 12, 13 in the table, [illegible]) but [illegible] Lunge.

[illegible] of the American Chemical Society in Cincinnati, April, [illegible]

[illegible] (1[illegible]).

[illegible] has illustrated the last variation that was destined to be widely used. He precipitated lead chloride by an alkali persulfate, using silver ions as a catalyzer. While [illegible] eliminated from the procedure the filtration [illegible] the [illegible]. It had the further advantage that the [illegible] could easily [illegible] for the manganese.

The persulfate method has long been recognized as being erratic; the true permanganate color is not always obtained and oxidation is often incomplete. The color frequently fades after a short time. Fairly close control of the time of heating and of the concentration of acid and of manganese is necessary to obtain accurate results and to prevent the formation of manganese dioxide. Traces of chloride give an opalescence of silver chloride which must be coagulated and filtered off before comparison can be made. In spite of these disadvantages the method is widely used and the Chemists' Committee of the U. S. Steel Corporation[3] included it among the standard methods for iron and steel analysis.

Another oxidizing agent which has been much used and which is more reliable than persulfate is sodium bismuthate. Its use for this purpose was first proposed by Blundstone and Rumage[4] in 1895, six years before the permanganate reaction was known, but it seems to have been unnoticed or forgotten until Dietz[5] in 1901 again called attention to it. The excess of this reagent must, however, be separated before comparison.

A complete bibliography of the literature on the colorimetric determination of manganese is given at the end of this article.

The New Method.

The method described in the following pages is free from all the faults of previous methods and is based upon an entirely new reaction—the oxidation in acid solution of manganous salts to permanganate by means of periodic acid or its salts; the latter are reduced to the form of iodate. The reaction is represented by the following equation:

$$2Mn(NO_3)_2 + 5KIO_4 + 3H_2O = 2HMnO_4 + 5KIO_3 + 4HNO_3$$

Only a small excess of periodate is required, but the success of the reaction depends upon the presence of a sufficient concentration of acid, otherwise precipitation occurs, and the [illegible] varies widely according to conditions. This accounts for the conflicting results reported by previous investigators. The minimum concentration of acid required increases with the concentration of manganese.

[1] *Chem. News*, 83, 71 (1901).

[2] [illegible] and Rose, *J. Soc. Chem. Ind.*, 4, 502; [illegible] *Chem. Analysis*, [illegible] 17, 22.

[3] *J. Ind. Eng. Chem.*, 4, 807 (1912).

[4] *J. Chem. Soc.*, 67, 172 (1895), [illegible].

[5] *Chem. News*, 84, 258 (1901).

[illegible] stated that periodic acid was reduced to iodic acid by man-[illegible] salts and that a precipitate of manganic iodate was formed.

[illegible] found that a precipitate of hydrated manganese dioxide [illegible] in neutral solution.

[illegible] Price[illegible] and analyzed several brick-red precipitates formed [illegible] nitric or sulfuric acid solution. From his results he calculated formulas for complex sodium, potassium and possibly hydrogen, man-[illegible] (trivalent) periodates.

[illegible] Benedict[1] suggested the use of potassium periodate in neutral or [illegible] solution as a reagent for the detection of manganese. The [illegible] already described by Price[2] served to indicate manganese [illegible] present in considerable amounts, while in very dilute solutions of [illegible] a red coloration appeared, which Benedict designated as "[illegible] characteristic color of manganese periodate," although he mentioned no [illegible] in support of this view.

[illegible] studying the reactions between periodate and salts of manganese the qualitative effects described earlier were, in general, found to be [illegible]. However, the coloration produced in solutions of low man-[illegible] content, ascribed by Benedict to a compound containing iodine, [illegible] to be due solely to the permanganate ion, and this reaction always [illegible] if sufficient acid is present, even with considerable amounts of [illegible].

[illegible] of this reaction led to the idea that periodate might, with [illegible], be substituted for persulfate in the colorimetric determination [illegible] manganese. To test the method the effects of temperature and of [illegible] concentrations of sulfuric, nitric and phosphoric acids, of ammo-[illegible] chloride, manganese and periodate were observed by com-[illegible] solutions containing only chemically pure substances [illegible] known amounts. These experiments demonstrated the reliability [illegible]. It was then applied to steels and iron ores, since stand-[illegible] samples of these materials were available. The results in all [illegible] were satisfactory. Attempts to utilize this reaction in a [illegible] manganese, similar to the persulfate-arsenite [illegible], since the excess of the reagent could not be re-[illegible] [illegible] to titrate the permanganate [illegible].

Experimental.

[illegible] and kind of [illegible] used, the figures represent-[illegible] of [illegible] having the same intensity of [illegible].

[illegible] (1909).

[illegible] 1909, 123 (1909).

[illegible] (1909).

standard permanganate with sulfur dioxide, expelling the excess by boiling, and, after cooling, diluting to the proper volume. The permanganate solution had been prepared by dissolving the "analyzed" salt of a well-known manufacturer in water which had been distilled from alkaline permanganate and redistilled. This solution, which was very stable, had been standardized against samples of sodium oxalate and iron ore of known iron content obtained from the Bureau of Standards, both methods showing excellent agreement.

The acids used—sulfuric, nitric, hydrochloric and phosphoric—were chemically pure products; ammonium sulfate and persulfate were the purest grade furnished by a well-known firm. All reagents were carefully tested for manganese and found to be free from it.

The periodate used was the potassium salt, KIO_4, furnished by a European manufacturer. This material showed by analysis the theoretical [illegible] of KIO_4 and qualitative tests indicated no impurities except a trace of sodium. The potassium salt is the only form in which this oxidizing agent is ordinarily sold by dealers. Sodium metaperiodate, $NaIO_4$, would, however, be more serviceable as a reagent on account of its greater solubility. Moreover, the latter can be produced more cheaply under [illegible] conditions and possibly at all times.

The Campbell-Hurley colorimeter[1] was used throughout this work.

The first comparisons made were between a solution containing pure potassium permanganate and those containing the same amount of manganese as sulfate, oxidized in one case by persulfate and in the other by periodate. The two latter solutions were also compared with each other. They were prepared as follows:

Pure permanganate: 4.30 cc. of 0.10428 N solution (containing 5 mg. of manganese) diluted to 250 cc.

Persulfate oxidation: 50 cc. manganese sulfate solution (containing 5 mg. of manganese), 15 cc. sulfuric acid (sp. gr. 1.84), 0.2 g. silver nitrate and [illegible] ammonium persulfate, heated to boiling, cooled at once and diluted to 250 cc.

Periodate oxidation: 50 cc. manganese sulfate solution, 10 cc. sulfuric acid, and 0.5 g. potassium periodate, boiled [illegible] minutes, cooled and diluted to [illegible] cc.

[illegible] were [illegible], the figures representing [illegible] having the same intensity of [illegible].

Table I.

[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	...
[illegible]	...	[illegible]
...	[illegible]	[illegible]

[1] Campbell and Hurley, This J., [illegible], [illegible] (1911).

The [illegible]lution oxidized by periodate had exactly the same color as pure [illegible]ganate, but the one oxidized by persulfate differed slightly in tint.[1] [illegible], however, the variation of 1.5 to 2 scale divisions in the readings [illegible] solution oxidized by persulfate is larger than the experimental error [illegible] question. [illegible] the periodate oxidized the manganese completely under these conditions.

[illegible] of Varying the Concentration of Acid.—The concentration of sulfur[illegible] acid was varied as indicated in Table II. The solution during oxid[illegible] had a volume of 50 cc. and contained 2.5 mg. of manganese as [illegible]. Each solution, after adding periodate, was boiled one minute, [illegible] on the sand bath for fifteen minutes, after which it was cooled [illegible] to 250 cc. The time required for the appearance of the full [illegible] color was noted. The solution containing 30 cc. of sulfuric acid per [illegible] cc. was taken as the standard and compared with each of the [illegible].

Table II.

Concentration of H_2SO_4 varied.

2.5 mg. Mn. Volume during oxidation, 50 cc.; 0.25 g. KIO_4 added.

Cc. conc. H_2SO_4 per 100 cc. solution during oxidation	5	10	15	20	25	30
[illegible] to full color, minutes	12	10	8	6	4	3
[illegible] reading	91	89	89	90	89	90

[illegible] allowed to stand 1½ hours at room temperature, gave [illegible] this reaction did not proceed further in 4 hours, [illegible] precipitate being thrown down instead.

Table III.

Concentration of HNO_3 varied.

Volume during oxidation, 100 cc.; final volume 250 cc.; 0.3 g. KIO_4 added. [illegible] 10 min. Standard contained 10 cc. H_2SO_4 but no HNO_3; oxidized in the same way.

[illegible] (g.) per 100 cc. solution					
[illegible]	Standard	2.6	16.5	33.7	50
[illegible] to full color, minutes	2	Precipitate formed	6	6	3
[illegible] reading	90	...	89	90	90

Table IV.

[illegible]

[illegible]

[illegible] with the concentration of manganese, and varies with different acids, [illegible] lowest for phosphoric acid. The well-known stability of manganic phosphate probably accounts for the non-appearance of precipitate [illegible] in solutions of very [illegible].

Effect of Varying the Concentration of Periodate.—In the following table the theoretical requirement is 0.052 g. Complete oxidation was obtained in every case, even with only 0.1 g. KIO_4, the lower readings corresponding to a greater depth of color. The variation represents the experimental error. The only effect observed was a slight increase in the speed of the reaction as the concentration of periodate was increased.

Table V.

5 mg. Mn, 10 cc. conc. H_2SO_4, volume during oxidation 100 cc. Heated 10 min. Full color developed in 3 or 4 min.

KIO_4 [illegible], grams	Standard 0.3	0.1	0.2	0.4	0.5	0.6
Colorimeter reading	90	88	88	89	90	90

Oxidation of Large Amounts of Manganese.—Calculated volumes of tenth normal permanganate were reduced with sulfur dioxide, boiled to remove the excess, and diluted to 100 cc. for oxidation. Afterwards they were diluted to the final volumes noted for comparison. The standard [illegible] before, containing 20 mg. of manganese per liter.

Table VI.

10 cc. H_3PO_4 (sp. gr. [illegible]) in 100 cc. of solution during oxidation.

[illegible]	KIO_4 G.	Time to full color. Min.		Diluted to final volume. Cc.	Reading. Solution.	Standard.
20	1		1–2	1000	90	90
60	2	apparently	instantaneous	2000	50	74.5

The complete oxidation of 0.06 g. of manganese in 100 cc. of solution, such as occurred above, cannot be accomplished by any of the other methods. Even larger amounts of manganese can be oxidized, but such solutions are so deeply colored as to be of no practical value.

[illegible] of Chlorides.—Since periodic acid [illegible] oxidizes hydrochloric acid [illegible] according to the equation

$$[illegible]HCl + [illegible] = Cl_2 + [illegible] + H_2O$$

[illegible] amounts of chloride ought to be possible if a [illegible] is added. That this is true is evident from the results given in Table [illegible].

[illegible] solutions were heated until the odor of chlorine disappeared.

[illegible] be removed is one of the [illegible] advantages of [illegible].

TABLE VII.

[illegible] 10 cc. [illegible] H_2SO_4 in 100 cc. of solution during oxidation. Diluted to 250 cc. for comparison.

[illegible] added. Cc.	KIO_4 G.	Time to full color. Min.	Time of heating.	Readings. Solution.	Readings. Standard.
0	0.3	2	15 min.	Standard	..
0.2	0.3	2	15 min.	88	90
0.5	0.3	2	15 min.	89	90
[illegible]	0.3	3	15 min.	87	90
17	0.5	precipitate	1½ hrs.	..	..
17	0.7	3	1¼ hrs.	88	90
30	2.0	3	2 hrs.	89.5	90

Effect of Ammonium Salts.—Although it hardly seemed possible that ammonium salts could interfere, the following experiments were made to [illegible] point: Solutions containing 5 mg. of manganese, 10 cc. of concentrated sulfuric acid, 0.3 g. of periodate, and amounts of ammonium sulfate varying from 5 to 35 g. in a volume of 100 cc. were heated for 20 minutes, cooled, diluted and compared with a standard containing no ammonium salt. In each case the readings showed a ratio of 90 : 89. The presence of the ammonium salt did not affect the intensity of the color of the solution or the speed of oxidation.

A [illegible] feature of the solutions oxidized by periodate is their [illegible] when a slight excess of the reagent is present. Such a solution [illegible] in a stoppered flask, when compared with a similar solution without excess, showed no change whatever. This makes it [illegible] the standard solution in the colorimeter, renewing it [illegible] instead of preparing a fresh solution each time, as is [illegible] permanganate is used.

[illegible] metals do not interfere in this method except insofar as [illegible] impart a color to the solution. The color due to ferric [illegible] removed by the addition of phosphoric acid, but to correct [illegible] due to other metals the same amount must be added to the [illegible] such as silver, lead, bismuth and mercury, [illegible] which are insoluble in dilute acids, but by using [illegible] of acid [illegible] remain in solution, since the amount of [illegible] is small.

[illegible] such as a ferrous salt is present the periodate [illegible] will color the solution and render it [illegible] kind are removed by boiling or evaporating [illegible].

[illegible] having thus been determined, the method was [illegible] by applying it to one of the most common analytical [illegible] of manganese in iron ore and steel. In [illegible] to the latter the silica was not removed, but the

proper correction was made by adding to the standard the same amount of iron in the form of ferric nitrate. This solution was prepared by dissolving [illegible] g. of electrolytic iron in nitric acid, evaporating nearly to dryness and diluting to one liter. It contained 1 g. of iron in 50 cc. and by colorimetric determination [illegible] mg. of manganese in the same volume. In comparing solutions of the various materials examined, after dilution to suitable volumes, the following standards were used:

TABLE VIII.

Mn added as $MnSO_4$. Fe as $Fe(NO_3)_3$.

Standard.	KIO_4 added. G.	Mn. Mg.	Fe. G.	Conc. H_2SO_4. Cc.	Conc. HNO_3. Cc.	Conc. $HClO_4$. Cc.	Final volume. Cc.
a	0.3	10	1	20	20	..	500
b	0.3	10	2	20	20	..	500
c	0.6	10	0.5	20	20	..	500
d	0.6	10	0	..	..	20	500
e	0.3	5	0	..	..	20	500
f	0.3	5	0	..	..	40	1000

In the series of determinations given in Table IX, samples of iron and steel from the U. S. Bureau of Standards were dissolved in dilute nitric acid containing, for 0.5 g. of metal, 15 cc. of concentrated acid; and for 1 g., 15 cc. Then cc. amounts of sulfuric acid and the amounts of KIO_4 indicated were then added to the solution, having a volume of about 100 cc., then boiled for a few minutes, cooled, diluted to 250 cc. and compared with the standard containing the same amount of iron.

TABLE IX.

No phosphoric acid added; same amount of iron in standard.

Steel No.	Description of material.	Wt. of sample. G.	KIO_4 added. G.	C present. G.	Mn present. %	Mn found. %
1	[illegible] Open Hearth 1% C. Sample 16	1.0000	0.5	1.05	0.405	0.403
2	[illegible] Open Hearth 0.1 C. Sample 18	1.0000	0.3	0.10	0.412	0.409
3	[illegible] 0.3 C. Sample 23	0.5000	0.5	0.81	0.775	0.771
4	[illegible] Hearth 0.2 C. Sample 19	0.5000	0.3	0.21	0.760	0.752
5	[illegible] 0.2 C. Sample [illegible]	0.5000	0.3	0.25	0.918	0.913
6	[illegible] 0.4 C. Sample [illegible]	0.5000	0.3	0.45	0.916	0.902
7	[illegible] Sample 6	0.5000	0.3	2.89	1.41	1.407
8	[illegible] Association [illegible]	1.0000	0.5	3.11	0.415	0.404

In the series of determinations given in Table X, the same steels were used as in Table IX and analyzed by the method only. They were dissolved in dilute HNO[illegible] as before, then 5 or 10 cc. of phosphoric acid (sp. gr. [illegible]) and [illegible] were added and the solution oxidized as before. The addition of phosphoric acid formed a colorless complex ferric iron, and the [illegible] solutions diluted to 250 cc. were compared with standard [illegible] with no iron had been added.

TABLE X.

Phosphoric acid added; no iron in standard.

Metal No.	Weight of sample. G.	KIO_4 added. G.	Mn present. %	Mn found. %
1	1.0000	0.5	0.405	0.402
2	1.0000	0.3	0.412	0.412
3	0.5000	0.5	0.775	0.770
4	0.5000	0.3	0.760	0.755
5	0.5000	0.3	0.918	0.905
6	0.5000	0.3	0.916	0.905

The solutions differed only slightly in tint from the pure permanganate standard used, and such comparisons are readily made after a little practice. When more than 1 g. of iron is present its color becomes approximate, but if the standard contains no phosphoric acid, the addition to it of 1% of the weight of the iron in the sample gives sufficient color to compare for this.

Occasionally difficulty was caused by variation in tint both with sulfuric and phosphoric acid solutions, arising from the brownish color of the carbon compounds after solution in nitric acid. This effect was especially noticeable where the carbon content was 0.8% or more. Since even a large excess of periodic acid oxidizes these compounds with difficulty, some means for distinguishing them before the periodate was added.

Three methods were found: (a) The sample was dissolved in nitric acid and then boiled in contact of solution with 10 cc. of sulfuric acid. (b) It was also found advantageous in a mixture of 10 cc. nitric and phosphoric acid, 10 cc. water and boiled until decolorized, the solution evaporated during this treatment. A little care was required to avoid drying further to a gummy mass that would be difficult to dissolve. The concentrated phosphoric acid solution was simply diluted, carefully due to incipient phosphorite. (c) The sample was dissolved in a mixture of 15 cc. nitric acid, 15 cc. water and 5 cc. sulfuric acid, 1 g. of ammonium persulfate was added and the solution boiled by heating 3 to 5 minutes. Finally, sulfuric acid was added to fumes. This method was found to be the most satisfactory for removing carbon compounds. The results are given in Table XI.

TABLE XI.

Description of ore.	Weight of sample. G.	Standard used.	Mn present. %	Mn found. %
[illegible]	[illegible]	d	[illegible]	[illegible]
[illegible]	0.5000	d	0.775	0.775
[illegible]	[illegible]	[illegible]	0.775	0.778
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	d	0.405	0.410

Finally, two determinations were made using iron ores of known manganese content. 1 g. samples were dissolved in platinum dishes with 15 cc. phosphoric acid, 5 cc. hydrofluoric acid and a little nitric acid and heated until all fumed, and had been volatilized. The resultant mass was dissolved in water, oxidized with 0.5 g. KIO_4 and diluted to 250 cc. The usual method of dissolving the ore in hydrochloric acid could have been used equally well. To expel all chloride and to oxidize ferrous salts it is necessary to add 10 cc. of nitric acid and 15 cc. of sulfuric acid and evaporate the solution to fumes of the latter. The results are given in Table XII.

TABLE XII.

Description of ore.	Weight of sample. G.	Standard used.	Mn present. %	Mn found. %
U. S. B. S. No. 28, Norris Ore	1.0000	d	0.465	0.460
U. S. B. S. No. 29, Magnetite	1.0000	f	0.07	0.068

In addition some twenty iron ores of known manganese content used as "unknowns" for students, were analyzed by the above procedure. All the common types of iron ores were represented in this series. The results obtained always agreed well with the values given by other methods.

General Procedure.

The material to be analyzed is brought into a solution containing in 100 cc. at least 10–15 cc. concentrated sulfuric, 20 cc. of nitric or 5–10 cc. of syrupy phosphoric acid, or mixtures of two or more acids. The solution should previously have been freed from reducing agents by boiling with nitric acid, adding a little persulfate if carbon compounds are present, as with steel; if chloride is present it should be evaporated with nitric and sulfuric acids to fumes of the latter. 0.2 to 0.4 g. of KIO_4 or HIO_4 is added or an equivalent amount of $Na_3H_2IO_6$, the solution boiled for a minute, kept hot 5 to 10 minutes, cooled, diluted to the proper volume, filtered and with a standard of known manganese content similarly prepared. When ready for comparison the solution should not contain much more than 1 mg. of manganese per 50 cc., otherwise the color will be too dark.

In the presence of considerable hydrofluoric acid or phosphoric acid manganese periodate is insoluble in fairly concentrated nitric acid but readily soluble in the other acids. A very large concentration of acid delays the hydrolysis, and thus does a longer time of heating.

The method is especially adapted for the determination of manganese in [illegible] where other [illegible] is present in small amounts. The application to [illegible] steels will be made the subject of a [illegible]

The [illegible] of the reagent [illegible] are relatively small amounts [illegible] required. It is less expensive than sodium bismuthate,

[illegible] is widely used. Simple methods for the preparation of periodic acid and its salts have been devised and will be discussed in a later paper.[1]

The method has been used by students in this laboratory during the [illegible] years for the determination of small amounts of manganese in steel, iron ore and other minerals, and has been found entirely satisfactory.

Summary.

1. The periodates of the alkali metals are excellent reagents for the oxidation of manganese to the form of permanganate in the colorimetric determination of this element. The oxidation with a small excess of permanganate is rapid and complete, providing sufficient free acid is present to prevent the precipitation of manganic periodates or oxides. This oxidation is more reliable than that with persulfate and silver nitrate, and, unlike this, it gives the true permanganate color.

2. A high concentration of acid, the presence of ammonium salts, and prolonged time of heating are all without effect. Traces of chloride [illegible] interference and even larger amounts may be expelled by heating [illegible] of the reagent. Of the common metals none interferes, [illegible] forms a colored ion.

3. The procedure involved is simple; no catalyzer is required, and the [illegible] may be kept unchanged for months.

4. Relatively large amounts of manganese may be oxidized.

5. [illegible] may be removed by suitable means, such as treatment with [illegible] acid. If much iron is present either oxalate or phosphate [illegible] should be employed to prevent precipitation of basic periodate.

6. [illegible] acid also serves to remove the yellow color of ferric salts, [illegible] especially phosphoric, are also more efficient in preventing [illegible] of manganese compounds.

7. [illegible] is described. This method is analyzed [illegible] and the necessary [illegible] are described.

8. [illegible] to use this on the basis of a volumetric method were un[illegible]

[illegible] is described.

[illegible]

[illegible]

[illegible] Chem. [illegible] (1903), [illegible] that the [illegible]

[illegible] J. Am. Chem. Soc., 24, 136

[illegible]

4. A. [illegible], Dingler's Ibid., [illegible] (1873), ferric [illegible] are oxidized by fusion with NaOH.

5. M. Kuppmayer, Dingler's Ibid., 211, 133 (1874), found useless the method described [illegible].

6. S. [illegible], Chem. News, 53, [illegible] (1886), uses PbO_2 as oxidizing agent.

7. A. [illegible], Zeitschr. Anal. Chem., 43, [illegible] (1892), uses [illegible].

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9. R. [illegible] and Barrage, J. Chem. [illegible], 17, 275 (1896), fortants. The use of sodium bismuthate is suggested.

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13. F. [illegible], Gesundh. Ing., 28, 197 (1905), also Chem. Zentralb., 1905, II, 74, precipitate Mn in alkaline tartrate solution by adding [illegible].

14. N. [illegible], Gaz. chim. Ital., 36, [illegible] (1906), oxidizes Mn to colored form in alkaline glycerine solution by air or hypochlorite.

15. [illegible], Chem. [illegible], [illegible], 19, 215 (1907), oxidizes Mn to $KMnO_4$ in slightly [illegible] solution by hypochlorite [illegible] $CuSO_4$ [illegible].

16. [illegible], Chem. News, 96, [illegible] (1907), uses persulfate and silver.

17. [illegible] and Riordan, Am. [illegible], 3, 370, [illegible] (1910), use persulfate with a drop of $Cr_2(SO_4)_3$.

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22. T. [illegible] and C. C. [illegible], Ind. Eng. Chem., 4, 522 (1912), use sodium [illegible].

23. M. [illegible], Z. f. [illegible] Chem., 80, 171 (1913), uses persulfate and silver.

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28. M. [illegible], Chem. Z., 38, [illegible] (1913), uses [illegible] and silver.

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30. [illegible], [illegible], 81 (1913), [illegible] sulfate and silver.

31. [illegible], Chem. [illegible], [illegible] (1913), [illegible] $Mn^{(III)}$; oxidize [illegible]

32. [illegible], [illegible], [illegible] of [illegible] and reaction [illegible]

33. [illegible], [illegible], [illegible] of [illegible] of test and reaction [illegible]

34. [illegible], Chem. [illegible], [illegible] (1914), [illegible] of [illegible] and [illegible]

[illegible]