MANGANESE SALTS VOL II #68
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Manganese Salts-Volume 2 #68 11/9/73

VOLUME 2

GRAS MONOGRAPH SERIES

MANGANESE SALTS

(COPIES OF ARTICLES CITED IN MONOGRAPH SUMMARY)

prepared for

THE FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION
AND WELFARE

NOVEMBER 9, 1973

This publication was prepared under Contract Number FDA 72-100
with the Public Health Service, Food and Drug Administration,
Department of Health, Education, and Welfare

prepared by

Tracor Jitco, Inc.

J. Biol. Chem. 164: 783-784, 1946

LETTERS TO THE EDITORS

THE LIPOTROPIC ACTION OF MANGANESE*

Sirs:

In the course of studies to determine the effect of manganese and choline on bone formation in the rat, we found that manganese as well as choline prevents the deposition of excess fat in the liver. At a given level of choline, more fat was observed to be present in the livers of manganese-deficient rats than in the livers of rats receiving adequate manganese. The lipotropic action of manganese was much greater when the choline content of the diet was low, thus indicating an interaction between manganese and choline.

The basal diet used in the studies was similar to the low manganese diet described by Amdur, Norris, and Heuser.[1] The choline content was reduced by omitting the supplement of 0.2 mg. of choline per gm. of diet and reducing the whole milk powder content from 20 to 10 per cent. The carbohydrate, protein, and fat were increased to compensate for the lower level resulting from the reduced whole milk powder content. This gave a low choline, low manganese diet containing 0.1 mg. of choline and 0.3 γ of manganese per gm. Choline was added to produce a medium choline (0.4 mg. per gm.), low manganese diet and a high choline (0.8 mg. per gm.), low manganese diet. A high manganese diet on each choline level was obtained by supplementing the corresponding low manganese diet with 0.25 per cent manganous chloride.

The fresh livers from each group were used as a composite sample. They were finely divided in a Waring blendor, ground with anhydrous sodium sulfate, and extracted for 48 hours with chloroform. The fat was expressed as the percentage in fresh liver.

The data from five experiments are summarized in the accompanying graph. An analysis of variance showed that the reduction of liver fat by manganese was highly significant. As expected, choline likewise markedly

* The authors wish to express their appreciation for the grant of the Snyder Fund and of a portion of the Sage Fund by the Graduate School of Cornell University in partial support of the research work presented in this report. They are also indebted to the Poultry Department of Cornell University for the use of their facilities in carrying out this investigation.

[1] Amdur, M. O., Norris, L. C., and Heuser, G. F., *Proc. Soc. Exp. Biol. and Med.*, **60**, 251 (1945).

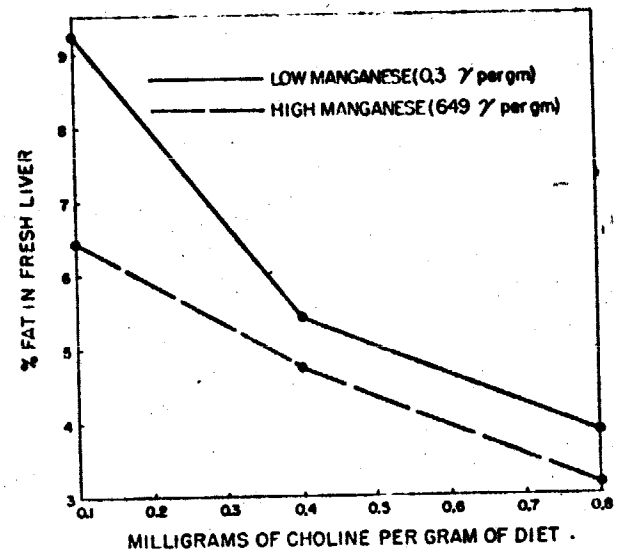

FIG. 1. The action of manganese and choline on liver fat. In all, 186 rats were used in these studies. Since there were three levels of choline and two levels of manganese used, each point on the graph represents a total of thirty-one animals. The pooled samples of four experiments contained the livers of six rats and that of the fifth experiment the livers of seven rats.

reduced liver fat. The manganese by choline interaction was found to be highly significant.

As further evidence that manganese has a lipotropic action, it was found that the presence of manganese in the diet caused a highly significant reduction in the percentage of fat occurring in the fresh bone. Choline also significantly reduced bone fat.

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Received for publication, June 17, 1946

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Federal Register - March 6: 2152-2155, 1962

Title 42—PUBLIC HEALTH

Chapter I—Public Health Service, Department of Health, Education, and Welfare

PART 72—INTERSTATE QUARANTINE

Drinking Water Standards

On July 27, 1961, notice of proposed rule making relating to the revision of the regulations in this Subpart J—Drinking Water Standards, and a related section was published in the FEDERAL REGISTER (26 F.R. 6737). After consideration of all relevant matter presented regarding the proposed revision, the regulations as so published are adopted, to become effective 30 days after the publication of this notice in the FEDERAL REGISTER, subject to the changes set out below.

1. Section 72.203: The words "Figure I" are added immediately below the graph in this section.

2. Section 72.205(b)(1): The word "fluoride" is substituted for the word "flouride" appearing in the table in paragraph 1.

3. Section 72.205(b)(2): The concentration in mg/l for chromium (Hexavalent) shown in the table is amended to read "0.05".

4. Paragraph (b) of § 72.206 is amended.

Dated: February 21, 1962.

[SEAL] LUTHER L. TERRY,
Surgeon General.

Approved: February 28, 1962.

ABRAHAM RIBICOFF,
Secretary.

§ 72.1 [Amendment]

1. Section 72.1(1) is amended to read:

(1) *Potable water.* Water which meets the standards prescribed in the Public Health Service Drinking Water Standards (see Subpart J of this part).

2. Subpart J is amended to read as follows:

Subpart J—Drinking Water Standards

Sec.
72.201 Definition of terms.
72.202 Source and protection.
72.203 Bacteriological quality.
72.204 Physical characteristics.
72.205 Chemical characteristics.
72.206 Radioactivity.
72.207 Recommended analytical methods.

AUTHORITY: §§ 72.201 to 72.207 issued under sec. 215, 58 Stat. 690, as amended; 42 U.S.C. 216. Interprets or applies sec. 361, 58 Stat. 703; 42 U.S.C. 264.

§ 72.201 Definitions of terms.

As used in this subpart, the following terms shall have the meanings set out below:

(a) "Adequate protection by natural means" involves one or more of the following processes of nature that produces water consistently meeting the requirements of these Standards: dilution, storage, sedimentation, sunlight, aeration, and the associated physical and biological processes which tend to accomplish natural purification in surface waters and, in the case of ground waters, the natural purification of water by infiltration through soil and percolation through underlying material and storage below the ground water table.

(b) "Adequate protection by treatment" means any one or any combination of the controlled processes of coagulation, sedimentation, absorption, filtration, disinfection, or other processes which produce a water consistently meeting the requirements of these Standards. This protection also includes processes which are appropriate to the source of supply; works which are of adequate capacity to meet maximum demands without creating health hazards, and which are located, designed, and constructed to eliminate or prevent pollution; and conscientious operation by well-trained and competent personnel whose qualifications are commensurate with the responsibilities of the position and acceptable to the reporting agency and the certifying authority.

(c) "Certifying Authority" means the Surgeon General of the United States Public Health Service or his duly authorized representatives. Reference to the certifying authority is applicable only for those water supplies to be certified for use on carriers subject to this part.

(d) "The coliform group" includes all organisms considered in the coliform group as set forth in Standard Methods for the Examination of Water and Wastewater, current edition, prepared and published jointly by the American Public Health Association, American Water Works Association, and Water Pollution Control Federation.

(e) "Health hazards" mean any conditions, devices, or practices in the water supply system and its operation which create, or may create, a danger to the health and well-being of the water consumer. An example of a health hazard is a structural defect in the water supply system, whether of location, design, or construction, which may regularly or occasionally prevent satisfactory purification of the water supply or cause it to be polluted from extraneous sources.

(f) "Pollution", as used in these Standards, means the presence of any foreign substance (organic, inorganic, radiological, or biological) in water which tends to degrade its quality so as to constitute a hazard or impair the usefullness of the water.

(g) "Reporting agencies" means the respective official State health agencies or their designated representatives.

(h) "The standard sample" for the bacteriological test shall consist of:

(1) For the bacteriological fermentation tube test, five (5) standard portions of either:

(i) Ten milliliters (10 ml)

(ii) One hundred milliliters (100 ml)

(2) For the membrane filter technique, not less than fifty milliliters (50 ml).

(i) "Water supply system" includes the works and auxiliaries for collection, treatment, storage, and distribution of the water from the sources of suply to the free-flowing outlet of the ultimate consumer.

§ 72.202 Source and protection.

(a) The water supply should be obtained from the most desirable source which is feasible, and effort should be made to prevent or control pollution of the source. If the source is not adequately protected by natural means, the supply shall be adequately protected by treatment.

(b) Frequent sanitary surveys shall be made of the water supply system to locate and identify health hazards which might exist in the system. The manner and frequency of making these surveys, and the rate at which discovered health hazards are to be removed shall be in accordance with a program approved by the reporting agency and the certifying authority.

(c) Approval of water supplies shall be dependent in part upon:

(1) Enforcement of rules and regulations to prevent development of health hazards;

(2) Adequate protection of the water quality throughout all parts of the system, as demonstrated by frequent surveys;

(3) Proper operation of the water supply system under the responsible charge of personnel whose qualifications are acceptable to the reporting agency and the certifying authority;

(4) Adequate capacity to meet peak demands without development of low pressures or other health hazards; and

(5) Record of laboratory examinations showing consistent compliance with the water quality requirements of these Standards.

(d) For the purpose of application of these Standards, responsibility for the conditions in the water supply system shall be considered to be held by:

(1) The water purveyor from the source of supply to the connection to the customer's service piping; and

(2) The owner of the property served and the municipal, county, or other authority having legal jurisdiction from the point of connection to the customer's service piping to the free-flowing outlet of the ultimate consumer.

§ 72.203 Bacteriological quality.

(a) *Sampling.* (1) Compliance with the bacteriological requirements of these Standards shall be based on examinations of samples collected at representative points throughout the distribution system. The frequency of sampling and the location of sampling points shall be established jointly by the reporting agency and the certifying authority after investigation by either agency, or both, of the source, method of treatment, and protection of the water concerned.

(2) The minimum number of samples to be collected from the distribution system and examined each month should be in accordance with the number on the graph in Figure I, for the population served by the system. For the purpose of uniformity and simplicity in application, the number determined from the graph should be in accordance with the following: For a population of 25,000 and under—to the nearest 1; 25,001 to 100,000—to the nearest 5; and over 100,000—to the nearest 10.

(3) In determining the number of samples examined monthly, the following samples may be included, provided all results are assembled and available for inspection and the laboratory methods and technical competence of the laboratory personnel are approved by the reporting agency and the certifying authority:

(i) Samples examined by the reporting agency.

(ii) Samples examined by local government laboratories.

(iii) Samples examined by the water works authority.

(iv) Samples examined by commercial laboratories.

(4) The laboratories in which these examinations are made and the methods used in making them shall be subject to inspection at any time by the designated representatives of the certifying authority and the reporting agency. Compliance with the specified procedures and the results obtained shall be used as a basis for certification of the supply.

(5) Daily samples collected following a bacteriological unsatisfactory sample as provided in paragraph (b)(1), (2), and (3) of this section shall be considered as special samples and shall not be included in the total number of samples examined. Neither shall such special samples be used as a basis for prohibiting the supply: *Provided*, That (i) when waters of unknown quality are being examined, simultaneous tests are made on multiple portions of a geometric series to determine a definitive coliform content, (ii) immediate and active efforts are made to locate the cause of pollution, (iii) immediate action is taken to eliminate the cause, and (iv) samples taken following such remedial action are satisfactory.

(b) *Limits.* The presence of organisms of the coliform group as indicated by samples examined shall not exceed the following limits:

(1) When 10 ml standard portions are examined, not more than 10 percent in any month shall show the presence of the coliform group. The presence of the coliform group in three or more 10 ml portions of a standard sample shall not be allowable if this occurs:

(i) In two consecutive samples;

(ii) In more than one sample per month when less than 20 are examined per month; or

(iii) In more than five percent of the samples when 20 or more are examined per month.

When organisms of the coliform group occur in three or more of the 10 ml portions of a single standard sample, daily samples from the same sampling point shall be collected promptly and examined until the results obtained from at least two consecutive samples show the water to be of satisfactory quality.

(2) When 100 ml standard portions are examined, not more than 60 percent

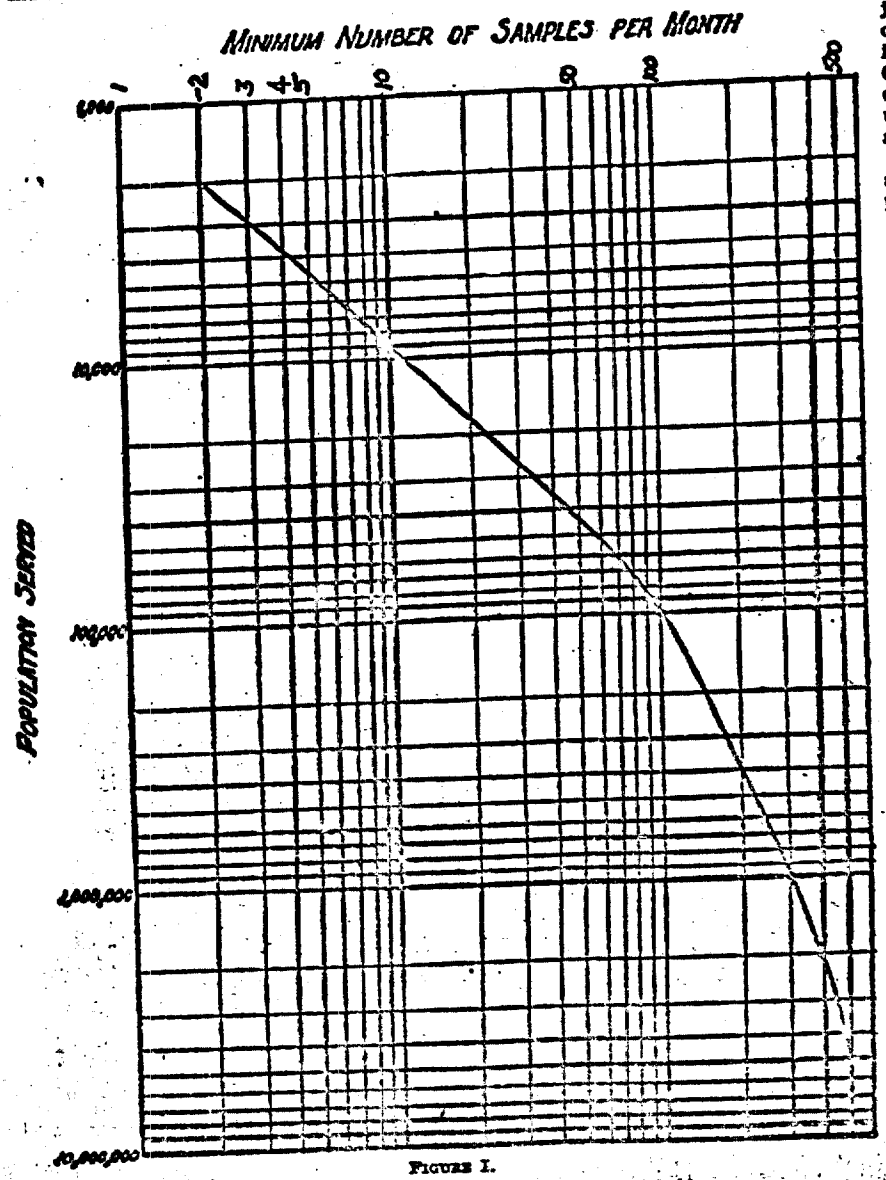

FIGURE I.

in any month shall show the presence of the coliform group. The presence of the coliform group in all five of the 100 ml portions of a standard sample shall not be allowable if this occurs:

(i) In two consecutive samples;

(ii) In more than one sample per month when less than five are examined per month; or

(iii) In more than 20 percent of the samples when five or more are examined per month.

When organisms of the coliform group occur in all five of the 100 ml portions of a single standard sample, daily samples from the same sampling point shall be collected promptly and examined until the results obtained from at least two consecutive samples show the water to be of satisfactory quality.

(3) When the membrane filter technique is used, the arithmetic mean coliform density of all standard samples examined per month shall not exceed one per 100 ml. Coliform colonies per standard sample shall not exceed 3/50 ml, 4/100 ml, 7/200 ml, or 13/500 ml in:

(i) Two consecutive samples;

(ii) More than one standard sample when less than 20 are examined per month; or

(iii) More than five percent of the standard samples when 20 or more are examined per month.

When coliform colonies in a single standard sample exceed the above values, daily samples from the same sampling point shall be collected promptly and examined until the results obtained from at least two consecutive samples show the water to be of satisfactory quality.

§ 72.204 Physical characteristics.

(a) *Sampling.* The frequency and manner of sampling shall be determined by the reporting agency and the certifying authority. Under normal circumstances samples should be collected one or more times per week from representative points in the distribution system and examined for turbidity, color, threshold odor, and taste.

(b) *Limits.* Drinking water should contain no impurity which would cause offense to the sense of sight, taste, or smell. Under general use, the following limits should not be exceeded:

Turbidity—5 units.
Color—15 units.
Threshold odor number—3.

§ 72.205 Chemical characteristics.

(a) *Sampling.* (1) The frequency and manner of sampling shall be determined by the reporting agency and the certifying authority. Under normal circumstances, analyses for substances listed below need be made only semi-annually. If, however, there is some presumption of unfitness because of the presence of undesirable elements, compounds, or materials, periodic determinations for the suspected toxicant or material should be made more frequently and an exhaustive sanitary survey should be made to determine the source of the pollution. Where the concentration of a substance is not expected to increase in processing and distribution, available and acceptable source water analyses performed in accordance with standard methods may be used as evidence of compliance with these Standards.

(2) Where experience, examination, and available evidence indicate that particular substances are consistently absent from a water supply or below levels of concern, semi-annual examinations for those substances may be omitted when approved by the reporting agency and the certifying authority.

(3) The burden of analysis may be reduced in many cases by using data from acceptable sources. Judgment concerning the quality of water supply and the need for performing specific local analyses may depend in part on information produced by such agencies as (i) the U.S. Geological Survey, which determines chemical quality of surface and ground waters of the United States and publishes these data in "Water Supply Papers" and other reports, and (ii) the U.S. Public Health Service which determines water quality related to pollution (or the absence of pollution) in the principal rivers of the Nation and publishes these data annually in "National Water Quality Network." Data on pollution of waters as measured by carbon chloroform extracts (CCE) may be found in the latter publication.

(b) *Limits.* Drinking water shall not contain impurities in concentrations which may be hazardous to the health of the consumers. It should not be excessively corrosive to the water supply system. Substances used in its treatment shall not remain in the water in concentrations greater than required by good practice. Substances which may have deleterious physiological effect, or for which physiological effects are not known, shall not be introduced into the system in a manner which would permit them to reach the consumer.

(1) The following chemical substances should not be present in a water supply in excess of the listed concentrations where, in the judgment of the reporting agency and the certifying authority, other more suitable supplies are or can be made available.

Substance	Concentration in mg/l
Alkyl Benzene Sulfonate (ABS)	0.5
Arsenic (As)	0.01
Chloride (Cl)	250
Copper (Cu)	1.0
Carbon Chloroform Extract (CCE)	0.2
Cyanide (CN)	0.01
Fluoride (F)	(*)
Iron (Fe)	0.3
Manganese (Mn)	0.05
Nitrate (NO_3)	45
Phenols	0.001
Sulfate (SO_4)	250
Total Dissolved Solids	500
Zinc (Zn)	5

*See 72.205(b)(3).

[1] In areas in which the nitrate content of water is known to be in excess of the listed concentration, the public should be warned of the potential dangers of using the water for infant feeding.

(2) The presence of the following substances in excess of the concentrations listed shall constitute grounds for rejection of the supply:

Substance	Concentration in mg/l
Arsenic (As)	0.05
Barium (Ba)	1.0
Cadmium (Cd)	0.01
Chromium (Hexavalent) (Cr^{+6})	0.05
Cyanide (CN)	0.2
Fluoride (F)	(*)
Lead (Pb)	0.05
Selenium (Se)	0.01
Silver (Ag)	0.05

*See 72.205(b)(3).

(3) (i) When fluoride is naturally present in drinking water, the concentration should not average more than the appropriate upper limit in Table I. Presence of fluoride in average concentrations greater than two times the optimum values in Table I shall constitute grounds for rejection of the supply.

(ii) Where fluoridation (supplementation of fluoride in drinking water) is practiced, the average fluoride concentration shall be kept within the upper and lower control limits in Table I.

TABLE I

Annual average of maximum daily air temperatures *	Recommended Control Limits (Fluoride concentrations in mg/l)		
	Lower	Optimum	Upper
50.0–53.7	0.9	1.2	1.7
53.8–58.3	0.8	1.1	1.5
58.4–63.8	0.8	1.0	1.3
63.9–70.6	0.7	0.9	1.2
70.7–79.2	0.7	0.8	1.0
79.3–90.5	0.6	0.7	0.8

* Based on temperature data obtained for a minimum of five years.

(iii) In addition to the sampling required by paragraph (a) of this section, fluoridated and defluoridated supplies shall be sampled with sufficient frequency to determine that the desired fluoride concentration is maintained.

§ 72.206 Radioactivity.

(a) *Sampling.* (1) The frequency of sampling and analysis for radioactivity shall be determined by the reporting agency and the certifying authority after consideration of the likelihood of significant amounts being present. Where concentrations of Ra-226 or Sr-90 may vary considerably, quarterly samples composited over a period of three months are recommended. Samples for determination of gross activity should be taken and analyzed more frequently.

(2) As indicated in § 72.205(a), data from acceptable sources may be used to indicate compliance with these requirements.

(b) *Limits.* (1) The effects of human radiation exposure are viewed as harmful and any unnecessary exposure to ionizing radiation should be avoided. Approval of water supplies containing radioactive materials shall be based upon the judgment that the radioactivity intake from such water supplies when added to that from all other sources is not likely to result in an intake greater than the radiation protection guidance[1]

[1] The Federal Radiation Council, in its Memorandum for the President, September 13, 1961, recommended that "Routine control of useful applications of radiation and atomic energy should be such that expected

recommended by the Federal Radiation Council and approved by the President. Water supplies shall be approved without further consideration of other sources of radioactivity intake of Radium-226 and Strontium-90 when the water contains these substances in amounts not exceeding 3 and 10 μμc/liter, respectively. When these concentrations are exceeded, a water supply shall be approved by the certifying authority if surveillance of total intakes of radioactivity from all sources indicates that such intakes are within the limits recommended by the Federal Radiation Council for control action.

(2) In the known absence[3] of Strontium-90 and alpha emitters, the water supply is acceptable when the gross beta concentrations do not exceed 1,000 μμc/liter. Gross beta concentrations in excess of 1,000 μμc/liter shall be grounds for rejection of supply except when more complete analyses indicate that concentrations of nuclides are not likely to cause exposures greater than the Radiation Protection Guides as approved by the President on recommendation of the Federal Radiation Council.

average exposures of suitable samples of an exposed population group will not exceed the upper value of Range II (20 μμc/day of Radium-226 and 200 μμc/day of Strontium-90)."

[3] Absence is taken here to mean a negligibly small fraction of the above specific limits, where the limit for unidentified alpha emitters is taken as the listed limit for Radium-226.

§ 72.207 Recommended analytical methods.

(a) Analytical methods to determine compliance with the requirements of these Standards shall be those specified in Standard Methods for the Examination of Water and Wastewater, Am. Pub. Health Assoc., current edition and those specified as follows:

(1) Barium: Methods for the Collection and Analyses of Water Samples, Water Supply Paper No. 1454, Rainwater, F. H. & Thatcher, L. L., U.S. Geological Survey, Washington, D.C.

(2) Carbon Chloroform Extract (CCE): Manual for Recovery and Identification of Organic Chemicals in Water, Middleton, F. M., Rosen, A. A., and Burttschell, R. H., Robert A. Taft Sanitary Engineering Center, PHS, Cincinnati, Ohio.

(3) Radioactivity: Laboratory Manual of Methodology, Radionuclide Analyses of Environmental Samples, Technical Report R59-6. Robert A. Taft Sanitary Engineering Center, PHS, Cincinnati, Ohio, and Methods of Radiochemical Analysis, Technical Report No. 173, Report of the Joint WHO-FAO Committee, 1959, World Health Organization.

(4) Selenium: Suggested Modified Method for Colorimetric Determination of Selenium in Natural Water, Magin, G. B., Thatcher, L. L., Rettig, S., and Levine, H., J. Am. Water Works Assoc. 52, 1199 (1960).

(b) *Organisms of the coliform group.* All of the details of techniques in the determination of bacteria of this group, including the selection and preparation of apparatus and media, the collection and handling of samples and the intervals and conditions of storage allowable between collection and examination of the water sample, shall be in accordance with Standard Methods for the Examination of Water and Wastewater, current edition, and the procedures shall be those specified therein for:

(1) The Membrane Filter Technique, Standard Test, or

(2) The Completed Test, or

(3) The Confirmed Test, procedure with brilliant green lactose bile broth,[3] or

(4) The Confirmed Test, procedure with Endo or eosin methylene blue agar plates.[3]

[F.R. Doc. 62-2191; Filed, Mar. 5, 1962; 8:49 a.m.]

[3] The Confirmed Test is allowed, provided the value of this test to determine the sanitary quality of the specific water supply being examined is established beyond reasonable doubt by comparisons with Completed Tests performed on the same water supply.

Voprosy Revmatizma
No. 5(1), 1965: 57-60

MANGANOUS CHLORIDE THERAPY OF NONSPECIFIC INFECTIOUS POLYARTHRITIS

M. P. Artamonova

One of the most difficult problems of internal medicine is still the treatment of chronic nonspecific infectious polyarthritis. Many drugs have been suggested for this purpose, but none of them is wholly efficacious and some produce undesirable side effects. For example, the salicylates are far from having a complete therapeutic effect. Pyrazolone preparations appeared to be more promising in this respect, but they produced good results in only 50 to 57% of rheumatoid arthritis patients (N. D. Beklemishev; L. J. Michotte). The corticosteroids now widely used in most cases quickly suppress the inflammatory process in the joints, but the suspension of therapy gives rise to the "withdrawal syndrome" in one-third of the patients, relapses occur within a week in one-half, and a remission lasting a year or so is achieved in only 30% of the cases (M. G. Astapenko). Gold preparations result in remissions in only 50 to 60% of the cases (A. I. Nesterov, Yu. Vakhtengeym). Similar data are to be found in the statistical studies of Moll and Forestier.

Comprehensive therapy, including various combinations of the above agents and quinine derivatives, is being increasingly recommended in order to obtain better results (M. G. Astapenko, and T. M. Trofimova; V. P. Krikunov et al.; others).

Of considerable interest from the standpoint of the search for and clinical trial of new preparations are the reports of Picard, Drsinich, and Harmath on the efficacy of manganous chloride in the treatment of rheumatoid arthritis. These investigators attribute the therapeutic effect of manganese to its significance for bone and some enzymes. The fact is manganese is very abundant in bones (a deficiency of the

element may result in deformity), where it stimulates phosphatase, especially alkaline, activity, thereby affecting the process of ossification. It also plays an important role in stimulating the oxidation process (F. K. Men'shikov; A. O. Voynar; Yu. P. Kolesnikov). However, the most intimate mechanism of action of manganous chloride in nonspecific infectious polyarthritis is still obscure. There are no references in the Soviet literature to the use of manganese for joint diseases. This prompted us to test the therapeutic value of this agent in the treatment of nonspecific infectious polyarthritis.

Under our observation were 30 patients, 23 female, with 6 under 30, 7 between 30 and 40, 13 between 40 and 50, and 4 over 50. All suffered from constant pain in the joints, constraint and limited movements in the joints, and general malaise. The duration of the disease was less than 5 years in 15, 5 to 10 years in 8, and over 10 years in the others. All had at one time been treated with salicylates and pyrazolone preparations; 22 received steroid hormones and a few received crysanol [oleochrysine] and chloroquine. Focal infection was detected in the majority of cases. For example, chronic tonsillitis was found in 17, carious teeth in 4, and chronic otitis in 1. Two women had a history of postpartum sepsis. Three patients had frequent colds. Upon admission to the clinic, 28 were suffering from swelling and deformity of the joints. Joint involvement was of an acute exudative character in 4, exudative-proliferative in 11, while productive changes were pronounced in the others. In 28 patients, X rays of the affected joints revealed such pathological changes as osteoporosis, narrowing of the interarticular clefts, destructive changes in the joint surfaces, etc. Many patients exhibited significant shifts in laboratory indices, e.g., an accelerated ESR in 18, increase in the gamma globulin fraction in 17, positive Vaaler-Rose test in 27. The diseased joints limited work capacity in 16 and led to disability in the others.

All these patients were treated with manganous chloride after the infection subsided. Other forms of therapy, including exercise, were completely excluded at this time. The agent was prepared by sterilizing a 1% aqueous solution of manganeus chloride in a water bath at 100° for 30 minutes. The treatment schedule was as follows. The solution was injected intramuscularly every day starting with 0.1 ml which was increased by 0.1 ml every day or every other day to 1.5 to 2 ml. The course of treatment consisted of 15 to 20 injections. The first injections produced a reaction in some patients in the form of brief intensification of the pain in the joints (5 cases) and slight rise in temperature on the second or third day (10 cases). In these patients, the preparation was administered every other day and the amount increased somewhat more slowly. We observed the general condition of the patients during the course of therapy, assessed changes in the joints, and monitored a number of laboratory indices (blood, protein fractions, fibrinogen, deiphenylamine test, antistreptohyaluronidase, antistreptolysin O, Vaaler-Rose test). We did not detect any toxic side effects from the preparation.

Good or satisfactory results were obtained in many rheumatoid arthritis patients. Pain in the joints ceased completely in 10, swelling disappeared and almost the full range of movements was restored. In 12, pain diminished considerably, edema in the vicinity of the joints largely subsided and their function improved. The other patients showed no improvement. These persons, we wish to stress, had had the disease 10 years or more and their joints were markedly deformed. We present below an excerpt from the case history of patient B. in whom manganous chloride treatment was efficacious despite the long duration of the disease.

Patient B., age 42, was admitted to the clinic on June 10, 1963 with complaints of constant aching pain in all the large and small joints. The pain intensified when she moved. She could move only with difficulty, holding onto the wall, and was completely unable to climb stairs. She could not hold anything in her hands. She had the disease since 1950. Following postpartum sepsis, pain appeared in the talocalcaneal, humeral, and interphalangeal joints along with swelling. In 1952 the pain spread to the knee, ulnar, and coxofemoral joints. Exacerbations were invariably accompanied by elevation of the temperature and swelling of the joints. The interphalangeal joints of the hands became deformed and stiff in 1956.

She was treated every day in a hospital. She took salicylates, pyrazolone and antimalarial preparations, and corticosteroids and spent time at a health resort. Prednisolone brought about temporary improvement. For the last 5 months she took prednisolone constantly, 2 or 3 tablets a day.

The case history included references to frequent bouts of sore throat, a tonsillectomy in 1952, dental caries, chronic periadnexitis, and perimetritis since 1956.

Physical findings: subfebrile temperature. General condition fair. Knee joints swollen. Talocalcaneal and interphalangeal joints deformed. No pathology in the viscera. X-ray examination: pronounced osteoporosis, narrowing of the joint clefts, sclerosis of the closing plates of the joint surfaces. Laboratory findings: WBC 8700, ESR 14 mm/hour, gamma globulins 26%, fibrinogen 0.483%, diphenylamine test 0.447 units, antistreptolysin O 63 units, antistreptohyaluronidase 330 units, Vaaler-Rose test 1:128.

Treated with 1% manganous chloride solution (17 injections). Marked improvement resulted, with complete disappearance of pain in the coxofemoral, knee, and humeral joints; pain in the other joints greatly diminished and became intermittent. The range of movements increased considerably. She

was able to make a fist and hold objects tightly and move her arms freely to the side and upward. Movements in the knee joints were normal. She walked freely unassisted and could take care of herself.

Laboratory findings: WBC 7200, ESR 3 mm/hour, gamma globulins 22%, fibrinogen 0.384%, diphenylamine test 0.389 units, antistreptolysin O not found, antistreptohyaluronidase 825 units, Vaaler-Rose test 1:64.

Despite the distinct clinical effect achieved in most (22 of 30) patients with chronic nonspecific infectious polyarthritis, no definite changes were noted in the laboratory indices during the course of manganous chloride treatment. While these tests became normal or improved in several patients, they remained unchanged or even deteriorated slightly in the others. No parallelism was detected in the dynamics of the laboratory indices, for an improvement in some was associated with a deterioration in others, and vice versa.

Thus, our observations indicate that the administration of manganous chloride mitigates the clinical course of nonspecific infectious polyarthritis. In most of the patients, the preparation brought about a general improvement, disappearance or sharp diminution of pain in the joints, subsidence of inflammatory phenomena, and increased range of movements. However, this distinct clinical improvement was not always accompanied by analogous changes in the parameters characterizing the activity of the infectious-allergic process. Manganous chloride should obviously used in conjunction with other preparations. It may be that the comprehensive therapy which we are now using will bring about not only clinical but laboratory remission.

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УДК 616.72-002-022.6-085-777.954

К ЛЕЧЕНИЮ ИНФЕКЦИОННОГО НЕСПЕЦИФИЧЕСКОГО ПОЛИАРТРИТА ХЛОРИСТЫМ МАРГАНЦЕМ

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Терапия больных хроническим неспецифическим инфекционным полиартритом до настоящего времени остается одним из сложных вопросов внутренней патологии. Для лечения этого заболевания предложено много средств, однако ни одно из них не является абсолютно эффективным, а некоторые обладают нежелательным побочным действием.

Так, далеко не полный терапевтический эффект дают салицилаты. Более обнадеживающими в этом отношении оказались пиразолоновые препараты, однако и с их помощью хороший результат удалось достичь лишь у 50—57% больных ревматоидным полиартритом (Н. Д. Беклемишев; Michotte L. J.). Широко применяемые в настоящее время кортиконды в большинстве случаев быстро подавляют воспалительный процесс в суставах, и вместе с тем прекращение терапии у 2/3 больных ведет к появлению «синдрома отмены», у 1/2 больных возникает рецидив заболевания уже в течение ближайшей недели и лишь в 30% случаев достигнутая ремиссия сохраняется до года (М. Г. Астапенко). Препараты золота при ревматоидном полиартрите позволяют достичь ремиссии также лишь у 50—60% больных (А. И. Нестеров, Ю. Вахтенгейм). Данные, близкие к указанным, представлены в статистических разработках Moll, Forestier.

Для достижения большего терапевтического эффекта все шире рекомендуется комплексная терапия ревматоидного полиартрита, включающая различные комбинации упомянутых выше средств, а также производных хинина (М. Г. Астапенко, Т. М. Трофимова; В. П. Крикунов с соавторами и др.).

Определенный интерес с точки зрения поисков и клинической апробации новых препаратов представляют сообщения Picard, Drsinich и Harmath об эффективности при ревматоидном полиартрите хлористого марганца. При объяснении терапевтического эффекта марганца авторы ссылаются на его значение для костной ткани и ряда ферментов.

Действительно, марганец весьма богато представлен в костях (при его дефиците возможна их деформация), он активирует фосфатазу, в частности щелочную, влияя тем самым на процессы оссификации; существенно его значение и в активировании окислительных процессов (Ф. К. Меньшиков; А. О. Войнар; Ю. П. Колесников).

Однако более интимный механизм действия хлористого марганца при инфекционном неспецифическом полиартрите остается все же не-

ясным. В отечественной литературе не удалось встретить работ о применении марганца при заболевании суставов. Это обстоятельство и побудило нас проверить терапевтическую ценность этого препарата при инфекционном неспецифическом полиартрите.

Под нашим наблюдением находилось 30 больных, из них 23 женщины. В возрасте до 30 лет было 6 больных, от 30 до 40 лет — 7, от 40 до 50 лет — 13 и старше 50 лет — 4 больных. Все больные отмечали постоянные боли в суставах, скованность и ограничение движений в них, общее недомогание.

Длительность заболевания менее 5 лет была у 15 лиц, 5—10 лет — у 8, а у остальных превышала этот срок. В прошлом все больные лечились салицилатами и пиразолоновыми препаратами, 22 из них получали стероидные гормоны и немногие — кризанол и хлорохин. У значительного большинства выявлена очаговая инфекция. Так, у 17 человек имелся хронический тонзиллит, у 4 — кариозные зубы, у 1 — хронический отит; 2 женщины перенесли в прошлом послеродовой сепсис, 3 больных часто подвергались влиянию простудного фактора. При поступлении в клинику припухлость и деформация суставов выявлены у 28 человек. Поражение суставов у 4 человек носило острый экссудативный характер, у 11 — экссудативно-пролиферативный, у остальных были выраженные продуктивные изменения. У 28 больных на рентгенограммах пораженных суставов выявлены такие патологические изменения, как остеопороз, сужение межсуставных щелей, деструктивные изменения суставных поверхностей и т. д. У многих больных имелись существенные сдвиги со стороны лабораторных показателей. Так, ускоренная РОЭ была у 18 человек, увеличение фракции γ-глобулинов — у 17, положительная реакция Ваалер—Роузе — у 27 человек. Заболевание суставов ограничило трудоспособность у 16 человек, а у остальных привело к инвалидности.

Всем этим больным после ликвидации инфекции проведено лечение хлористым марганцем. Другие виды терапии, включая и физиотерапию, в этот период полностью исключались.

Методика приготовления препарата: 1% водный раствор хлористого марганца стерилизуют в водяной бане при 100° в течение 30 мин.

Схема лечения была следующей. Ежедневно вводили внутримышечно 1% раствор хлористого марганца, начиная с 0,1 *мл* и увеличивая каждый день или через день на 0,1 *мл* до 1,5—2 *мл*. Курс лечения состоял из 15—20 инъекций препарата. Первые введения хлористого марганца у ряда больных сопровождались реакцией в виде кратковременного усиления болей в суставах (5 человек) и небольшого подъема температуры на 2—3 дня (10 человек). В этих случаях препарат вводили сначала через день и повышение его дозы несколько замедляли. В ходе лечения хлористым марганцем мы наблюдали за общим состоянием больных, оценивали изменения со стороны суставов, а также следили за рядом лабораторных показателей (анализ крови, белковые фракции, фибриноген, дифениламиновая проба, антистрептогиалуронидаза, антистрептолизин-О, реакция Валлер—Роузе). Каких-либо проявлений токсического действия этого препарата мы не отметили.

При лечении хлористым марганцем у многих больных ревматоидным полиартритом удалось достичь хороших и удовлетворительных результатов. Так, у 10 человек совершенно прекратились боли в суставах, исчезла их припухлость, почти полностью восстановился объем движений. У 12 больных боли резко уменьшились, отечность в области суставов в значительной мере сократилась, улучшилась их функция. У остальных лиц улучшения состояния достичь не удалось. Следует подчеркнуть, что это были в основном больные с большой давностью заболевания — 10 лет и более, с выраженной деформацией суставов.

Ниже мы приводим выдержку из истории болезни больной Б., у которой лечение хлористым марганцем даже при большой длительности заболевания все же оказалось эффективным.

Больная Б., 42 лет, поступила в клинику 10.VI 1963 г. с жалобами на постоянные ноющие боли во всех крупных и мелких суставах. Боли усиливались при движении. Передвигалась с трудом, держась за стену, совершенно не могла ходить по лестнице. Была не в состоянии держать в руках какие-либо предметы. Больна с 1950 г. После перенесенного послеродового сепсиса появились боли в голеностопных, плечевых и межфаланговых суставах с их отечностью. С 1952 г. боли распространились на коленные, локтевые и тазобедренные суставы. Обострение всякий раз сопровождалось повышением температуры, припухлостью суставов. С 1956 г. развилась деформация межфаланговых суставов рук с их туговодвижностью.

Ежегодно лечилась в стационаре. Принимала салицилаты, пиразолоновые и антималярийные препараты, кортикостероиды и санаторно-курортное лечение. Было кратковременное улучшение от преднизолона. Последние 5 месяцев больная постоянно принимает преднизолон по 2—3 таблетки в день.

В анамнезе у больной частые ангины. В 1952 г. тонзиллэктомия, с 1956 г. — кариес зубов, хронический пернадиексит, пер. метрит.

Объективные данные: температура субфебрильная. Общее состояние средней тяжести. Коленные суставы опухшие. Голеностопные и межфаланговые суставы кистей деформированы.

Со стороны внутренних органов патологии не выявлено.

На рентгенограммах: резко выраженный остеопороз, сужение суставных щелей, склерозирование замыкающих пластинок суставных поверхностей.

Лабораторные данные: лейкоциты 8700, РОЭ 14 мм в час, γ-глобулины 26%, фибриноген 0,483%, дифениламиновая проба 0,447 ед., антистрептолизин-О 63 ед., антистрептогиалуронидаза 330 ед., реакция Ваалер—Роузе 1 : 128.

Проведено лечение 1% раствором хлористого марганца (17 инъекций). Состояние больной значительно улучшилось. Полностью исчезли боли в тазобедренных, голеностопных и плечевых суставах. В других суставах боли стали непостоянными и значительно уменьшились. Объем движений существенно увеличился. Сжимает пальцы в кулак, крепко удерживает предметы, свободно отводит руки в стороны, поднимает их вверх. В голеностопных суставах движения в полном объеме. Свободно без посторонней помощи ходит, полностью обслуживает себя.

Лабораторные данные: лейкоцитов 7200, РОЭ 3 мм в час, γ-глобулинов 22%, фибриноген 0,384%, дифениламиновая проба 0,369 ед., антистрептолизин-О не обнаружен, антистрептогиалуронидаза 825 ед., реакция Ваалер—Роузе 1 : 64.

Несмотря на явный клинический эффект, достигнутый у большинства (у 22 из 30) больных хроническим инфекционным неспецифическим полиартритом, в лабораторных показателях не удалось отметить каких-либо определенных сдвигов в ходе лечения хлористым марганцем. Если у ряда больных эти тесты нормализовались или улучшились, то у других они не изменились либо даже несколько ухудшились. Следует подчеркнуть, что какого-либо параллелизма в динамике самих лабораторных показателей также не было: улучшение одних из них сопровождалось ухудшением других и наоборот.

Таким образом, проведенные наблюдения все же дали нам основание считать, что при инфекционном неспецифическом полиартрите хлористый марганец облегчает клиническое течение заболевания. У значительного большинства больных улучшилось общее состояние, исчезли или резко уменьшились боли в суставах, сократились в них воспалительные явления, увеличился объем движений. Однако это явное клиническое улучшение далеко не всегда сопровождалось аналогичными сдвигами со стороны показателей, характеризующих активность инфекционно-аллергического процесса. Видимо, хлористый марганец нужно применять совместно с другими препаратами. Можно думать, что именно такая комплексная терапия, к которой мы сейчас и приступили, позволит достичь не только клинической, но и лабораторной ремиссии.

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Поступила 30/XI 1963 г.

THE TREATMENT OF RHEUMATOID POLYARTHRITIS WITH MANGANESE CHLORATE

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Summary

Basing on literature data concerned with the efficacy of manganese chlorate in the treatment of rheumatoid arthritis the author has underaken treatment of 30 patients with this remedy. Improvement was achieved in 22 patients. This was manifested by a complete disappearance or marked decrease of pains and swelling of joints, restoration or increase in the mobility of joints.

However despite the evident clinical effect, laboratory tests characterizing the activity of the inflammatory process showed no changes. The author recomends manganese chlorate for the treatment of rheumatoid arthritis.

Proc. Soc. Exp. Biol. Med., 38(5): 740-742, 1938

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Toxicity of $MnCl_2.4H_2O$ when Fed to Rats.

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Findlay[1] claims that addition of manganese chloride to the "ordinary" diet of rats produces cirrhosis of the liver and death in from 6 to 10 weeks. He fed 0.3 to 0.4 g manganese chloride per rat per day but failed anywhere to indicate the composition of the diet used. McCarrison[2] fed rats 0.889 mg of MnO per day. He compared their weights with those of a similar group receiving no manganese other than that contributed by the salt mixture and the other constituents of the diet. He states, "There was no difference in the percentage weight curves of the 2 groups up to the 32nd day. Thereafter the curves diverged gradually and increasingly; that of the group receiving manganese being at the lower level." The experi-

1 Findlay, G. M., *Brit. J. Exp. Path.*, 1924, **5**, 92.

2 McCarrison, R., *Ind. J. Med. Res.*, 1926-27, **14**, 641.

ment lasted 135 days. To another group of rats he fed $MnCl_2$ at a level of .0327 mg per rat per day. A control group received no $MnCl_2$. He states, " . . . from the outset the rate of growth of the group receiving 0.327 mg $MnCl_2$ daily per rat surpassed that of the control group." The experiment lasted 53 days. Unfortunately, the diets used in these 2 sets of experiments were not identical either in the source of the manganese, amounts of manganese, or sources of fat or vitamins.

Orent and McCollum[3] demonstrated the need of the rat for manganese for normal reproduction. On manganese-free diets females fail to suckle their young, and males show testicular degeneration after 100 days or less. The addition of .005 to .05% manganese in the form of $MnCl_2.4H_2O$ promotes normal functioning of males and females.

As a result of these observations further experiments were conducted where manganese was added to the basal ration at the following levels of $MnCl_2.4H_2O$: 0.18%, 0.36%, 0.9%, 1.8%, 3.6%. Thus there was added to the diets .0499, .0998, .2495, .4990, and .9980 g of manganese respectively. The basal ration had the following composition:

	%
Yeast	10.0
Casein	18.0
Salts (No. 51)	6.1
Dextrin	57.9
Butterfat	8.0
Viosterol	15 drops per kilo of ration

Young animals weighing 40 to 50 g were taken from our stock diet and placed on the experimental rations.

On all the diets except where $MnCl_2.4H_2O$ was fed at 3.6% the animals grew well and produced healthy young. On the latter diet there was some depression of growth but here, as at the lower levels of manganese feeding, reproduction was excellent. The experiments on the 4 lower levels of manganese were terminated after the animals had been on the diet 240 days. Only the group receiving 3.6% $MnCl_2.4H_2O$ was kept for longer observation. At this level marked rancidity of the fat developed. When the ration was kept on ice no such changes occurred. Hence this practice was followed. As mentioned above, there were no indications of toxicity on this diet other than slight depression of growth. After 730 days on the diet 2 males still show normal testes and motile sperm as de-

[3] Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, 1931, **92**, 651.

termined by examining epididymal smears obtained by aspiration. Each of these males has subsequently been bred to 4-month-old virgin females from our stock colony. One sired 40 young when mated with 8 females, the other 25 young when mated with 5 females. Of 50 males on similar diets but without added manganese, other than that found as a contaminant of certain inorganic constituents and natural foods as shown by Orent and McCollum,[3] testicular atrophy and tubular degeneration was marked in 45 males after they had been on the rations from 117 to 833 days.

On the type of diet employed, food consumption averages from 10 to 15 g per adult rat per day. Animals on the above rations were thus consuming about .018, .036, .09, .18, and .36 g of $MnCl_2.H_2O$ per day, or .00449, .00998, .02495, .0499, and .0998 g of manganese. The rations used contained .63 g calcium and .72 g phosphorus per 100 g. Evidently the high level of phosphorus effectively prevented symptoms of toxicity by reducing the amount of absorbable manganese.

It is thus apparent that the conditions in the intestines which influence the absorbability of manganese constitute the controlling factor in determining the level at which it is toxic. From our observations on a small number of rats it also seems clear that when a high intake of this element is provided sexual function is retained in males decidedly longer than is the case in our rats on similar diets without added manganese. This observation is being checked on a larger group of male rats.

Am. J. Physiol., 211: 217-224, 1966

.nterdependence of routes excreting manganese[1]

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BERTINCHAMPS, A. J., S. T. MILLER, AND G. C. COTZIAS. *Interdependence of routes excreting manganese.* Am. J. Physiol. 211(1): 217–224. 1966.—Radiomanganese (Mn^{54}) was excreted by all intestinal segments investigated at several exponential rates. Yet the cephalad segments showed much higher excretion, which could be accelerated briskly by giving manganous sulfate, whereas excretion via the ileum could not. Intestinal excretion contrasted to biliary excretion: the tracer entered the bile in two successive "waves." The first wave reflected the initial plasma clearance, signaling direct passage of some manganese in the direction of a steep concentration gradient between plasma and bile. This concentration gradient became even steeper during the second wave. This wave preceded the one reported by others for bile acids. It could be abolished by feeding low-manganese milk, but it was reinduced promptly by injecting manganous sulfate into the duodenal lumen. Increasing the manganese loads increased the duration rather than the height of this wave. Since both the artificial and the natural manganese isotopes reflected these phenomena, it appeared that small amounts of the essential trace metal had saturated its enterohepatic circulation. The excess was apparently excreted via the duodenum and the jejunum.

enterohepatic circulation; bile; isotopes; neutron activation analysis

THE PRECEDING PAPERS SHOW that the concentration of manganese in tissues is quite stable due to controlled excretion rather than to regulated absorption. Several excretory routes were found for this metal, all of which were linked to the gastrointestinal tract. Among these routes, the bile flow seemed to participate in an enterohepatic circulation of this essential element. Interruption of this circulation abolished or diminished the capacity of animals to accelerate the excretion of manganese after a metabolic load of this metal (3, 14, 18).

The experiments presented here had two aims: first, to identify various routes which excrete manganese; second, to assess their possible interdependence in response to metabolic loads. The first of these aims was pursued by means of observing the rate of excretion of Mn^{54} in the effluents from these routes, the second by means of observing changes of these rates following the administration of a manganous salt, enterally or parenterally.

By contrast to intestinal excretion, biliary excretion showed a most unusual pattern in the anesthetized rat. The origin of the metal participating in the various phases of this pattern was investigated in some detail.

METHODS

The rats, diets, housing, and isotopes used are described in the accompanying papers, as is the concentration of manganese in these diets (3, 14, 18).

Operations

All animals were studied shortly after laparotomy and while under ether anesthesia. Their bile ducts were catheterized over the uppermost third by means of no. 20 or no. 50 polyethylene tubing. The various segments of the gut were provided with in- and outgoing catheters constructed of no. 120 polyethylene tubing. The abdomen was covered with moist gauze.

Collections of Blood, Bile, and Intestinal Washings

Radioactive blood was collected from nicks in the tail veins into tared, heparinized capillary tubes. The radioactivity was measured and expressed per unit weight of whole blood.

Radioactive bile was routinely collected into tared, numbered, capped glass vials in which it was weighed and tested for radioactivity. After the initial phases of the biliary excretion were found to be exceedingly rapid the following collection schedule was adhered to: first four collections, 2.5 min; next four, 5 min; subsequent ones, 10 min. The bile was ascribed a specific gravity of 1 in converting weight to volume.[3]

The intestinal segments were rinsed through every

Received for publication 8 October 1965.
[1] This work was supported by the U.S. Atomic Energy Commission and, in part, by National Institutes of Health Grant OH 59-03.
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[3] ALTMAN, P. L., AND D. S. DITTMER (editors). *Blood and Body Fluids.* Washington, D.C.: Fed. Am. Soc. Exptl. Biol., 1961, p. 412.

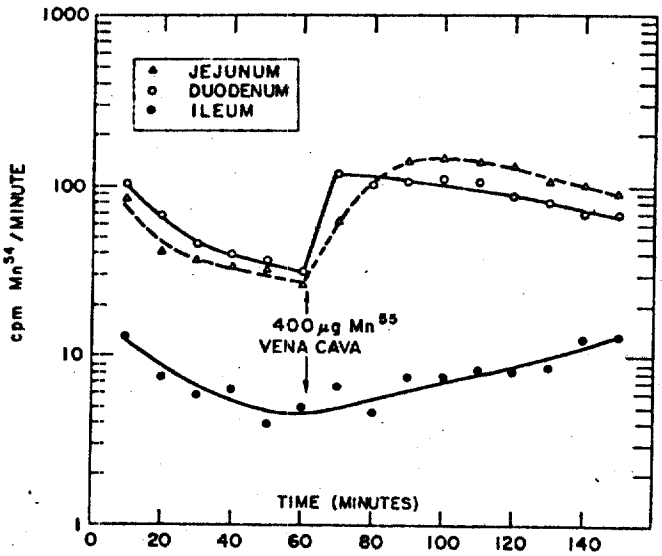

FIG. 1. Excretion of Mn^{54} into duodenum, jejunum, and ileum of an anesthetized rat. Note that on injecting stable manganese there occurred a sharp increase in excretion by the duodenum and the jejunum as contrasted to the ileum.

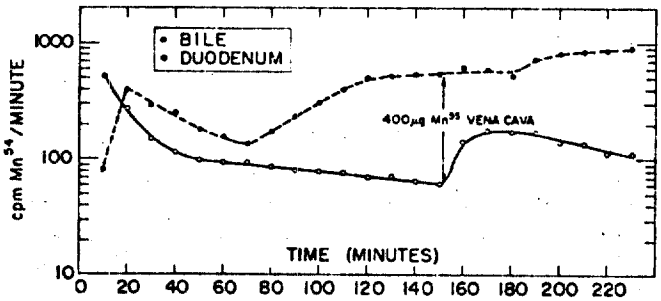

FIG. 2. Excretion of Mn^{54} into bile and duodenum of a Purina-fed anesthetized rat. Note that on injecting stable manganese there occurred a sharp increase in the excretion by the duodenum but no marked increase in the bile.

2.5 min with 1 ml normal saline solution. Four pooled rinses represented each 10-min collection.

The bile or plasma to be analyzed by neutron activation was collected with the precautions listed elsewhere (4a, 5, 7, 8, 17). Manganese 55 (stable) was measured together with the long-lived Mn^{54} (radioactive) as follows: on exiting the reactor the short-lived Mn^{56} ($T_{1/2}$ = 2.58 hr) produced from the stable metal was measured together with the long-lived Mn^{54} ($T_{1/2}$ = 314 days) since the photopeak used (840 kev) is common to both. After the passage of at least 24 hr the Mn^{54} was measured alone and Mn^{56} was quantified by difference. The standard error of the measurement by difference has not been assessed as yet, but as a rule the amountof Mn^{54} was less than that of Mn^{56}.

Administration of Isotopes

The isotopes administered included the radioisotope Mn^{54++} ($T_{1/2}$ = 314 days) discussed earlier (3, 14, 18) and the natural, stable Mn^{55++}. The first was injected in calibrated doses either with saline solution or with rat plasma as the vehicles. The latter was given in the form of $MnSO_4$ parenterally, into the lumen of the exposed intestine, via the diet, or via the drinking water as indicated.

Ultrafiltrations were performed with the aid of a micromethod to be discussed elsewhere.

Dialysis was performed with agitation in the cold after placing the bile in sacs constructed of viscose tubing.

Sodium and potassium concentrations were generously measured by Mrs. L. Tassinari of this department, with the aid of flame photometry.

RESULTS

Three rats kept for 1 week on milk were given Mn^{54++} intravenously. Two were sacrificed 10 min later and one at 72 hr. The gastrointestinal tract from the pylorus to the ileocecal valve was stretched to about 110 cm and was cut into 1-cm segments which were assayed for radioactivity individually. The values were plotted as a function of distance from the pylorus. Regardless of the time elapsing after sacrifice, the most cephalad segment contained the highest amount of radioactivity. This declined smoothly as the distance from the pylorus increased. At a distance of 10–15 cm the concentration of the radioactivity declined to about 1/10 of the initial level and remained constant in all the more caudally located segments of the gut. Two Purina-fed animals showed a similar but less steep diminution of radioactivity.

Identification of Intestinal Excretory Routes for Manganese

In view of the above, the cephalad segments of the intestine were studied first in one Purina-fed animal. The bile duct was ligated and two intestinal segments were provided with ingoing and outgoing catheters, corresponding to the duodenum and the jejunum. They measured 5 cm in length and were located at a distance of 7 cm from each other. The rat was injected intravenously with 9 μc Mn^{54++} and the segments were rinsed as described above. The effluents from both contained identical amounts of radioactivity. These declined smoothly by more than one exponential rate over the 150 min of observation.

In another similar animal, excretion via the terminal ileum was compared to that of the duodenum and the jejunum. All three segments measured 3.5 cm. Again, the radioactivity excreted by the duodenum was not markedly higher than that excreted by the jejunum, although the former must have included the pancreatic juice. Figure 1 shows that the level of radioactivity discharged by these segments was initially 10 times higher in the case of the duodenum and the jejunum than in the case of the terminal ileum. Yet, the radioactivities from all three segments declined in parallel during the next 60 min of observation, suggesting that one might be measuring leached rather than excreted isotope. Therefore, the response of these segments was tested to the administration of manganese carrier (400 μg Mn^{55})

identical in valence and injected in the same manner as the radioisotope (Mn^{54}). Figure 1 shows the rapid parallel increases of the radioactivity in the effluents from the duodenum and the jejunum as contrasted to the questionable response of the terminal ileum. This experiment was repeated twice on animals consuming Purina chow, whereas the comparison between duodenum and ileum was repeated once. In this latter case the smooth, exponential decline of the radioactivity in the respective effluents was followed for 110 min before the carrier was injected, but the response of the ileum, if present, was even less obvious than in Fig. 1.

Comparison Between Biliary and Intestinal Excretion

In one animal, also maintained on Purina chow, bile collections were performed together with washings of the duodenum after the intravenous injection of 9 μc Mn^{54}. Figure 2 shows the behavior of the radioactivity recovered from the respective routes. Surprisingly, the radioactivity appeared in the bile in the form of two distinct waves, in contrast to that in the duodenal washings which showed a continuous decline, again at more than one exponential rate. Furthermore, on iv injection of the 400 μg Mn^{55}, the output of Mn^{54} kept slowly increasing in the bile, whereas a sharp increase occurred in the Mn^{54} of the duodenal washings. Repetition of this experiment gave similar results.

Another experiment was identical in all details to the preceding ones except that the 400 μg Mn^{55++} were given into the duodenum instead of intravenously. The jejunal effluents showed no change in the rate of decline of the Mn^{54}, whereas the second wave of the radioactivity in the bile showed a prolonged plateau, by sharp contrast to experiments like that on Fig. 3.

Gross differences from these Purina-fed animals were encountered in two rats maintained on low-manganese milk for the 5 days preceding the experiment. In these latter instances, the original output of radioactivity in the bile declined faster than that in the jejunal washings and continued declining for the next 60 and 90 min, respectively. Indeed no second wave of radioactivity became evident in the bile of either animal. Introduction of 400 μg Mn^{55} into the lumen of the duodenum was followed this time by an immediate sudden rise in the output of radioactivity in the bile only, which reached within 20 min a level 15 times higher than the preinjection one. The jejunal output of radioactivity continued declining in both experiments.

The presence of unexpected components in the excretion curves of Mn^{54} in the bile, their relatively fixed timing, and the apparent reciprocity between biliary and intestinal excretion, led to detailed study of the excretion of manganese in bile. These experiments are presented as follows: *1*) comparisons are drawn between the biliary excretion of intravenously injected tracer and its blood clearance; *2*) contrasts are drawn between conditions which failed to affect the tracer's excretion pattern and those which permitted imposition of selective

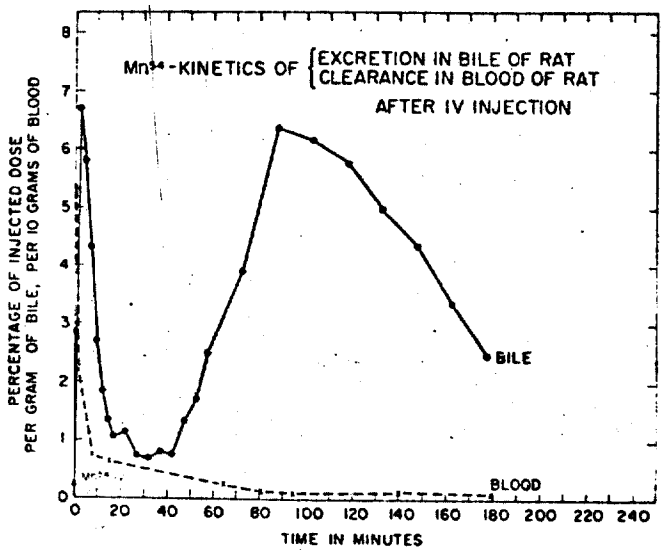

FIG. 3. Change in concentration of Mn^{54} in blood and bile of an anesthetized rat as a function of time elapsing from injection of tracer.

changes at will; *3*) experiments are presented indicating that this pattern indeed reflects the metabolism of this essential metal rather than simply that of the tracer used here arbitrarily.

Time Sequences in Excretion of Mn^{54} Into the Bile

An effort was now made to sample the bile rapidly by adhering to the schedule discussed under METHODS unless indicated otherwise.

The sequence shown in Fig. 3 was encountered in the 10 Purina-fed rats injected with tracer as soon as the bile flow was exteriorized. The radioactivity increased sharply in the bile to reach a peak 5–7 min after injection of the tracer. A rapid decline followed thereafter and a plateau was reached about 20–30 min after injection of Mn^{54}, which lasted for about 30 min. When the radioactivity was measured concomitantly in blood (sampled from the vena cava) and the bile, the blood clearance curve preceded by a few minutes the first wave of radioactivity in the bile. However, the concentration of the tracer was 10 times higher in the bile than in the corresponding blood samples. Approximately 40 min after the injection of the tracer the two curves became totally dissociated; while the blood-bound isotope continued on its decline the biliary concentration began to increase.

In conformity with the results shown in Fig. 3, all Purina-fed animals tested showed two waves in the concentration of the radioactivity in the bile. The first one reached a peak 7.5–10 min after the injection of the tracer, depending on the length of the catheter. The second wave reached a peak 60–120 min after the tracer's injection, followed immediately by a decline to the levels of the plateau encountered between the first and second waves. Extension of two experiments to approximately 8 hr revealed no third wave.

These findings made it necessary that the blood

clearance be defined in the rat as it had in man (2). The data shown in Fig. 4 were normalized at the 6-min point.

As expected, the half-life of the initial components of the blood clearance curve was less than 1 min. The rapidity of sampling displayed in Fig. 4 could not be duplicated with bile collections without administering prohibitive amounts of radioactivity. Therefore only a gross intercomparison between the blood clearances and the excretion curves could be made. For this, data from all animals discussed in this paper and obtained shortly after injection of the tracer were plotted as "cumulative excretion." Over the first 6 min this showed a doubling time of somewhat less than 1 min. As an illustration, Fig. 5 represents animals consuming *a*) milk; *b*) a commercial low-manganese diet (13); and *c*) this diet plus manganous sulfate in the drinking water. It is evident that the cumulative excretion did not reflect the dietary supply of this metal over the first 6 min. The latter parts of these curves, however, seemed to reflect the level of dietary manganese, but only the duration, not the height, of the second wave was affected by feeding massive amounts of metal.

Conditions That Did Not Affect Biliary Excretion Pattern of Mn^{54}

The initial bile flow in these experiments varied between 6 and 12 mg/min and declined slowly thereafter. Both waves of radioactivity seemed independent of the bile flow, regardless of whether the tracer was dissolved in rat serum or in saline. The following variables, evaluated for their effects on these two waves gave negative results. In one rat, distending the bile duct was avoided by entering the unclamped duct; in one the portal instead of a peripheral vein was used for the injection of the isotope. In one there was injected 0.5 ml 0.9% NaCl solution into the duodenal lumen every 10–20 min; in three, the saline solution was given subcutaneously; in another, the bile samples were injected into the duodenum after their weight and radioactivity were determined. In others, the adrenal glands or the thyroid and parathyroids were removed prior to the injection of the isotope. Two Wistar strain rats instead of Sprague-Dawley rats were tested.

Conditions That Affected Biliary Excretion Pattern of Mn^{54} Selectively

Changes of the first wave. The previous experiments suggested that the first wave might reflect, at least in part, the initial events of the blood clearance of injected Mn^{54}. This was supported by experiments in which the bile flow was exteriorized after sufficient time had elapsed for the injected isotope to reach negligible levels in the blood stream (Fig. 4). Five Purina-fed rats were studied 16 hr to 5 days after the injection of Mn^{54}. In all of these the initial decline of radioactivity was very small if present at all, but the second wave was invariably present; after about 30–50 min the concentration of

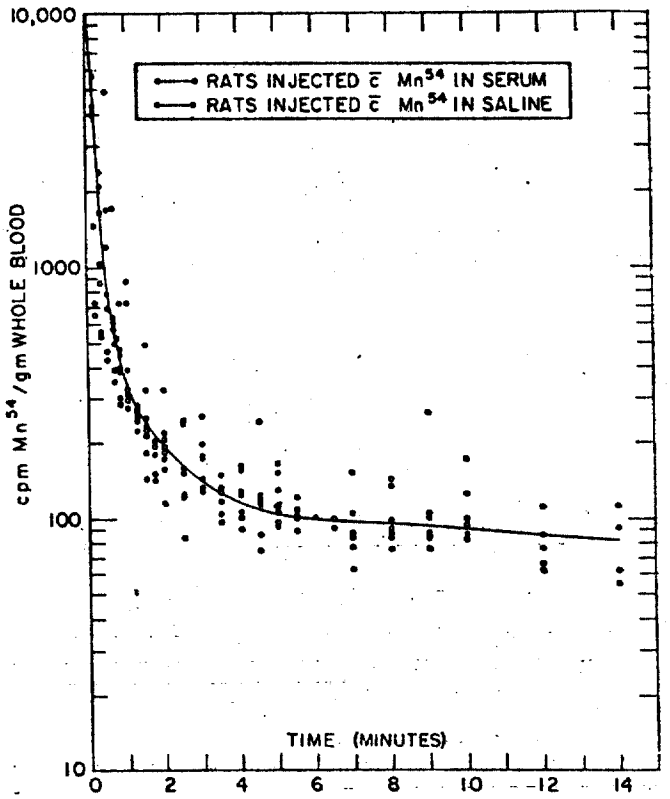

FIG. 4. Change in concentration of Mn^{54} in the blood of seven anesthetized rats as a function of time elapsing from injection of the tracer. Concentrations were normalized at the 6-min point. Note that the blood clearance appears to be independent of the two vehicles used for injecting the tracer.

Mn^{54} in the bile samples began to increase sharply and reached levels between 10 and 35 times higher than the level present at the beginning of these waves. Yet, neither the height of this second wave nor its duration could be correlated with the time which had elapsed between injection and observation.

Changes of the second wave. Since injecting the isotope before the initiation of the bile collections affected only the first wave of Mn^{54}, it was thought that injecting the isotope after the bile had been allowed to flow freely might affect only the second wave. The bile was permitted to flow in two freshly operated rats for 3 hr preceding the injection of the isotope. The first wave of radioactivity was present but the second was absent in both animals.

The experiment suggested that the second wave might signal the discharge of stable, natural manganese into the liver from some unidentified source. To test the soundness of this assumption, four rats were injected with Mn^{54} 24 hr before canulation of the duct and their bile was permitted to flow freely for about 3 hr. After the initial concentrations of Mn^{54} in bile were established, these animals were injected intravenously with 0.02, 0.1, or 2.0 mg nonradioactive manganese. The injection of the stable metal was followed by a rapid increase of the concentration of the radioactive isotope in the bile.

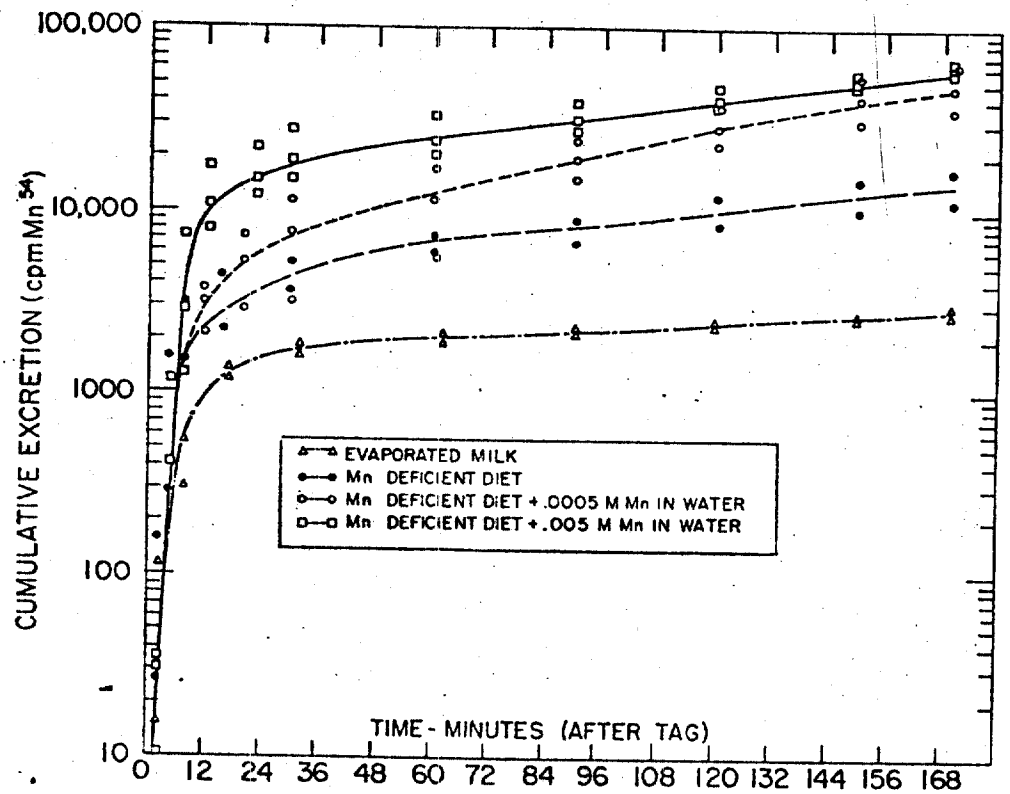

FIG. 5. Cumulative excretion of Mn^{54} in bile of anesthetized rats as a function of time elapsing from injection. Note that the early phases do not reflect the concentration of manganese in diets.

This was roughly proportional to the amount of carrier metal injected, as shown primarily by the duration of the ensuing waves of radioactivity. In one of these animals, the radioactivity was measured in the blood together with the bile. Both pools showed an increase in the isotope's concentration following administration of 2.0 mg manganous sulfate, but the increase amounted to only 25% in the blood while a 10-fold level was reached in the bile. Furthermore the ensuing waves lasted for 20 vs. 80 min, respectively.

In view of the above, it was thought that depleting the intestinal contents of manganese would abolish this wave. Three rats were fed the low-manganese diet for 14 days and received injections of Mn^{54} as soon as the catheter was inserted. The first wave was clearly evident in the bile samples of all these animals but the second wave was absent in all.

These data suggested that the second wave of radioactivity in the bile indicated dietary manganese being absorbed from the gut. Confirmatory evidence was gathered in two experiments generously performed by Dr. G. Gerber of the Euratom Laboratory, Brussels, Belgium. In these the perfused-liver technique was used in vitro. Injection of Mn^{54} into the arterial side of the perfusion system was followed by an early, single wave of the radioactivity in the bile, regardless of whether the donor animals had been kept on milk or on milk plus 0.005 M Mn^{55++}. Plotting these data as cumulative excretion revealed an initial doubling time of somewhat less than 1 min, as in Fig. 5.

If the second wave of the radioactivity in the bile was indeed linked to the absorption of the dietary manganese, the concentration of the natural Mn^{55} should vary in a manner similar to the tracer. This was investigated in the experiments which follow.

Biliary Excretion of Mn^{55}

Duplicate analyses of the serum manganese (Mn^{55}) concentration were performed on three Purina-fed rats and three animals maintained on milk for about 2 weeks. The respective mean serum concentrations (and ranges) were: 3.19 (3.00–3.52) μg/liter vs. 3.10 (2.70–3.24) μg/liter. These concentrations markedly contrasted to our previously published values for rat bile, which were both higher and much more variable (17). This suggested that the concentration of natural manganese in bile might also depend on the time which had elapsed between catheterization and sampling. Therefore the sequential analyses for Mn^{55} and Mn^{54} were performed.

In two animals reared on the Purina diet, the bile duct was entered and the radioisotope was injected intravenously. Bile collected over 10-min periods was analyzed both for the natural metal and for the radioisotope. The results were the same in both experiments; Fig. 6 shows the sequences found in one of them. The initial concentration of the natural isotope (Mn^{55}) in the bile was about 20 μg/liter. This declined together with the concentration of the radioisotope but not as rapidly. About 50 min after initiation of these experiments the customary second wave was again present and now it involved both the stable and radioactive isotopes.

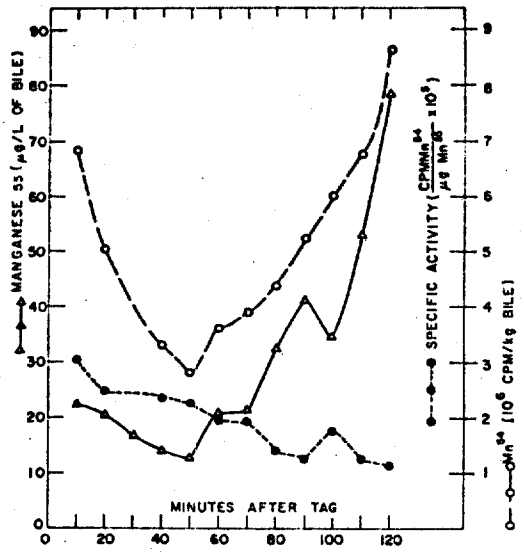

FIG. 6. Changes in concentrations of Mn^{54} and Mn^{55} in bile of a Purina-fed anesthetized rat. Note the continuous decline of the specific activity during both the fall and rise of the concentrations of these isotopes.

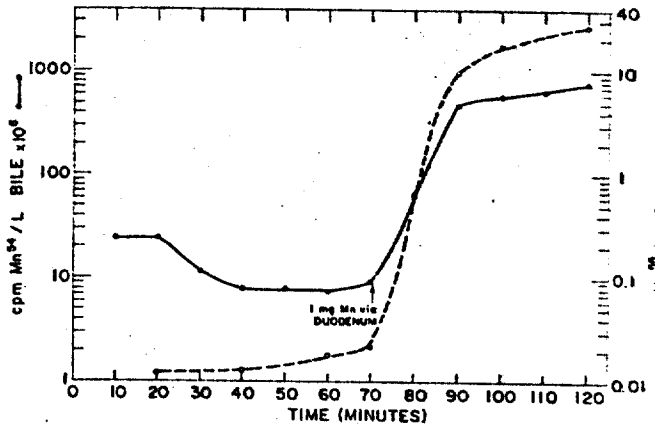

FIG. 7. Changes in the concentrations of Mn^{54} and Mn^{55} in th bile of an anesthetized rat consuming milk for 3 days preceding th experiment. By contrast to Fig. 3, this animal received 1 mg stable Mn^{++} in the duodenum. Note the immediate, sharp rise the concentrations of both isotopes in the bile.

Still the specific activity of the radioactivity declined gradually with a half-life of 60–70 min, proving that the tracer was tracing.

This experiment was repeated on an animal maintained for only 3 days on the milk diet (Fig. 7). The initial concentration of the stable manganese isotope was 12 μg/liter and slowly doubled itself over the next 50 min. At that time 1 mg Mn^{55++} (as $MnSO_4$) in 1 ml water was injected into the duodenal lumen. An immediate sudden rise of the manganese concentration in the bile occurred, which reached 27.516 mg/liter at the end of 120 min (Fig. 7). The total output of Mn^{55} in the bile during this observation amounted to only 0.3% of the dose injected into the duodenum.

Since the concentrations of manganese showed a sharp gradient between plasma and bile, a comparison was made with the respective concentrations of sodium and potassium in both pools. The plasma concentrations of these alkali metals in our rats have ranged, respectively, between 135 and 145 mEq/liter for sodium and 4.0–5.0 mEq/liter for potassium. In three rats the sodium concentration in bile ranged between 162 and 167 mEq/liter, whereas the potassium concentration ranged between 5.3 and 5.4 mEq/liter. These concentrations did not change with passing time.

State of Mn^{54} in Bile

The presence of these two waves suggested the existence of two states of the metal in bile. Hence several tests were performed in vitro on representative samples.

In four electrophoretic experiments, the mobility of the bile-bound isotope was identical to that of an aqueous solution of carrier-free Mn^{54} used as control, whether the bile had been tagged in vitro or in vivo. No significant differences could be elicited between bile samples collected immediately or 2 hr after the injection of th Mn^{54} and run concomitantly. However, in one bil sample collected during the preponderance of the secon wave, there was encountered in addition a negativel charged component. This was noteworthy because had the same electrophoretic mobility as the β-globuli of serum, known to contain a metal-binding fraction.

Ultrafiltration of bile samples collected during th first and second waves showed that 16% of the tota isotope was ultrafilterable in both. Dialysis of suc samples against triple-distilled water showed the radio activity of bile to be fully dialyzable, whereas only abou 4% of the radioactivity in plasma was ultrafilterabl The only clear-cut, reproducible difference was encoun tered when bile from Purina-fed rats was compared t that from similar animals that had been injected with mg $MnCl_2$. After freezing overnight and thawing, th samples obtained from the injected rats showed a gros precipitate which was fine in texture and grayish i color. This precipitate contained 90% of the Mn present in bile and was visible only after freezing an only after injection of this manganous salt. Its natur has not been investigated.

DISCUSSION

The excretion of manganese via several routes wa suggested by other communications (4, 12, 18) in dicating possible similarities with the excretion of zin (6). The present work focused on the cephalad segment of the intestine, and the tributaries emptying therein, a the significant excretory routes. This localization seem provocative in view of the common embryologica origin of these structures from the primordial gut (18) It would be intriguing if the similarities demonstrate among these routes prove to reflect common origin bu the differences prove linked to differentiation. Indeed the similarities were restricted primarily to the shapes c the respective excretory curves, whereas the difference were several and worthy of discussion.

The various curves representing excretion of manganese from the gut showed that much higher amounts of tracer were excreted by the duodenum and jejunum than by the terminal ileum. Furthermore, the cephalad segments responded briskly to manganese loading whereas the terminal ileum did not, suggesting that the cephalad segments might be homeostatic end organs auxiliary to the liver.

Animals reared on a standard laboratory diet excreted the radioisotope into the bile in the form of two distinct waves. The first of these appeared to be linked to the blood clearance of Mn^{54}, and must have signaled a direct passage of tracer from the plasma into the bile (Figs. 3–5). The manganese thus transferred did not constitute a major portion of the metal in bile, since there was found a steep gradient between plasma and bile. This sharp gradient was evident regardless of whether the radioisotope, Mn^{54}, or the stable isotope, Mn^{55}, were quantified. This gradient was much steeper in the case of manganese than in the case of either sodium or potassium, suggesting a different transport mechanism from those of alkali metals (21).

This gradient became even steeper during the preponderance of the second wave. It appears that this wave signaled that exteriorization of the bile flow had set off a marked acceleration of the enterohepatic circulation of this metal. The existence of such a circulation was proposed earlier (18), and was directly documented here (Fig. 7). That the second wave reflected an acceleration of this circulation is summarized on the basis of the following evidence: *1)* This wave was present when animals were consuming a high-manganese diet. *2)* It was absent when the animals ate a low-manganese diet for several days, when it was most unlikely (3, 14) that they could have developed manganese deficiency. *3)* In Purina-fed animals, manganese loads increased the duration of this wave. *4)* In milk-fed animals this wave was induced readily by manganese loads, given enterally or parenterally. *5)* The bile from isolated, perfused livers did not show this second wave. *6)* No second wave occurred spontaneously in the intestinal effluents but it could be induced here also, by means of loading with manganese.

The experiments in which the bile and intestinal effluents were analyzed concomitantly showed the following: animals consuming a high-manganese diet (Purina) responded to parenteral manganese loads primarily by producing a radiation wave in the intestinal effluents, but not by discernible increases in the height of the second wave in the bile (Fig. 2). By contrast, animals consuming a low-manganese diet (milk) seemed to respond by producing a second wave of the radioactivity in the bile but not in the jejunal effluents. Hence it appears that, when the excretion by the liver became saturated, auxiliary excretion occurred via the gut. This seems to explain the bypass of the liver encountered earlier (18).

The mechanism by which this set of events was brought about remains obscure. It must be re-emphasized that these animals were freshly laparotomized and under ether anesthesia. This circumstance does not jeopardize the validity of comparisons made on the same animal but it detracts seriously from extrapolations to intact animals (13, 14, 18). It is highly desirable that surgery and observation be temporally separated in future experiments for the following reasons: Another set of essential nutrients, the bile acids, are known to undergo enterohepatic circulation in a manner similar to manganese. Exteriorization of the bile flow has induced fluctuations of the bile acid concentration similar to, but out of phase with, the fluctuations of the manganese concentration encountered here (11, 15, 20). In the rat, the bile acids are formed directly from cholesterol and their enterohepatic circulation acts as a feedback control mechanism inhibiting cholesterol synthesis (16). In this context, it is most intriguing that manganese is established as an important cofactor in cholesterol synthesis (1, 9, 10, 19). Hence it is possible that the unusual excretory pattern of manganese in the bile may have physiological implications transcending the laparotomized, anesthetized animals studied here.

The authors thank Miss Judith Edwards for expert assistance with analyses and Mrs. Lily Tang for help with some of the observations.

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J. Clin Invest 37: 1269-1278
1958

MANGANESE METABOLISM IN MAN: RAPID EXCHANGE OF Mn^{56} WITH TISSUE AS DEMONSTRATED BY BLOOD CLEARANCE AND LIVER UPTAKE [1]

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(Submitted for publication February 10, 1958; accepted May 2, 1958)

Despite the well-known physiological importance of manganese (1–7) and its implication in some disease states (2, 8–10), the exact nature of its metabolic role remains undefined (11, 12). In mammals manganese concentrates in organs rich in mitochondria, notably the liver (2–7, 11, 13, 14); and previous work from this group (11) has shown these organelles to be the major pool of radiomanganese turnover shortly after injection into animals. *In vitro* traces of the element have widespread biochemical activity (2, 3, 15–19), especially in relationship to mitochondria (20) and those of their enzyme systems that carry out oxidative phosphorylation (16, 21–26).

The present study of blood clearance and of radiation measured at the body surface following Mn^{56} injection in man is an attempt to describe and to determine the kinetics of this turnover, which appears to correlate with mitochondrial function. From the analysis of these data in relation to the work of others it is also possible to gain insight into the mechanisms of transport directly from the blood. Our results further suggest that most of the normal body manganese is readily exchangeable and is not bound in metalloproteins or other highly stable configurations.

EXPERIMENTAL

Radioisotopic tracer. Mn^{56} (half-life: 2.58 hr.) [2] was generated in the Brookhaven research reactor. One ml. aliquots containing 11.1 μg. of $MnSO_4 \cdot H_2O$ (3.6 μg. of Mn^{++}) in saline and representing 15 to 20 microcuries of Mn^{56} [3] were injected into patients. The radiation decay of other aliquots was followed for 48 to 72 hours with a well-type NaI(Tl) scintillating crystal and scaling circuit. The resultant activity curves indicated the only contaminating radioelement to be a small amount of Na^{24} (about 0.5 per cent of the Mn^{56} activity at injection time), which was ignored in the subsequent treatment of the data.

Route of administration and withdrawal of blood samples. Following an overnight fast, patients received Mn^{56} tracer intravenously. Thereafter serial venous blood samples of approximately 3 ml. volume were withdrawn from the contralateral arm. Samples were promptly assayed for radioactivity and weighed. Corrections for radioactive decay were then made. Frequent samples were obtained during the first few minutes after injection, but later sampling frequency was decreased gradually to intervals of approximately five minutes.

Surface counting technique. Concurrently with the withdrawal of blood samples, radiation was monitored over the body surface with collimated detectors whose outputs were transcribed automatically.[4] As anticipated from animal data (2, 4–7, 11), preliminary scanning following Mn^{56} injection revealed localization of the greatest radioactivity in the region of the liver. Accordingly, one scanner was positioned on the skin surface over the liver area, usually about 3 cm. above the costal margin in the right midclavicular line ("liver area" counter). A second counter was located over the right femur about 18 cm. above the patella ("thigh" counter). This was taken to be a "reference area" that would not reflect active visceral metabolism or selective manganese uptake.

The exact distribution of Mn^{56} *in situ* was unknown. Thus absolute calibration of the body surface counters with laboratory standards was impossible. Furthermore, two scanners of different sensitivity were used. Empirically, the response of one specific counter in a fixed position and orientation was taken as standard. The two scanners then were intercalibrated at late times, when the levels of external radioactivity had reached virtual equilibrium.

Standardized units of radioactivity. Intercomparison

[1] This research was supported by the United States Atomic Energy Commission.

[2] The radioemission spectrum of Mn^{56} is complex, but the decay scheme includes several energetic gamma rays (most prominent at 845 Kev) (27) of such penetrating power that correction for self-absorption of radiation emanating from within the body was unnecessary. Bremsstrahlung associated with abundant high-energy negatrons provides a further source of externally detectable radiations.

[3] This presented to the recipient a total body radiation dose of less than 4 m-rad (28).

[4] Voltage signals from conventional scaling circuits were recorded on separate channels of a standard polygraph, and the resulting frequency record was integrated manually and plotted as an amplitude curve (cf. Figure 1)

TABLE I

Patient data

Experiment	Date	Patient	Unit no.	Sex	Age	Major diagnosis
1	11 21 55	F. McD.	7090R	M	60	Cancer with metastases (some to liver)
2	11 22 55	F. McD.*				
3	12 19 55	R. E.	7107R	F	40	Wilm's tumor with metastases (some to liver)
4	3 30 56	F. M.	7275R	M	42	Cancer with metastases (none apparent in liver)
5	5 24 56	M. M.	7376R	M	65	Paralysis agitans—? idiopathic
6	6 8 56	A. B.	7343R	F	60	Paralysis agitans—postencephalitic; diabetes
7	7 26 56	J. McK.	7284R	F	42	Paralysis agitans—? postencephalitic
8	8 28 56	A. B.)				
9	9 11 56	F. N.	7785R	F	51	Paralysis agitans—? idiopathic; diabetes
10	9 20 56	B. S.	7826R	F	71	Hypertensive vascular disease—recent cerebral vascular accident
11	9 21 56	L. M.	7656R	M	67	Polycythemia vera—old cerebral vascular accident
12	10 2 56	R. B.	7159R	M	48	Essential hypertension
13	10 9 56	G. M.	7835R	M	48	Rheumatoid arthritis
14	11 30 56	(T. D.	7853R	M	60	Diabetes—peripheral vascular insufficiency
15	1 25 57	J. B.	8062R	F	47	Paralysis agitans—? postencephalitic
16	2 1 57	(T. D.)				
17	2 15 57	(B. S.)				
18	2 28 57	H. C.	8103R	F	57	Paralysis agitans—? idiopathic
19	3 5 57	(H. C.)				

* ( ) indicates a second entry of a patient previously cited.

of data was facilitated by the reduction of all radioactivity values to standardized units (29, 30). Experimental readings were adjusted for ambient background and then were corrected for radioisotopic decay, variations in tracer dosage and difference in body weight. Thus blood radioactivity is in units of counts per minute per gram of whole blood per microcurie of administered isotope per 70 kilograms of body weight, while external radiation (radioactivity) is in different units of counts per minute per microcurie per 70 kilograms. The empirical ratio relating the two different units remained fixed within the range of experimental reproducibility, so relative changes in the time courses of blood and body surface data could be compared, as in Figure 1.

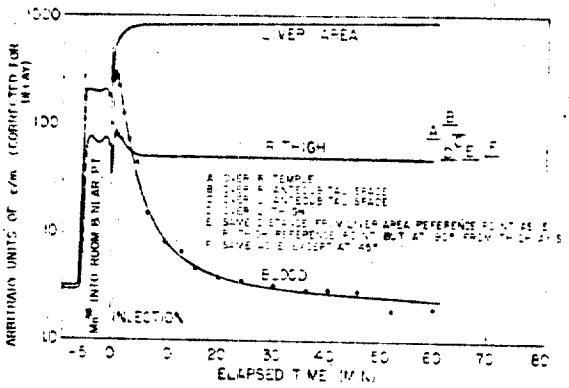

FIG. 1. REPRESENTATIVE EXPERIMENTAL DATA: PATIENT J. B.

Since the injection site is closer to one counter than the other, note the different readings when Mn^{56} is in position for infusion.*

Radiation scatter. External radioactivity over the liver usually reached values 10 to 20 times greater than those over the thigh (*cf.* Figure 1), indicating that radiations scattered from Mn^{56} outside the field of view of this counter were relatively insignificant. Therefore, analyses of liver area data neglected scatter.

RESULTS

Blood and liver area measurements were performed on 18 occasions in 14 individuals (Table I). In one additional instance (Experiment 17) only blood data were obtained.

A. The disappearance of injected Mn^{56} from peripheral venous blood

Compiled blood disappearance data are presented in the scattergram of Figure 2. Beyond 22.5 minutes along the abscissa the regression represented by the data is regarded as diminishing exponentially. A straight line has been fitted to the points in this interval by the method of least squares, using logarithms of the blood radioactivity values.[5] Data from times less than one

[5] The resultant least squares regression line of the logarithms (base 10) of the radioactivity against time has a zero-intercept of 0.6375 (log of 4.340), a slope of 0.01007 (log of exp-0.0232), a standard error of estimate

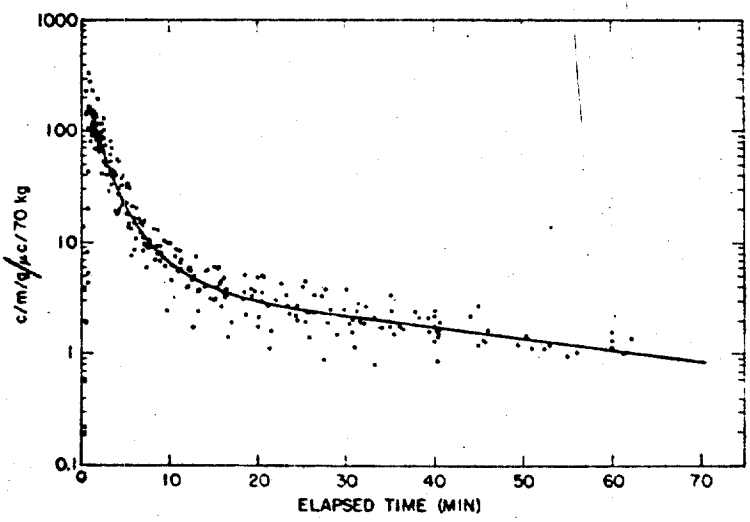

FIG. 2. CLEARANCE OF MN^{56} FROM THE BLOOD

minute are excluded from the calculated curve, because in individual experiments the peak of radioactivity in the peripheral blood occurs at about one minute or less, with the precise time depending on factors pertinent only to the circulatory mixing process. The remaining blood data between 1 and 22.5 minutes of time have been approximated by a best eye-fit curve, which is drawn to fit smoothly with the least squares regression line at later times. The resultant curve (Figure 2) is termed the "blood clearance curve," whose ordinate values are in standardized blood radioactivity units, as defined earlier. These units have dimensions equivalent to the fraction of administered radioactivity remaining in a gram of blood, after adjustment for variations in body weight.

B. The appearance of Mn^{56} radiation over the body surface

Data from a representative experiment are presented in Figure 1.

1. Thigh area radioactivity. The characteristic record of radiation over the thigh reveals an early rise and decay followed by a plateau of activity essentially unchanged for the remainder of the observation period.[6]

2. Liver area radioactivity. The radioactivity in the liver region shows a rapid early rise to a high value which increases only very slightly thereafter. A composite representation of the liver data has been compiled (Table II and Figure 3), using the arithmetic means of the values of individual records at various times after injection.

CALCULATIONS AND DISCUSSION

Analysis of the blood clearance and liver uptake data reported here allows characterization of some important aspects of the metabolic behavior of body manganese.

A. The dynamic metabolic state of manganese

In this portion of the discussion the active turnover of manganese from the systemic circulation will be identified with a rapid transport within the liver and more generally throughout the body.

1. Rapid movement of manganese from the blood. The carrier manganese injected along with the Mn^{56} was less than 3 per cent of the normal plasma or blood content (31). Hence it represented an insignificant perturbation of the endogenous physiology. Therefore, a steep decline in

(σ) of 0.1377 (*i.e.*, plus 37.1 per cent and minus 27.2 per cent about the mean), and a product moment coefficient of correlation (r) of minus 0.632 ($p < 0.001$).

[6] The early rise and fall of thigh radioactivity is compatible with the presence of blood-borne Mn^{56} within the limb. The succeeding plateau appears to reflect radiation scattered largely from the accumulation of tag in the liver and other viscera. This conclusion was based on the virtual lack of change in the "plateau" reading of Figure 1 when the counter was moved in a circle with the liver as center (Curves D, E, and F of Figure 1).

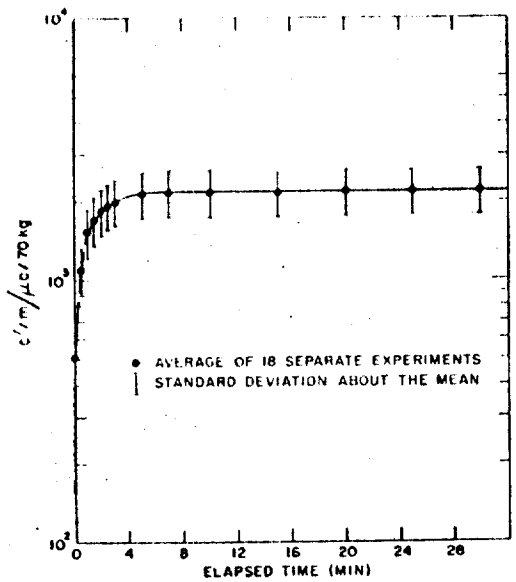

FIG. 3. RADIOACTIVITY OVER THE LIVER AREA AFTER INTRAVENOUS Mn^{54}

Note that the arbitrary counting units of the reference external counter are designated by C' to distinguish them from the readings, C, of the well-counter utilized in the blood work. The length of the bar represents plus-minus one standard deviation about the mean.

blood radioactivity would reflect the rapid movement of both tracer and endogenous circulating manganese to extravascular sites.

Description and significance of the blood clearance curve. Radioactive tracers generally mix with substantially the entire blood volume well within one minute following intravenous injection (29, 30, 32). Figure 2 indicates that much of the radiomanganese initially in the blood is cleared within a minute of virtual mixing, with very little remaining by 45 minutes. However, manganese is excreted much more slowly than this (12, 33–37): Hence the high dilution of initial blood radioactivity implies rapid concentration of the element at certain sites. From partition studies of endogenous and tracer manganese (11, 14), these sites can be presumed to be intracellular.

The blood clearance curve of Figure 2 has been analyzed into three exponentially decaying components (38). It is well represented analytically by the equation

$$a(t) = 265e^{-0.77t} + 44.1e^{-0.26t} + 4.34e^{-0.023t}, \quad 1)$$

where a(t) is radioactivity in standardized units (Figure 4). The first exponential component is comparable to the aperiodic time relation of Sheppard, Overman, Wilde and Sangren describing the rapid blood clearance of indicators (30). The last component is identical with the exponential (antilogarithmic) form of the least squares regression line previously calculated. Although the experimental curve has not been determined for $t < 1$ minute, the "virtual" blood clearance can be extrapolated to zero (injection) time.

Rapidity of transcapillary exchange. The steep decline of blood radiomanganese represented by the first term of Equation *1* implies that in reality the tracer never quite achieves uniform mixing with the entire blood volume, even after several minutes, because along each capillary there must be a strong gradient of radioactivity (29). Furthermore, should this gradient reach zero before the entire length of the capillary be traversed, the transendothelial passage of manganese would actually be more rapid than the calculated value represented by the exponential coefficient, 0.77-minute^{-1}. Keeping in mind the distinction between the net movement of small molecules produced by large concentration gradients and other influences and the rates of transfer *in both directions* through a membrane (39), it is likely that the transcapil-

TABLE II

External radioactivity of the liver (18 experiments)

Elapsed time *min.*	Mean value *C'*/min./μc./70 Kg.*	Standard deviation *C'/min./μc./70 Kg.*
Injection time	507	152
0.5	1,096	223
1.0	1,528	323
1.5	1,698	352
2.0	1,830	362
2.5	1,916	374
3.0	1,978	383
5.0	2,104	418
7.0	2,138	427
10	2,143	427
15	2,132	411
20	2,157	419
25	2,163	415
30	2,171	410
40	2,188	400
50	2,213	373
60	2,216	265

* The units C' refer to counts recorded by the external radiation monitor. These units are to be distinguished from the units, C, which refer to counts recorded by the counter used to determine blood radioactivity.

lary movement is in reality exceedingly rapid, because for water and certain other small molecules the transfer in both directions through the vessel wall occurs at rates that are prodigious in comparison with their rates of *net* transport from the vascular space (39).

The overall turnover rate of blood manganese, therefore, has also been estimated for comparison with that of other small ions which have been studied in detail. R is defined as the fraction of the total intrinsic blood manganese that leaves the vascular compartment in one minute, where

$$\frac{[d\,a(t)]}{(dt)_0} \Big/ a_0 = R = (-\lambda_1 a_1 - \lambda_2 a_2 - \cdots - \lambda_n a_n)/a_0, \quad 2)$$

with a_0 equal to the sum of a_i (29, 32). After substitution from Equation *1*, R becomes about 69 per cent.

This value of R calculated for manganese is roughly the same as for other electrolytes, except potassium, as measured in various species (29). Since diffusion or simple filtration, acting alone, would pass all electrolytes at nearly the same rates (29), this similarity is consistent with a diffusion mechanism of transcapillary transport. By contrast, potassium is exchanged much more rapidly than manganese and other ions (R = 225 per cent in the rabbit), and for this case more complex transfer mechanisms have had to be invoked (29, 40).

2. Important role of the liver. Prompt hepatic uptake and retention of injected radiomanganese demonstrate that the liver partakes importantly in the swift exchange of circulating manganese with intracellular sites; however, significant extrahepatic influx of radiomanganese occurs also. This conforms with the organ distribution of Mn^{56} in animals (11, 36, 41).

Intraportal injection studies with tracer manganese in animals demonstrate nearly complete removal of tag in a single transhepatic passage (42). In such a case the fraction of tracer disappearing from blood per minute approaches f/T, where f is the fraction of the entire systemic arterial outflow traversing the liver and T is the time for a single blood volume of tracer to circulate through the heart and appear in the arterial supply or the portal inflow of the liver. Then when other symbols are employed as before,

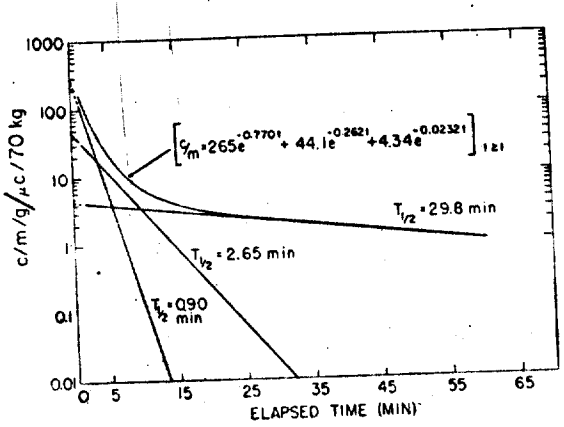

FIG. 4. COMPONENT ANALYSIS OF BLOOD CLEARANCE OF Mn^{56}

$$a(t)/a_0 = e^{-(f/T)t} = e^{-\lambda_1 t}, \quad 3)$$

since f/T is equal to λ (43).

In man the resting cardiac output has been reported variously as about 3 to 6 liters per minute (44, 45), with a total blood volume of 5.0 to 5.5 liters (44, 46). Therefore, one minute represents an apparent average circulation time, T.[7] λ_1 can be taken from Equation *1*, and f then becomes $\lambda_1 T$, or 0.77. However, it is apparent that 77 per cent of the cardiac output cannot traverse the liver, whose total blood flow has been calculated by several methods as about 1,100 to 1,800 ml. per minute (48, 49). It is more reasonable to assume that Mn^{56} is removed from the blood by capillary beds elsewhere equally as fast as it is cleared by the liver.[8]

[7] The exact determination of T is impossible, since its physical meaning is obscure. Different circulatory pathways may not possess identical circulation times, so several circuits of blood over some shorter routes may occur while a single circuit is being made through some longer pathway (29, 47). For example, the shortest complete circulation time in man is about 21 seconds (range of 12 to 26 seconds) (44); whereas the apparent mean circulation time approximates one minute.

[8] In fact, assuming the turnover value of manganese to be about 70 per cent (Equation *2*) with an hepatic blood flow of 1.5 liters per minute (44, 49) out of a total cardiac output of 4 liters per minute (44, 45), it is found that 0.70(1.5/4.0), or about 25 per cent of injected Mn^{56} should accumulate in the liver, provided the intrinsic hepatic clearance is efficient. As a check upon the present calculations, this figure agrees reasonably with the distribution found by Maynard and Cotzias (11, 42) and with that estimated from the data of others (34, 41)

Homogeneity of all pathways of rapid exchange of manganese. The blood clearance of an intravenously injected radioisotope reflects its movement to *all* organs with which there is significant exchange. An identical time-dependence of radiation recorded over a body surface would imply that these same mechanisms of exchange also produced isotope retention within the tissues whose uptake was thereby being measured. This hypothesis has received general experimental support (48, 50–52).

Blood and liver kinetics of Mn^{56} can be correlated by expressing the liver uptake curve as an enantiogram of the difference between liver activity at a given moment and the final equilibrium ("plateau") value, plotted against time (48) (Figure 5). Then over the first four minutes or so Figure 6 shows the blood and liver kinetics to be virtually identical, and even at later times the differences are small. The clearance of the bulk of injected Mn^{56} from the peripheral blood therefore reflects the same means of manganese transport that account for most of its accumulation by the liver.

Extension of the above reasoning implies that most of the influx of manganese into other organs also proceeds in the same fashion as in the liver. Hepatic uptake has been shown to account for the disappearance of well under half of the Mn^{56} from blood. The parallel activity curves can be explained alternatively only by the existence of extrahepatic pathways of rapid manganese interchange which are significantly different in nature from those existing in liver but which possess, nevertheless, virtually identical overall kinetic behavior. This is unlikely.

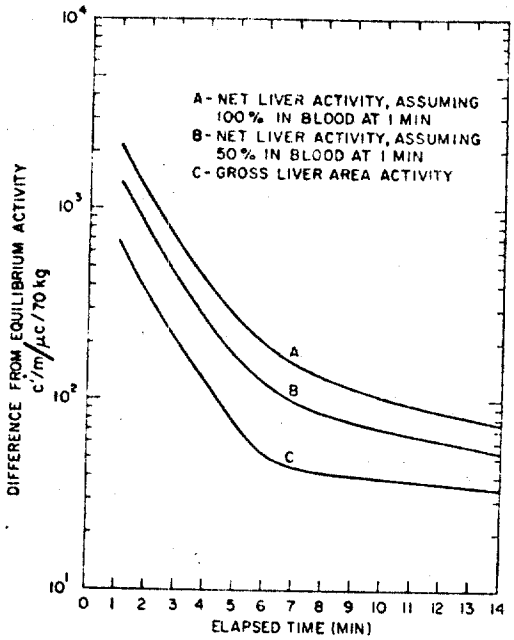

FIG. 5. APPROACH OF LIVER ACTIVITY TOWARD EQUILIBRIUM

The liver area counter also detects Mn^{56} in tissue adjacent to the liver. At the time of maximum blood radioactivity (*ca.* one min.) Curve A assumes all detected activity to derive from tracer still circulating, while B represents a less severe assumption. A and B are calculated from Figure 3, taking circulating Mn^{56} to decline as in Figure 2. Intrinsic hepatic radiomanganese uptake presumably lies within the range A-C.

B. *The active role of mitochondria in manganese metabolism*

In animals early radiomanganese distribution is characterized by preferential mitochondrial accumulation (11, 42). In the present experiments a single mode of manganese metabolism has been

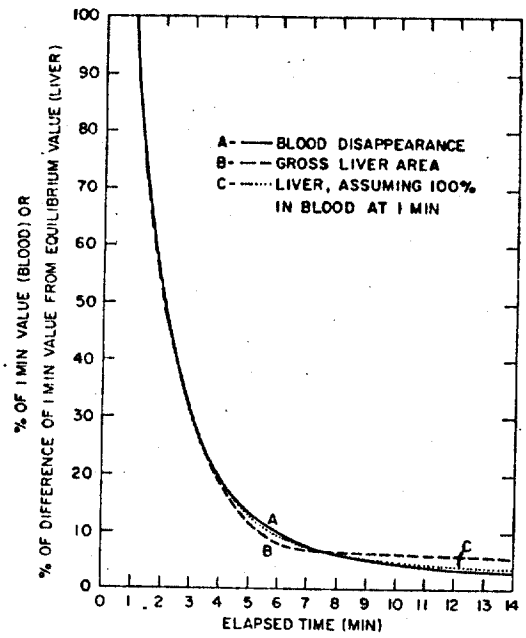

FIG. 6. COMPARISON OF EARLY BLOOD AND LIVER KINETICS

The kinetics of Mn^{56} movement in blood and liver are nearly congruent regardless of whether liver area (B) or calculated organ uptake data (C) are employed.

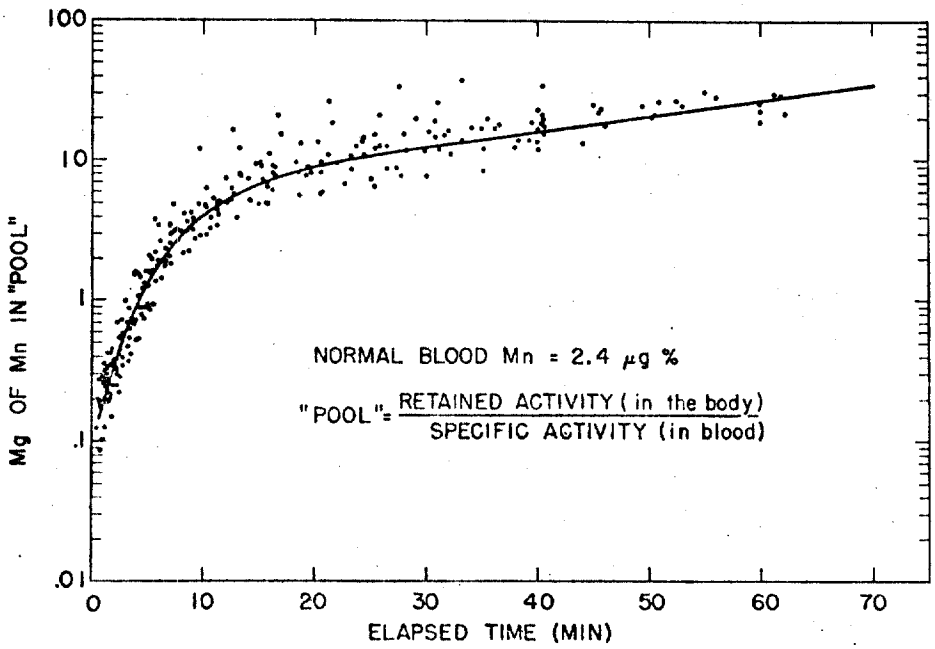

FIG. 7. "APPARENT" EXCHANGEABLE MN "POOL"

shown to determine overall tracer distribution in the body shortly after injection. It therefore appears that in man the mitochondria of all organs participate importantly in the rapid interchange of endogenous tissue manganese with that circulating in the blood.

It has been shown earlier that the first of the analyzed exponential components of the Mn^{56} blood disappearance curve probably represents transcapillary exchange, and later it was inferred that mitochondrial assimilation dominates the transfer of tracer during the first hour. Since the first term of the blood curve has been otherwise identified, whereas the third term does not suggest an exchange as rapid as the rate of mitochondrial uptake, it is probably the second component of the clearance curve (*cf.* Figure 4 and Equation *1*) that reflects strongly the movement of injected radiomanganese into the mitochondria themselves. However, that component's slope and intercept cannot as yet be interpreted in a quantitative fashion.

Insofar as rapid manganese exchange in tissue signifies mitochondrial function, some measure of this intracellular parameter may be determined in man, using the methods reported here. The total mass or activity of mitochondria [9] may be indicated by the initial portion of the radiomanganese blood clearance curve, and particularly by the second exponential component analyzed from it; and the liver area uptake may provide a similar measure of hepatic mitochondria. Independent substantiation would be highly desirable, but present cellular fractionation techniques (11) lack sufficient time resolution to confirm this relationship directly in animals.

C. The labile physico-chemical status of body manganese

Each of the recognized essential mineral micronutrients other than manganese [10] has been demonstrated as a strongly bound intrinsic component of one or many naturally occurring metalorganic compounds (2, 16, 18, 19, 53). In contrast, no manganese metalorganic component has yet been definitely identified in animal tissues (2, 16, 18, 19, 53).[11]

[9] Exclusive of brain, because of the intervening blood-brain barrier.

[10] Iron, cobalt, copper, zinc, and molybdenum.

[11] Since this paper was submitted for publication, evidence has been educed that strongly suggests the presence

The mass of manganese apparently interchanging with Mn^{56} can be represented by a dilution "pool" (54) (Figure 7). Manganese excretion is negligible over the first hour (12, 33–37). Retained activity (Figure 7) is therefore taken as the entire radioisotope dose.

Data on manganese in blood are meager (3); but recently Bowen has perfected a technique of improved resolution and sensitivity wherein small blood samples are radioactivated by reactor neutrons, followed by conventional carrier chemical separation processes and then by counting the isolated Mn^{56} (31). The concentration of manganese has been determined thereby to be 2.4 ± 0.8 μg. per 100 ml., divided roughly equally between cells and serum. This represents the lowest reported value (3, 4, 55, 56).

In the absence of individual values, that of 0.024 μg. per gram from Bowen (31) has been assumed. Thence from the individual data of Figure 2 a dilution "pool" was calculated (Figure 7).

Recent reports cite a total body manganese content of 11 to 20 mg. (2, 46). Extrapolation from human (7) and animal (13) tissue measurements places the body manganese at 9.5 to 12.0 mg. and 22.5 to 27.2 mg., respectively. In any case, by 45 to 60 minutes the Mn^{56} interchanges with a pool representing roughly all the endogenous body manganese (Figure 7).[12]

The rapid dilution of tracer (Figure 7) indicates the absence of a significant nonexchangeable portion of the total body manganese, and conversely, that most body manganese must reside in relatively labile metal-protein complexes or similar configurations compatible with its known chemistry (13–17).

SUMMARY AND CONCLUSIONS

Combined studies of blood clearance and radiation measured over the liver in humans following the intravenous injection of tracer doses of Mn^{56} showed a consistent pattern. The transfer of manganese from blood into intracellular sites was rapid throughout the body.

of a small amount of stable manganese-porphyrin complex in man (Borg, D. C., and Cotzias, G. C. Submitted for publication).

[12] Utilization of the lowest value cited for blood manganese yields a minimum estimate of exchangeable pool size.

The data showed further that most or all of the endogenous manganese became available for interchange with tracer within an hour. Hence most of the element within the body must exist in dissociable chelates or other relatively labile intracellular combinations. Considering the constancy of tissue concentrations of manganese, this demonstrates that manganese metabolism is under homeostatic control.

At least three, and probably four, apparent body compartments participate to some extent in the distribution of Mn^{56} within the first hour. The first component analyzed from the blood disappearance curve probably reflects the net transcapillary passage of the element.

Tracer concentration falls steeply in the capillaries, and about 70 per cent of the normal blood manganese leaves the circulation each minute. The transendothelial transport of manganese appears to be by passive diffusion. It follows that net turnover from the blood is perfusion-limited, despite subsequent intracellular concentration.

Identical mechanisms appear responsible for most of the swift inflow of manganese into liver and simultaneously into other organs. Mitochondrial uptake throughout the body is regarded as underlying the bulk of this rapid tissue retention, which largely determines a second component analyzed from the blood data as well.

A mitochondrial role is in accord with the known biochemistry of manganese *in vitro*. Therefore, the procedures reported here are proposed as a method for measuring mitochondrial mass or function in patients.

ACKNOWLEDGMENTS

The authors wish to acknowledge the expert assistance in this work of J. J. Greenough and H. Hamel.

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Am. J. Physiol., 211: 203-206, 1966

Dependence of manganese turnover on intake[1]

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BRITTON, A. A., AND G. C. COTZIAS. *Dependence of manganese turnover on intake.* Am. J. Physiol. 211(1): 203-206. 1966.—The turnover of parenterally administered Mn^{54} was found to be directly related to the level of stable manganese in the diet of mice over a wide range. The concentration of Mn^{54} in various tissues was also directly related to the level of Mn^{55} in the diet, suggesting that a variable excretion rather than a variable absorption regulates the concentration of this metal in tissues. The whole-body turnover of Mn^{54} can be approximated roughly by two exponential functions suggesting that manganese is distributed in at least two sets of compartments with different sizes and transfer constants. The relative size and transfer constant of the compartment with the slower turnover was linearly related to the concentration of manganese in the diet.

opes; total-body counting

EARLIER STUDIES of the metabolism of the transition metal, zinc, indicated that the absorption, distribution, and excretion of the tracer, Zn^{65}, were sensitive to metabolic burdens with stable zinc (3, 5, 8). Conversely, Cd^{109} showed rather striking insensitivity when investigated in a similar fashion (4). Zinc is accepted as an essential element, whereas cadmium is considered a nonessential one. Hence, this difference in their behavior was thought to reflect differences in the efficacy of the mechanisms which control their respective metabolism.

The transition metal, manganese, is an essential element, but little is known concerning the control of its metabolism. Therefore, it was of interest to investigate whether its absorption, distribution, and excretion might follow the precedent of zinc. Furthermore, the apparent constancy of the tissue concentrations of manganese indicated that a search for possible homeostatic mechanisms might be rewarding.

The present paper reports experiments on manganese similar to those conducted with the two metals alluded to above: long-range observations were made on the rate of elimination and tissue distribution as affected by a single variable, the intake of stable manganese. The striking dependence of the metal's rate of excretion on the variable under study suggested that the concentrations of manganese in tissues must remain essentially stable over a wide range of metabolic burdens with this metal. This is confirmed in an accompanying paper (9).

METHODS

Animals. A total of 180 Swiss albino mice of the Hale-Stoner strain was studied. These were 4-5 weeks old at the start; groups of six were housed in steel cages. Because occasional animals were excluded from the final analysis by virtue of accidental injection of the tracer into the bladder or the gut, some groups consisted of five, rather than six animals.

Diets. On arrival in the laboratory all animals were maintained on Purina laboratory chow, which is a high-manganese diet (about 32.5 mg manganese/kg). During the actual tests the animals ate a manganese-poor diet, namely evaporated milk, diluted with an equal volume of demineralized water. The milk offered to the animals contained about 18 μg manganese/liter, as determined by neutron activation analysis (7, 10). Between the end of the period on Purina and the injection of the tracer, the animals ate the diluted milk without added manganese. This period is referred to as "prefeeding period."

The milk diet was supplemented as indicated with manganous sulfate in final molar concentrations ranging between 2.3×10^{-7} and 1.25×10^{-1} M (from 18 μg to 6.9 g Mn^{++}/liter, respectively). The concentrations offered to each group are listed in Table 1. As estimated from the milk dispensers, all animals appeared to consume similar amounts of diet except the group on the highest manganese intake, which consumed less. Due to some leakage, only the maximum intake could be estimated, and this approximated 15 ml/animal per day. All animals received distilled demineralized water ad lib.

Isotopes. Carrier-free $Mn^{54}Cl_2$ dissolved in approximately 0.5 N HCl was received from either the Nuclear Science and Engineering Corp. or the U.K. Atomic Energy Authority. Standardized doses of 4 or 5 μc in 0.1 ml 0.9% NaCl solution were injected intraperitoneally.

Radioactivity measurements. A Baird Atomic instrument

Received for publication 8 October 1965.
[1] This work was supported by the U.S. Atomic Energy Commission.
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TABLE 1. *Concentrations of Mn in diets of various groups*

Exp. No.	Group	Basal Diet, Days	Supplement (Molarity $MnSO_4 \cdot H_2O$)
1	1–3	5	4.69×10^{-7} M; 1×10^{-3} M; 2×10^{-3} M
2	1–4	8	4.69×10^{-7} M; 5.01×10^{-4} M; 4×10^{-3} M; 8×10^{-2} M
3	1–3	11	4.69×10^{-7} M; 1.6×10^{-2} M; 3.2×10^{-2} M
4	1–12	19	4.69×10^{-7} M; 5.01×10^{-4} M; 1×10^{-3} M; 2×10^{-3} M; 4×10^{-3} M; 8×10^{-3} M; 1.6×10^{-2} M; 2.4×10^{-2} M; 3.2×10^{-2} M; 4.8×10^{-2} M; 6.4×10^{-2} M; 1.25×10^{-1} M
5	1–12	26	4.69×10^{-7} M; 7.89×10^{-7} M; 2.07×10^{-6} M; 8.47×10^{-6} M; 4.05×10^{-5} M; 2.01×10^{-4} M; 1×10^{-3} M; 5×10^{-3} M; 2.5×10^{-2} M

was used for the measurements of both the total-body radioactivity and of that located in the organs of these animals. This consisted of a NaI, thallium-activated crystal measuring 3 x 3 inches containing a well measuring 1.5 x 2.5 inches, connected to a preamplifier, amplifier, and scaler. Intact animals were inserted into a ventilated plastic tube and introduced into the counter. The counts were corrected as necessary for background, coincidence, and decay, but not for geometry. The first measurement was used as the 100% retention in the plotting of all subsequent counts, except for *experiment 5* (Table 1), in which the total-body radioactivity at the time of the addition of the manganous supplements (16th day after the injection of Mn^{54}) was used as the point of reference. A standard was counted during each session to determine the stability of the instrument and to correct for radioactive decay. Standard error of counting was ordinarily within 1%.

The data for each mouse were plotted on semilogarithmic paper as percent retention vs. time. The mean values were computed for each group of mice and a best-fit curve was drawn visually through the means. The final four points of these whole-body turnover curves fell on a straight line in the majority of the groups. However, in the experiments with the three highest concentrations of dietary manganese only the two last points could be used to delineate a straight line. The slope and intercept of this line represented the "slow" component discussed in the text. The values of the slow component were subtracted from each retention curve and the remainder is referred to as the "fast" component. Further analysis of the fast component into constituent ones was inaccurate due to scarcity of experimentally determined points. Therefore, it was treated as if it constituted a single component.

Experimental design. The period of prefeeding was varied from 5 to 26 days and was followed by two separate types of experimentation: *1*) The different amounts of $MnSO_4$ were offered immediately to the respective groups (*exps. 1–4*, Table 1). *2*) The basal low-manganese diet was continued for 16 days following the injection of the isotope, before the various concentrations of $MnSO_4$ were added to the diets (*exp. 5*). In both cases no further changes of the regimen were made. In *experiments 1* and *4* of Table 1, the animals were not decapitated until their total-body radioactivity had reached about 1.5% of the initial level, to determine the period of time necessary for the excretion of almost all of the tracer. Conversely, all animals in *experiment 5* were sacrificed 57 days after the isotope had been injected.

Following decapitation the mice were dissected, the carcasses were assayed for radioactivity separately, but the livers, gastrointestinal tracts, brains, and "small viscera" from each group were pooled. Small viscera included the heart, lungs, spleen, testes, and thymus. The data are reported as average values per mouse. The sum of the radioactivity recovered with the tissues exceeded that recorded within the intact animals by about 20% because of factors alluded to elsewhere (11).

RESULTS

Manganese deprivation and total-body loss of Mn^{54}. All animals maintained on the low-manganese milk showed significant losses of their total-body Mn^{54} over the entire period of observation (i.e., Figs. 1 and 2). However, the total amount of tracer excreted by these animals decreased linearly as a function of time on manganese deprivation. The animals fed milk for only 5 days prior to injecting the tracer excreted only 35% of their total body radioactivity at the slow rate as determined by extrapolation to zero time. With increasing time on the low-manganese diet the percent of the isotope excreted at the slow rate increased linearly and reached 95% when the prefeeding time was extended to 26 days. The half-life of this slow component seemed to be affected to a lesser extent: it decreased from about 50 to about 35 days over the stated range of prefeeding periods. The changes in the half-life of the fast component were not deemed significant.

Acceleration of the total-body loss of Mn^{54} following adminis-

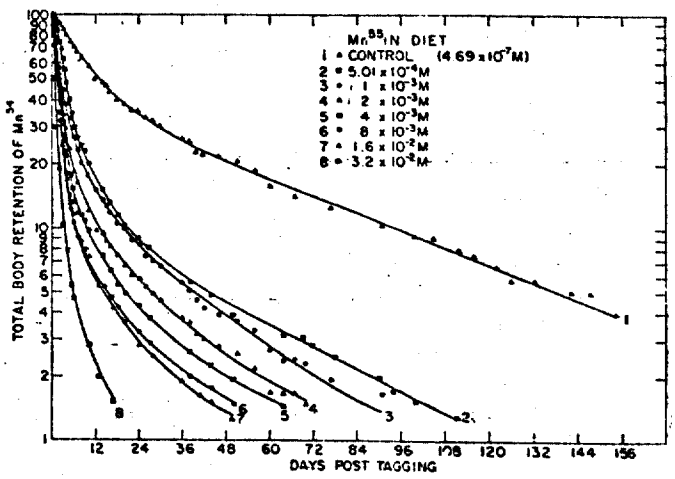

FIG. 1. Change in turnover of Mn^{54} in mice as a function of the concentration of Mn^{55} in the diet. The stable metal salt was added to the diet at the time that the radioisotope was injected.

ation of Mn^{55++}. Addition of the manganous sulfate to ne milk immediately after injection of Mn^{54} accelerated the rate of elimination of the injected tracer by these mice. The mean values from *experiments 1–3* (Table 1) are shown in Fig. 1. *Experiment 4* (not illustrated because of higher ranges of the data) confirmed these findings: the ranking of the total-body retention curves had duplicated the ranking of the concentrations of manganese in the milk. Furthermore, when the time necessary for excretion of 98% of the radioactivity was plotted against the dietary concentration of Mn^{55++}, there became apparent a linear, inverse relation between these two parameters. The slope of this line indicated that 10-fold increments in dietary manganese diminished the time necessary to excrete 98% of the isotope by roughly one-half. An identical line with an identical slope was obtained when the intercepts of the slow components were plotted against the dietary concentrations. Concordantly the half-life of the fast components diminished from about 10 to 2 days with increasing concentration of manganese in the diet.

In order to define these total-body retention curves, it was necessary to observe these animals for long periods of time (Fig. 1), during which the isotope remaining in the tissues might have become progressively less available for exchange with the stable manganese absorbed from the diet. Hence these measurements might have underated the initial effect of stable manganese on the excre-on of the tracer. It was necessary therefore to demonstrate: *1*) whether the isotope became less exchangeable with passing time; and *2*) whether, in spite of this, it could be shown that progressively larger manganese concentrations in the diet would progressively accelerate the

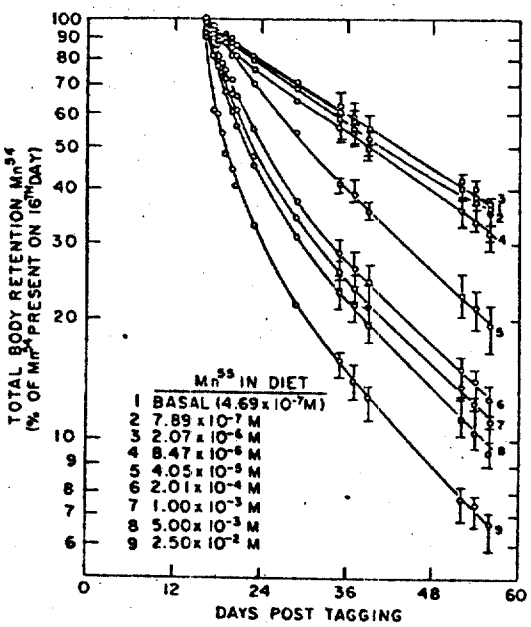

FIG. 2. Change in turnover of Mn^{54} in mice as a function of ne concentration of Mn^{55}. The stable metal salt was added to the diet 16 days after the isotope was injected. Brackets indicate the range of data.

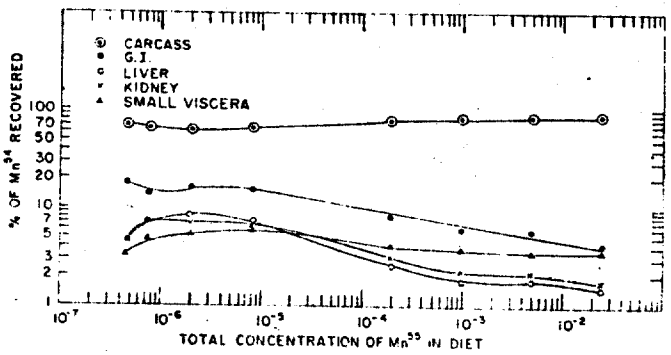

FIG. 3. Change in the distribution of Mn^{54} as a function of the concentration of Mn^{55} in the diet.

tracer's excretion. In *experiment 5*, 44% of the tracer was excreted during the 16 days which followed its injection and the remainder was being eliminated at the slow rate.

The total-body retention curves for each group in *experiment 5* are shown in Fig. 2. This time the three lowest concentrations of manganese in the milk had no definite effect on the turnover rate of the metal. However, with higher manganese concentrations (ranging from 8.47×10^{-6} M to 2.5×10^{-2} M $MnSO_4$) the ranking of the total-body retention curves again duplicated the ranking of the diets. Furthermore, increments of the concentration of manganese in the diet resulted in linear decreases in both the intercept and the half-life of the slow components of the total-body retention curves. This indicated only one sharp contrast to the experiments discussed above, namely, that the changes in turnover brought about by changes in the diet were now smaller than when the salt was fed immediately after the isotope had been injected.

The sum of these experiments indicated that the excretion of the isotope by these mice was proportional to the amount of metal supplied in their diet. It was deemed interesting to identify the individual tissues which had participated in this exchange.

Tissue distribution of the radioactivity. In spite of its low levels, the radioactivity remaining in the animals from *experiments 1–4* was of interest since its distribution contrasted to that of the animals discussed immediately below. In *experiments 1–4* the radioactivity remaining in the carcass ranged between 78 and 93% of the total. The gastrointestinal tract contained 2–13%, the pooled small viscera 1.5–6%, the liver 0.5–5%, and the brain 1–3%. These large ranges did not permit conclusions with regard to effects of the varying dietary concentrations of manganese, but the data were worth comparing to the distribution of the isotope shortly after its injection. A group of five animals kept on the milk for 26 days was injected intraperitoneally with Mn^{54}. At sacrifice 2 hr later, the mean and range of the total radioactivity recovered among the tissues were as follows: carcass 16% (14–20); gastrointestinal tract 55% (53–60); liver 16% (14–19); kidney 5% (4–6); small viscera 7% (6–8); and brain 0.2% (0.05–1.0). By contrast, the first points in Fig. 3 indicate the change in this distribution after the

lapse of 56 days in the animals from *experiment 5*: at that time the highest proportion of the recovered radioactivity was located in the carcass. This increased linearly with increasing concentration of manganese in the diet, whereas all other tissues tested showed a linear decrease in the proportion of the radioisotope recovered at sacrifice. The brain contained only 1–2 % of the remaining radioactivity. It is evident that the smaller of these manganous loads had not affected materially the tissue distribution of this metal, as they had failed to affect its total-body turnover rate.

DISCUSSION

The observations made on animals consuming only the low-manganese milk suggest the existence of an impressive obligatory loss for this metal: highly significant excretion occurred even during long periods of manganese intakes low enough to cause manganese deficiency (2). The total isotope lost during these observations depended on the time the animals had spent on the low-manganese diet prior to having been injected with tracer: while the prefeeding periods were being increased, a progressively larger percent of the total isotope became excreted at the slow exponential rate. It was indeed necessary to maintain milk feeding for about 1 month before the excretion of the isotope proceeded almost solely at a single, slow exponential rate. These findings argued against the existence of a mechanism which rapidly shuts off manganese excretion in the presence of deficiency.

It was known from earlier work (6) that gross metabolic loads of a manganous salt would accelerate the excretion of injected Mn^{54}. The present experiments extend these observations over a wide range of metabolic loads, including toxic levels, suggesting that the limit of absorption of manganese from the gut was probably not reached in the present experiments. It is evident that extensive isotopic exchange had taken place between the parenterally injected radiomanganese (Mn^{54}) and the orally administered stable metal (Mn^{55}). Indeed, there was demonstrated a linearity between the rate of excretion of the tracer and the level of manganese in the diet of these mice. This linearity held also when the distribution of Mn^{54} among various organs was studied and suggests that when excess manganese was fed, the metal was being absorbed from the diet in proportion to the amount presented for absorption. Hence the metabolism of this metal appears to be regulated primarily by means of its excretion rather than by both regulated absorption and excretion as had been the case with zinc (3, 5, 8).

The present experiments show that the radioisotope retained became progressively less available for exchange with the metal's stable isotope as a function of time after administration. Therefore the rapidity with which the excretory routes respond to the administration of excess manganous sulfate was underestimated in these long-lasting experiments. This is further borne out elsewhere (1). Furthermore, the relative contribution of various tissues to the excretion of this metal can be tentatively assessed by these experiments. Two hours after tagging the radioactivity was located primarily in the parenchymatous organs, whereas with passing time the carcass contained increasingly higher proportions of the radioactivity present at sacrifice. This shift was accentuated linearly as a function of the manganous sulfate added to the diet. Hence it appears that the viscera contained readily exchangeable, rapidly excretable manganese in much higher proportion than did the carcass. The latter represents in all likelihood the incorportion of manganese into bone.

Thanks are due to Miss Carol Rozanski for expert assistance with the preparation of the diets, some of the assays for Mn^{54}, and the extensive processing of the data.

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THE EFFECT OF COPPER SULPHATE AND MANGANESE SULPHATE ON THE TOXICITY AND TREMOR OF TREMORINE AND ON SOME PERIPHERAL RESPONSES INDUCED BY ACETYLCHOLINE, NORADRENALINE, DOPAMINE AND 5-HYDROXYTRYPTAMINE

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(*Received* 11 *March* 1966; *accepted* 14 *June* 1966)

Abstract—Copper sulphate and manganese sulphate markedly potentiated the toxicity of tremorine in mice. Neither metal salt caused tremor on its own. Copper sulphate, administered prior to tremorine, increases the tremor and appears to prolong it. Manganese sulphate caused a significant increase in tremor only in the early stages of the experiment.

Copper sulphate, at a dose of 20 μg/ml significantly potentiated the response of guinea pig ileum to acetylcholine, whereas manganese sulphate inhibited the standard response at all dose levels. On the same preparation, both metal salts inhibited the responses to a standard dose of 5-hydroxytryptamine. All doses of copper sulphate used inhibited the noradrenaline standard response of guinea pig vas deferens and also caused inhibition of the response to dopamine at the 2 and 20 μg/ml dose levels of metal salt. However, at a high dose of 200 μg/ml copper sulphate there was non-significant potentiation of the dopamine response. Manganese sulphate, in doses of 2 and 20 μg/ml potentiated responses to both catecholamines, but markedly inhibited responses at high doses of metal salt.

CURZON AND SCHNIEDEN[1] showed that copper sulphate potentiated both the toxicity and tremor of NN-diethylcysteamine. Tremorine (1, 4-dipyrrolidino-2-butyne) can also cause tremor.[2] In Wilson's disease and Manganism excess amounts of copper and manganese occur in the body and are associated with extrapyramidal disorders in which tremor is a common feature. In view of this, the effect of copper sulphate and manganese sulphate on the toxicity and tremor caused by tremorine in mice has been investigated.

Tremorine is also known to increase the concentration of brain acetylcholine,[3, 4] and to affect brain monoamine levels.[5, 6] It was therefore of interest to examine the effects of a copper and manganese salt on the responses of some isolated tissues to acetylcholine, noradrenaline, dopamine and 5-hydroxytryptamine.

METHODS

Male albino mice (T T strain), weighing between 17 and 30 g, were used for the determination of the effects of copper sulphate and manganese sulphate on tremorine toxicity and tremor.

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Acute toxicity determination

The mice were placed singly in glass jars. Experiments were conducted in a constant temperature room at 25·5° ± 1°. Injections were given by the intraperitoneal route and the animals were allowed free access to food and water up to the time of the injection. One hour LD_{50} values were determined for copper sulphate, manganese sulphate and tremorine. The effect of a dose of copper sulphate and manganese sulphate, below the theoretical LD_0 of these drugs, on the LD_{50} value of tremorine was then determined. A control group of mice were injected with 0·25 ml of 0·9% sodium chloride solution (saline). The LD_{16}, LD_{50} and LD_{84}, and estimate of relative potency, were calculated by the method of Litchfield and Wilcoxon.[7]

Tremor studies

The tremor recording apparatus was a modification of that described by Ahmed and Taylor.[8] The output from the gramophone pick-up was fed to a frequency selective amplifier, the output of which charged a capacitor. The amount of movement of a mouse registered as a reading on an arbitrary scale, between 0 and 50, on a micro-ammeter. The amplifier was most sensitive to frequencies of about 11 c/s and thus amplified frequencies of the order of those found in tremor to a greater extent than those of ordinary walking movements. Experiments were performed at a temperature of 25·5° ± 1°. A mouse was placed in each of four plastic containers which were then suspended from the needle-hole of a gramophone pick-up. The animals were allowed to settle down for about 30 min, after which they were treated with test drug or control. All injections to the mice were given by the intraperitoneal route. The mice were injected with either 0·9% saline as control, tremorine alone, copper sulphate plus tremorine or manganese sulphate plus tremorine. The first tremor recording was taken 10 min after injection of tremorine and thereafter every 5 min over a period of 1 hr. Where a metal salt was given, it was injected 15 min before the tremorine. The tremor in a mouse occurred in short bursts. Therefore, to obtain a satisfactory recording of the tremor, six readings were taken over a period of 1 min. Each reading lasted 5 sec e.g., for a recording of tremor at the 10th minute after injection of tremorine, readings were taken from 0 to 5 sec, 10 to 15 sec, 20 to 25 sec, 30 to 35 sec, 40 to 45 sec and 50 to 55 sec. The mean of these six readings during the 10th minute was taken as the tremor score for that minute.

Isolated tissues

Acetylcholine and 5-hydroxytryptamine. Guinea pig ileum. Guinea pigs, of either sex and weighing 300–600 g were used. A length of gut of about 4 cm was removed from the distal end of the ileum. This was immersed in Tyrode solution contained in a 20 ml organ bath at a temperature of 33·5° ± 0·5°. The solution was aerated by a mixture of 95% oxygen and 5% carbon dioxide. Contractions of the gut were recorded with an isotonic frontal lever on a smoked paper kymograph. Magnification of the response was about six times. Loads of 0·5–1·0 g and 0·5 g were placed at the point of attachment of thread to the lever for the experiments with acetylcholine and 5-hydroxytryptamine respectively.

For both sets of experiments, standard doses of either acetylcholine or 5-hydroxytryptamine were added to the bath at 5-min intervals. The method used was to obtain three standard contractions of the ileum about 50 per cent of the maximum, to either

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acetylcholine or 5-hydroxytryptamine. The former was added to the bath for a contact time of 30 sec and the latter for a contact time of 25 sec. In the next cycle, copper sulphate or manganese sulphate was added 30 sec before the drug. The drug was then added to the bath for a contact time similar to that used during the control period. The preparation was washed several times, at constant intervals, after addition of acetylcholine and 5-hydroxytryptamine.

Noradrenaline and dopamine. Guinea pig vas deferens. The isolated guinea pig vas deferens was prepared as described by Leach.[9] Male guinea pigs of 300–600 g were used. Conditions were similar to those described for the ileum experiments but a temperature of 31·5 ± 0·5° was used. For noradrenaline experiments the response was magnified about seven times with a 0·5-g load on the tissue at the point of attachment to the lever. Corresponding figures for dopamine were eight times magnification and 1-g load.

Five minutes was found to be a satisfactory cycle time for the noradrenaline experiments, with a drug contact time of 1 min. Three standard submaximal contractions were established for the noradrenaline standard dose. Then the effect of either copper sulphate or manganese sulphate on the standard noradrenaline response was examined, the metal salt being added 30 sec before the catecholamine. In all experiments the tissue was washed several times at constant intervals following addition of drugs to the bath.

The dopamine experiments were conducted in a similar manner to those involving noradrenaline, except that a 7-min cycle was used, and a contact time of 2 min, 15 sec was required owing to a lag period before the appearance of the dopamine response.

Drugs used

Copper sulphate ($CuSO_4,5H_2O$), Analar grade. Manganese sulphate ($MnSO_4$, $4H_2O$). Analar grade. Tremorine dihydrochloride* (1,4-dipyrrolidino-2-butvne dihydrochloride). L-Noradrenaline bitartrate.* Serotonin creatinine sulphate.* Acetylcholine chloride* and 3-hydroxytyramine hydrochloride.*

In the text above, items marked with an asterisk* will be referred to as tremorine, noradrenaline, 5-hydroxytryptamine, acetylcholine and dopamine respectively. All concentrations of drugs are expressed in terms of drug *salt* not base e.g., 1 μg noradrenaline refers to 1 μg L-noradrenaline bitartrate. Copper sulphate and manganese sulphate refers to their respective hydrated salts.

RESULTS

The 1 hr i.p. toxicities of tremorine, copper sulphate and manganese sulphate in the mouse are shown in Table 1. No mice died in the control group of mice injected with normal saline.

The theoretical maximum LD_0 of copper sulphate was calculated to be 39 mg/kg body wt. and that of manganese sulphate to be 370 mg/kg body wt. Doses of metal salt below these calculated LD_0's i.e. 30 mg/kg copper sulphate or 300 mg/kg manganese sulphate were injected into mice 25 min before injecting the same animals with the LD_{50} dose of tremorine, i.e. 315 mg per kg body wt.

From Table 2 it can be seen that administration of copper sulph tentiates the oxicity of tremorine, increasing the percentage death from 50 to 100 per cent. Manganese sulphate also has a marked potentiating effect on the toxicity of tremorine.

The effects of copper sulphate (30 mg/kg) and manganese sulphate (100 mg/kg) on tremorine tremor in mice is shown in Table 3. The metal salt was injected 15 min prior to tremorine. Neither salt caused tremor on its own nor appeared to interfere with the characteristic autonomic effects of tremorine. Tremor could not be evaluated when higher doses of manganese sulphate were used (i.e., 300 or 200 mg/kg) as the combination of these doses with the tremorine proved too toxic to the mice.

TABLE 1. THE 1-HR TOXICITIES OF INTRAPERITONEAL TREMORINE, COPPER SULPHATE AND MANGANESE SULPHATE IN THE MOUSE

Drug	No. of animals used	LD_{16}	LD_{50}	LD_{84}
Tremorine	106	255	315 (296–335)	389
Copper sulphate	90	51	89 (75–106)	155
Manganese sulphate	54	414·5	534 (479–595)	684

The doses are given in terms of mg/kg body wt. The figures in brackets represent the 95 per cent confidence limits for the LD_{50}.

TABLE 2. THE EFFECT OF COPPER SULPHATE AND MANGANESE SULPHATE ON THE 1-HR LD_{50} OF TREMORINE

Treatment	Dose	No. of animals used	No. dead after tremorine	% Death
0·9% saline; tremorine 25 min later	0·25 ml 315 mg/kg	16	8	50
Copper sulphate; tremorine 25 min later	30 mg/kg 315 mg/kg	16	16	100
Manganese sulphate; tremorine 25 min later	300 mg/kg 315 mg/kg	16	15	93·75
Copper sulphate alone	30 mg/kg	6	0	0
Manganese sulphate alone	300 mg/kg	6	0	0

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Isolated tissue experiments (*see Tables* 4 *and* 5 *and Figs.* 1 *and* 2)

Copper sulphate, in a dose of 20 μg/ml produced some potentiation of the response of the ileum to a standard dose of 0·02 μg/ml acetylcholine, but, at 2 μg/ml, it had no significant effect. Following the addition of 200 μg/ml copper sulphate to the bath potentiation occurred but the baseline did not return to the control position after washing.

TABLE 3. THE EFFECT OF A PREVIOUS INJECTION OF COPPER SULPHATE OR MANGANESE SULPHATE ON TREMORINE TREMOR

Treatment No.	Mean tremor score at times after tremorine or saline			
	10 min	15 min	20 min	40 min
Tremorine (30 mg/kg) (1)	28·30	22·49	20·43	11·33
Copper sulphate (30 mg/kg) 15 min later tremorine (30 mg/kg) (2)	29·45	30·96	29·03	17·98
0·25 ml 0·9% saline (3)	11·43	9·80	9·98	5·42
Tremorine (30 mg/kg) (4)	19·52	14·89	17·67	6·87
Manganese sulphate (100 mg/kg) 15 min later tremorine (30 mg/kg) (5)	26·10	21·30	21·53	15·08
0·25 ml 0·9% saline (6)	6·63	7·80	9·08	7·75
Mann-Whitney Test[10] comparing (1) and (2)	N. S.	*	*	*
comparing (1) and (3)	†	†	*	N. S.
comparing (4) and (5)	*	N. S.	N. S.	†
comparing (4) and (6)	†	*	*	*

* Significant at 5 per cent level, † at 1 per cent level. N.S.—not significant. Each reading is the mean of ten experiments.

TABLE 4. THE EFFECT OF COPPER SULPHATE ON STANDARD SUBMAXIMAL CONTRACTIONS INDUCED BY ACETYLCHOLINE (0·02 μg/ml) AND 5-HYDROXYTRYPTAMINE (0·2 μg per ml) ON ISOLATED GUINEA PIG ILEUM AND BY NORADRENALINE (2 μg/ml) AND DOPAMINE (5 μg/ml) ON ISOLATED GUINEA PIG VAS DEFERENS

	Concentration of copper sulphate (μg/ml)	No. of animals used	Mean % increase (+) or decrease (−) in response ± S.E. produced by metallic salt	*P* (Mann-Whitney[10] Test)
Acetylcholine	2	12	(−) 3·91 ± 4·30	N.S.
	20	12	(+)31·94 ± 6·33	< ·0014
	200	10	(+)14·27 ± 6·84	< ·0054
5-Hydroxy-tryptamine	2	6	(−)14·26 ± 4·32	< ·002
	20	7	(−)29·36 ± 5·83	< ·00014
	200	5	(−)49·78 ± 9·32	< ·002
Noradrenaline	2	6	(−)24·35 ± 5·65	< ·002
	20	5	(−)64·44 ± 10·49	< ·002
	200	8	(−)71·19 ± 5·94	< ·0003
Dopamine	2	5	(−)19·54 ± 8·52	= ·02
	20	6	(−)40·78 ± 12·31	< ·02
	200	5	(+)37·85 ± 14·32	> ·05 N.S.

N.S.—Not significant.

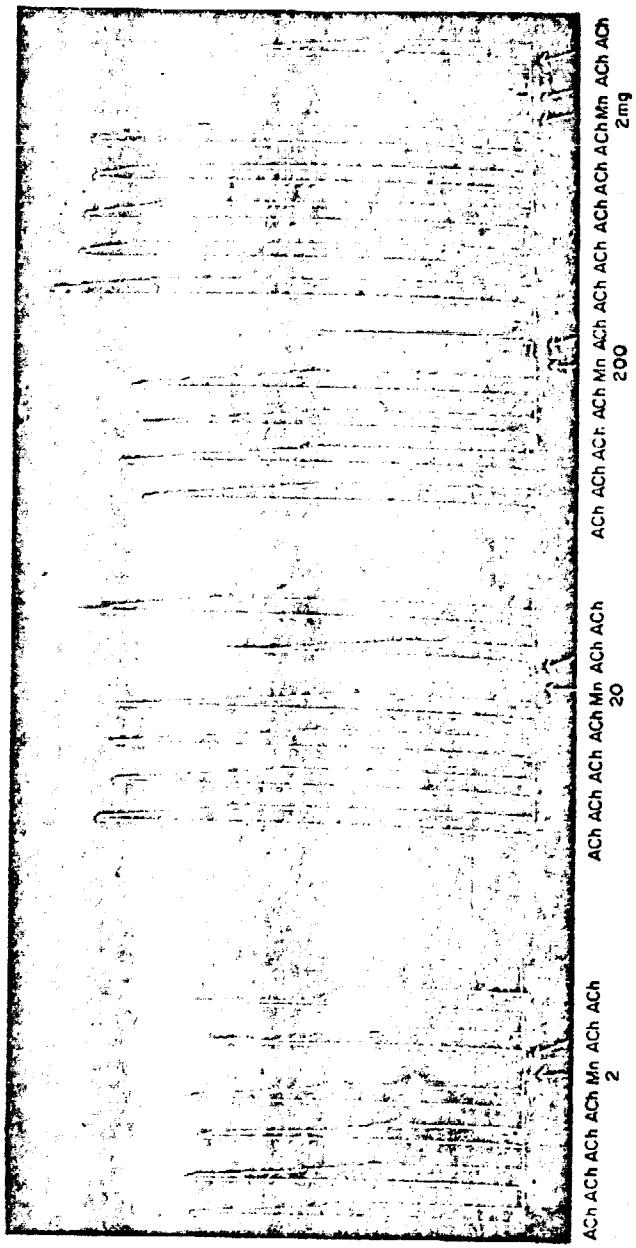

FIG. 1. Guinea pig ileum: The effect of manganese sulphate at four dose levels, on standard contractions induced by 0·4 μg/ml acetylcholine. Manganese sulphate added 30 sec before acetylcholine ACh—acetylcholine; Mn—manganese sulphate (doses in μg/ml or in mg/ml where shown).

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TABLE 5. THE EFFECT OF MANGANESE SULPHATE ON STANDARD SUBMAXIMAL CONTRACTIONS INDUCED BY ACETYLCHOLINE (0·4 μg/ml) AND 5-HYDROXYTRYPTAMINE (0·2 μg/ml) ON ISOLATED GUINEA PIG ILEUM AND BY NORADRENALINE (1 μg/ml) AND DOPAMINE 5 μg/ml) ON ISOLATED GUINEA PIG VAS DEFERENS

	Concentration of manganese sulphate (μg/ml)	No. of animals used	Mean % increase (+) or decrease (−) in response ± S.E. produced by metallic salt	P (Mann–Whitney[10] Test)
Acetylcholine	2 μg/ml	5	(−) 9·64 ± 3·35	< ·05
	20 μg/ml	5	(−)22·45 ± 3·53	< ·002
	200 μg/ml	5	(−)59·62 ± 5·27	< ·002
	2 mg/ml	5	(−)96·27 ± 0·53	< ·002
5-Hydroxytryptamine	2 μg/ml	5	(−)18·35 ± 5·89	< ·002
	20 μg/ml	5	(−)46·88 ± 5·08	< ·002
	200 μg/ml	4	(−)94·83 ± 1·77	< ·002
	2 mg/ml	5	(−)100	< ·002
Noradrenaline	2 μg/ml	8	(+)20·84 ± 7·24	< ·006
	20 μg/ml	9	(+)24·96 ± 27·39	N.S. > 0·3
	200 μg/ml	5	(−)92·49 ± 3·28	< ·002
	2 mg/ml	6	(−)95·62 ± 0·71	< ·002
Dopamine	2 μg/ml	8	(+) 7·94 ± 8·21	N.S. > 0·7
	20 μg/ml	9	(+)38·57 ± 9·84	< ·00032
	200 μg/ml	9	(−)95·42 ± 4·13	< ·002
	2 mg/ml	5	(−)100	

N.S.—Not significant.

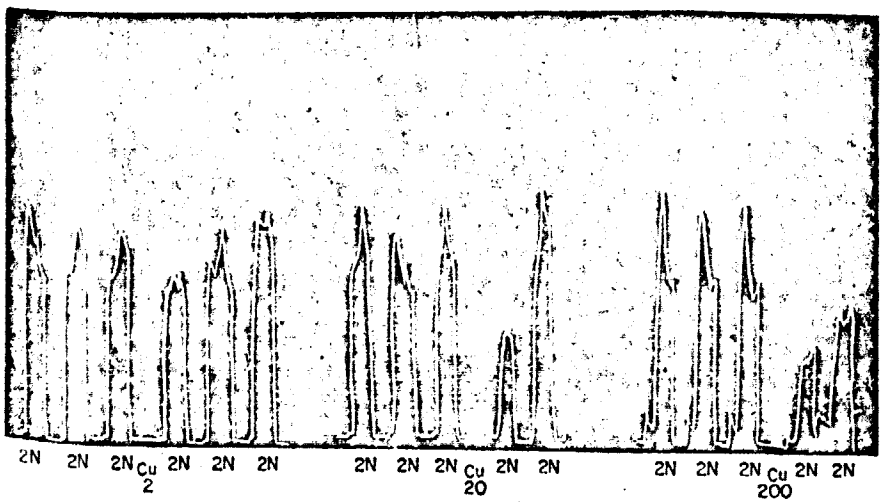

FIG. 2. Guinea pig vas deferens: The effect of copper sulphate at three dose levels, on standard contractions induced by 2 μg/ml noradrenaline. Copper sulphate added 30 sec before noradrenaline. N—noradrenaline; Cu—copper sulphate (μg/ml).

Inhibition of the responses to a standard dose of 0·4 μg/ml acetylcholine was observed at all dose levels of manganese sulphate used. The standard response to acetylcholine could not be restored following the addition of 2 mg/ml manganese sulphate to the bath.

The addition of 200 μg/ml copper sulphate and 2 mg/ml manganese sulphate to the bath always resulted in the appearance of a white precipitate in the Tyrode solution. This precipitate was revealed by chemical analysis to be due to precipitation of sparingly soluble metal phosphate. Only a small portion of the total amount of metal ion was involved in phosphate formation. Copper sulphate at dose levels of 2, 20 and 200 μg/ml inhibited the response of the ileum to a standard 0·2 μg/ml dose of 5-hydroxytryptamine. Manganese sulphate, at all the dose levels used, also inhibited the response of the ileum to the standard 0·2 μg/ml dose of 5-hydroxytryptamine. After addition of 2 mg/ml manganese sulphate to the bath the muscle would not respond to a standard dose of 5-hydroxytryptamine on washing. More washing than normal was required to bring the lever back to the baseline following addition of 200 μg/ml copper sulphate than at lower dose levels. The response to the standard dose of 5-hydroxytryptamine was then restored to normal.

Copper sulphate and manganese sulphate did not cause any contraction of the vas deferens in the 30-sec period prior to addition of noradrenaline or dopamine. Copper sulphate inhibited the response of the tissue to a standard 2 μg/ml dose of noradrenaline. This occurred at all the dose levels of copper sulphate used i.e., 2, 20 and 200 μg/ml. The response to the standard dose of noradrenaline after washing was found to be about 50 per cent of that prior to addition of 200 μg/ml copper sulphate.

A dose of 2 μg/ml manganese sulphate potentiated the response to the standard 1 μg/ml dose of noradrenaline. At the 20 μg/ml dose level the effect of manganese sulphate on the noradrenaline response was very variable, ranging from marked inhibition to marked potentiation. Very marked inhibition of the noradrenaline response occurred at the dose levels of 200 μg/ml and 2 mg/ml manganese sulphate. Following these doses the response to a standard dose of noradrenaline could not be restored.

When dopamine was added to the organ bath, a lag period of about 45 sec occurred before a contraction was observed. Inhibition of the standard response to 5 μg/ml dopamine occurred at the dose levels 2 and 20 μg/ml copper sulphate but some potentiation at the dose level of 200 μg/ml copper sulphate. At the latter dose a red coloration developed in the bath fluid on addition of dopamine, and several additional washes were required to bring the baseline back to its control position.

Manganese sulphate, at the dose levels of 2 and 20/μg/ml, potentiated the response to the standard 5/μg/ml dose of dopamine. Marked inhibition was caused by addition of 200/μg/ml and 2 mg/ml of manganese sulphate and a red coloration developed in the bath fluid when dopamine was added to the bath in the presence of 200/μg/ml manganese sulphate. The inhibitory effect of 2 mg/ml manganese sulphate was not reversible with washing.

DISCUSSION

The acute toxicity of tremorine was potentiated by both manganese sulphate and copper sulphate. Bienvenu *et al.*,[11] found that the sulphate radical has only a weak effect in the determination of the i.p. toxicities of these metal salts in mice, so the increase in toxicity is probably associated with the metal ions.

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The means whereby such ions could potentiate the toxicity and the tremorigenic effect of tremor is a matter for speculation. Two possibilities are inhibition of enzymes by the metals or chelation of the metals with aromatic amines.[12, 13] Though both possibilities are feasible there is evidence that changes in aromatic amine levels may be associated with tremor[6, 14, 15] and therefore chelation of amines with the metal appears the more likely explanation. Some support for this possibility is offered by the fact that some of the biologically important divalent metal ions frequently exhibit the following order of stability: Cu^{++}, Co^{++}, Fe^{++}, Zn^{++}, Mn^{++}, Mg^{++}, Ca^{++}.[16] One might therefore expect copper to form more stable complexes than manganese with biogenic amines and hence have a longer duration of action. It is of interest that in fact tremorine tremor was potentiated longer by the copper than by the manganese salt.

A potentiation of acetylcholine on the guinea pig ileum could be produced by certain concentrations of copper. This agrees with the findings of Mercier[17] and Godfraind and Godfraind De Becker.[18, 19] The latter workers also noted that the potentiation could occur following a short contact time between the metallic salt and the tissue.

The inhibition caused by manganese sulphate is in agreement with the results of Delaveau,[20] on the isolated intestine of the rat, the guinea pig and rabbit. He obtained similar results with the chloride and the sulphate, and Bohr,[21] has stated that substitution of sulphate or phosphate for chloride has little effect on the contractile response of smooth muscle. Hence it is likely that the metallic cations are important in the responses reported.

High concentrations of both metallic salts produced a red coloration with dopamine. However, it is unlikely that the cause of this coloration was responsible for the marked potentiation with copper sulphate since the opposite effect was produced in the vas deferens by manganese sulphate in the presence of dopamine. The potentiation of catecholamine response observed at the 2 and 20 μg per ml manganese sulphate levels is difficult to explain but for some enzyme systems *in vitro* manganese seems to be able to replace magnesium as an essential metal.[22] Magnesium, itself, has both an enhancing and depressing action on smooth muscle.[21] The effects of manganese ions *in vitro* might be of a similar nature.

On the ileum both copper sulphate and manganese sulphate inhibited the response to 5 hydroxytryptamine at all dose levels. As mentioned previously chelation may be implicated in these phenomena.

Acknowledgements--The authors wish to thank Mr. D. J. Simpson, Department of Medical Physics, University of Aberdeen, who designed the frequency selective amplifier and from whom details of the circuit can be obtained, and Dr. A. M. Adelstein, Medical Statistician, University of Manchester.

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established that manganese chloride does not precipitate in the albumins, but we injected this chloride mixed with soda thiosulfate in order to preserve the comparison to work done on the toxicological properties of metallic ions.

Our experiments on acute poisoning were done using dogs and rabbits. We have found that in the dog, the heart is first of all affected. The fall in blood pressure appears almost at the same time as cardiac paralysis. Only one time did we see a rise in blood pressure at the beginning of the injection. It is almost always the systole which is paralyzed; this phenomenon was observed only once for the diastole. It involves generally a sympathetic reaction. Towards the end of the poisoning, the cardiac contractions are similar to those produced by a faint. In the last phase of the poisoning, the blood pressure especially is affected. Breathing becomes increasingly shallow; the cessation of breathing may precede the collapse of the circulatory system. In the rabbit, the vaso-motor organs are first paralyzed. Also in this case, we noted a subtle fall in blood pressure, the cardiac rhythm is slowed, but the amplitude of the contractions increased. The animal is finally asphyxiated, but it seems that this a secondary consequence of the paralysis of the circulatory system. The result of the dissection was negative.

The average lethal dose for the dog is 0.056 g and for the rabbit 0.018 g of manganese per 1 kg of the living weight. According to Kobert, the lethal doses are the following: for the dog, 0.006 to 0.008 g; for the cat, 0.008 to 0.009; for the rabbit, 0.012 to 0.013 and for the guinea pig 0.028 to 0.030 g. We gave doses considerably

higher than those of Kobert to dogs; also for rabbits, we have exceeded the doses indicated by this author; we cannot explain this considerable difference between the lethal doses (the antitoxic effect of soda thiosulfate or the speed of the injection), because the literature does not give precise explanations of the methods used.

The chronic poisonings which we induced in three dogs and eight rabbits enabled us to establish the following facts: doses less than 0.0025 g of Mn per 1 kg of living weight were tolerated by rabbits; they caused no organic disturbance and could be repeated. A dose of 0.003 g of Mn per kg of living weight is the limit for the toxic dose, but this did not cause permanent disorders. A little stronger dose caused an upset of the digestive and urinary organs; the same doses were tolerated just as well by dogs.

In considering all the symptoms of acute and chronic poisoning, we believe that for the most part they can be explained by the paralysis of the sympathetic nervous system which brings about an upset of the digestive organs, an increase in urinary output, the lowering of the blood pressure, the slowing and the deepening of the cardiac function. Further, account must also be taken of the direct upset of the intestine during the elimination of the metal by the intestinal mucosa; this intestinal disturbance can be transmitted to the urinary organs by means of the reflexes. With high doses, an intense effect of manganese on the ganglions and the cerebral surface can be expected. Tonic and clonic convulsions, profound prostration which may be prevented by injections of morphine, are noted. Intravenous injection produces also a fall in temperature which in rabbits may go as low as 2°; in dogs,

the fall in temperature occurs just as often, but is not as great.

Repeated injection of the toxic dose leads to weakening, prostration, loss of weight and diarrhea. At autopsy, we regularly found acute catarrh or inflammation of the intestine, and sometimes also nephritis.

Comptes Rendus de la Société de Biologie,
102 : 262-264, 1929

Pharmacologie et toxicologie du manganèse,

par Fr. Cervinka.

Nous avons recherché la dose de manganèse que l'on pourrait employer en injection intraveineuse répétée sans conséquences nuisibles pour l'organisme. Nous avons aussi voulu essayer s'il était possible d'employer le manganèse dans le traitement de certaines maladies. Enfin, nous avons voulu vérifier les indications de la littérature concernant la toxicité du manganèse et la dose mortelle. La pharmacodynamie du manganèse a été étudiée par

plusieurs expérimentateurs qui ont constaté son action stimulante sur les organismes inférieurs (Levures) et chez les organismes supérieurs. D'après les recherches d'Uhlenhut, le manganèse active directement le protoplasme et ces effets catalytiques peuvent être employés pour détruire les toxines bactériennes, qui sont très sensibles aux processus d'oxydation. C'est sur ces effets qu'on a fondé la thérapeutique antitoxique de Walbum.

Injecté dans l'organisme, le manganèse se dépose surtout dans le système réticulo-endothélial du foie, des poumons, des reins, de la rate, de la moelle osseuse, etc. : c'est pourquoi on suppose que le manganèse possède une affinité particulière à l'égard de ce système.

Dans nos expériences nous avons employé le chlorure de Mn, qui est soluble dans l'eau. Les recherches sur les métaux lourds, qui sont poursuivies à l'Institut pharmacologique de Brno, constituent un ensemble d'études comparatives ; toutes nos expériences ont été faites d'après la méthode qui est mentionnée dans la communication de J. Lebduska (1*). Dans nos expériences préliminaires, nous avons établi que le chlorure de manganèse ne précipite pas les albumines, mais nous avons injecté ce chlorure mélangé au thiosulfate de soude pour conserver leur caractère comparatif aux travaux sur les propriétés toxicologiques des ions métalliques.

Nos expériences sur l'intoxication aiguë ont été faites sur des Chiens et sur des Lapins. Nous avons constaté que, chez le Chien, c'est d'abord le cœur qui est attaqué. La chute de la pression sanguine apparaît presque en même temps que la paralysie cardiaque. Une fois seulement nous avons constaté l'élévation de la pression sanguine au commencement de l'injection. C'est presque toujours la systole qui est paralysée : le phénomène a été observé une fois seulement pour la diastole. Il s'agit alors, en général, d'une réaction sympathique. Vers la fin de l'intoxication, les contractions cardiaques rappellent celles qui se produisent sous l'influence de l'excitation du vague. Dans la dernière phase de l'intoxication, c'est surtout la pression sanguine qui est touchée. La respiration devient de plus en plus superficielle ; l'arrêt de la respiration peut précéder la mort de l'appareil circulatoire. Chez le Lapin, c'est d'abord l'appareil vaso-moteur qui est paralysé. Dans ce cas aussi nous avons constaté une chute subite de la pression sanguine, le rythme cardiaque s'est ralenti, mais l'amplitude des contractions a augmenté. L'animal est finalement frappé d'asphyxie, mais il semble que ce soit une conséquence secondaire de la paralysie de l'appareil circulatoire. Le résultat de la dissection a été négatif.

(1*) C. R. de la Soc. de biol., 1909, t. 102, p. 969.

La dose mortelle moyenne est chez le Chien de 0,056 gr. et chez le Lapin de 0,018 gr. de manganèse pour 1 kgr. de poids vif. D'après Kobert, les doses mortelles sont les suivantes : chez le Chien, 0,006 à 0,008 gr. ; chez le Chat, 0,008 à 0,009 ; chez le Lapin, 0,012 à 0,013 et chez le Cobaye 0,028 à 0,030 gr. Nous avons appliqué, chez le Chien, des doses considérablement supérieures à celles de Kobert ; chez le Lapin, nous avons également dépassé les doses indiquées par le même auteur ; nous ne pouvons pas expliquer cette différence considérable entre la valeur des doses mortelles (l'action antitoxique du thiosulfate de soude ou la vitesse de l'injection), car la littérature ne fournit pas d'indications précises sur la méthode employée.

Les intoxications chroniques que nous avons pratiquées sur trois Chiens et sur huit Lapins nous ont permis d'établir les faits suivants : les doses inférieures à 0,0025 gr. de Mn pour 1 kgr. de poids vif sont supportées par les Lapins ; elles ne produisent aucune perturbation organique et peuvent être répétées. La dose de 0,003 gr. de Mn pour 1 kgr. de poids vif est à la limite de la dose toxique, mais elle ne suscite pas encore de troubles durables. Une dose un peu forte détermine l'excitation des organes digestifs et urinaires ; les mêmes doses sont aussi bien supportées par les Chiens.

En considérant tous les symptômes de l'intoxication aiguë ou chronique, nous croyons pouvoir les expliquer, pour la majeure partie, par la paralysie du système nerveux sympathique qui engendrerait l'excitation des organes digestifs, l'augmentation de la sécrétion urinaire, l'abaissement de la pression sanguine, le ralentissement et l'approfondissement de la fonction cardiaque. D'autre part, il faut cependant tenir compte de l'excitation directe de l'intestin pendant l'élimination du métal par la muqueuse intestinale ; cette excitation intestinale pourrait aussi être transmise par voie réflexe aux organes urinaires. Avec les doses élevées, on peut supposer une action intense du manganèse sur les ganglions et l'écorce cérébrale. On note des convulsions toniques et cloniques, une prostration profonde, qui peuvent être prévenues au moyen d'injections de morphine. L'injection intraveineuse produit aussi une chute de la température, qui peut atteindre, chez les Lapins, jusqu'à 2° ; chez les Chiens, l'abaissement de la température se présente aussi régulièrement, mais il est plus faible.

L'injection répétée de la dose toxique amène l'affaiblissement, la prostration, la diminution du poids et de la diarrhée. A l'autopsie on peut constater régulièrement le catarrhe aigu ou l'inflammation de l'intestin et quelquefois aussi de la néphrite.

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a p. ol. et toxicol. 1970, 28, 177–183.

From the Industrial Toxicology Research Centre, Lucknow, India

Experimental Production of Early Brain Lesions in Rats by Parenteral Administration of Manganese Chloride

By

Satya V. Chandra and S. P. Srivastava

(Received August 18, 1969)

Abstract: Attempts have been made to produce early brain lesions in rats by the daily intraperitoneal administration of manganese chloride (8 mg/kg) for a period of 180 days. Neuropathological examination performed at various intervals showed scattered neuronal degeneration in the cerebral and cerebellar cortex at 120 days which increased in intensity up to 180 days. The manganese concentration in the brain also showed a significant increase. The extent of the brain lesions was directly related to the amount of manganese in the brain tissue which increased with time.

Key-words: Brain – brain injury, acute – manganese.

Excessive manganese in the human body is known to cause degeneration of the basal ganglia of the brain (CANAVAN *et al.* 1934). It has also been established that manganese enters the body mainly via the pulmonary route and produces lesions in the extrapyramidal system, the base of the brain and the upper part of the spinal cord (WHITLOCK *et al.* 1966). The development of brain lesions and the mechanism responsible for their production is not understood. This experiment was undertaken in the rat in order to study early brain lesions produced by manganese and the relation between the manganese concentration and the development and severity of the pathological lesions.

Materials and Methods

Ninety male albino rats of I. T. R. C. colony (average weight 150) were reared under normal laboratory conditions and fed a stock diet which contained only small traces of manganese. The animals were divided into two groups. Group I consisted of 30 control animals into which 0.5 ml distilled water was injected and group II consisted of sixty animals into which manganese chloride in aqueous solution (8 mg/kg) was injected intraperitoneally daily for 180 days. Six animals from group I and 24 animals from group II died during the course of the experiment; the cause of their death however,

could not be ascertained. Four animals from group I and six from group II were sacrificed at 30 days intervals up to 180 days and during this period the animals did not develop any unusual neurological symptoms. Animals that died during the experiment and which were sacrificed at various intervals were autopsied and the brains removed and examined for gross pathological changes. The brains from half the animals from each group were kept in neutral buffered formalin for histopathological studies while in the remainder of the specimens estimation of the manganese content was done by the method of SRIVASTAVA *et al.* (1969). For histopathological studies, blocks of tissue were embedded in paraffin and sections were cut at 10 μ. The sections were stained with hematoxylin and eosin, carbol thionine for Nissl substance, Holzer's stain for glia fibres, Bielschowsky's stain for neurofibrils and Weil's stain for myelin sheath (MCMANUS & MOWRY 1960).

Estimations of the manganese content were done in brain tissue of experimental and control animals at 60, 120 and 180 days as well as in animals which died during the experiment. The results of manganese content in the animals dying up to 60 days are pooled together with the results of animals sacrificed at 60 days and similarly the results of dead animals up to 120 and 180 days have been pooled with the results of animals sacrificed at 120 and 180 days respectively.

To determine the number of degenerated neurons in the brain cortex, nerve cell counts were performed by the following method:

A paper diaphragm with a circular cut about 4 mm in diameter in the centre was inserted in a number 10 eye piece. Sections were put under magnification × 40, and 1000 degenerated and normal neurons were counted from one to the other side of the cortex, in the same way as in differential white cell counts. The average numbers of degenerated neurons were calculated from each group. Counts were performed at different periods of the experiment in both groups.

Results

Pathological findings.

Macroscopic. The meninges were smooth and shining; basal vessels and cranial nerves were normal in all the animals in groups I and II. No gross pathological change was observed in the brain and spinal cord of group I animals. In group II no gross pathological changes were seen in the brain and spinal cord up to 120 days; at 150 and 180 days, the brain appeared paler than normal.

Microscopic. Sections of the brain from group I animals did not reveal any pathological change (fig. 1). In group II (manganese chloride injection) sections of brain did not reveal any pathological change up to 90 days. At 120 days scattered neuronal degeneration in the cerebellar and cerebral cortex was seen. Nissl granules were absent in these cells. A few oligodendroglia appeared to be swollen. The blood vessels did not show any abnormality except a mild congestion in one rat. The ventricular ependyma and choroid plexus were normal in appearance while the white matter was found to be normal and the myelin stain revealed normal structure. Sections from the brain at 150 days showed vacuolated nerve cells in the cerebral and

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Fig. 1. Photomicrograph of cerebral cortex of a control rat, showing normal structure of brain tissue. Hematoxylin and eosin. Magnification × 400.

cerebellar cortex, and degenerative changes in the form of chromatolysis, the nuclei were shrunken and in some cases pyknotic. No myelin sheath degeneration was observed. There was swelling of the oligodendroglia. The meninges, ventricular ependyma, choroid plexus and blood vessels did not reveal any abnormality. Sections from the brain at 180 days showed a fairly large number of degenerated neurons scattered in the cortex. In some of these cells Nissl granules were absent and the cytoplasm was pale and vacuolated; the nucleus in degenerated neurons was shrunken and pyknotic and in some cases had completely disappeared. There was marked swelling of the oligodendroglia (fig. 2). The basal ganglia did not reveal any pathological change. No myelin sheath degeneration was noted. The meninges, ventricular ependyma, choroid plexus and blood vessels appeared to be normal.

The nerve cell count at various intervals showed that the percentage of degenerated neurons in the animals of group I was 5.4, and in group II this was almost the same as in group I up to 90 days; thereafter it increased to 17.3 at 120 days, 31.9 at 150 days and 43.3 at 180 days (table 1). This indicates that degenerative lesions in the brain due to manganese are present at 120 days and the severity increases with time, thus confirming the microscopic findings of maximum lesions in the brain at 180 days.

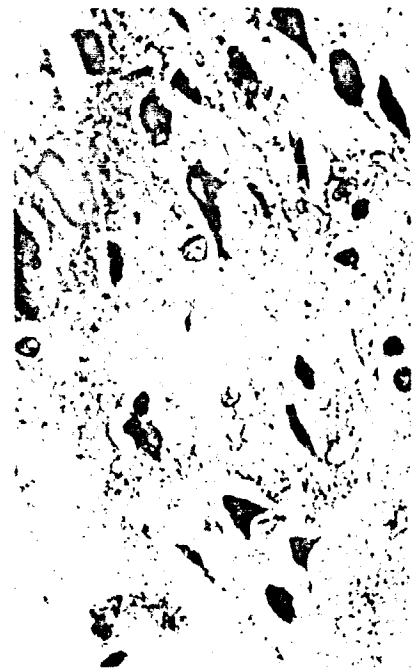

Fig. 2. Photomicrograph of cerebral cortex of rat after intraperitoneal injections of manganese chloride (8 mg/kg body weight) for 180 days, showing degenerated neurons and swollen oligodendroglia. Hematoxylin and eosin. Magnification × 400.

Manganese concentration in brain (mg/g dry weight of brain tissue).

The results on the manganese content in the brains of both the groups are shown in table 2. The average manganese content in brains of animals of

Table 1.

Average number of degenerated neurons in the cortex of brain in control and manganese injected rats.

Neurons	Control	Manganese injected rats					
		Days					
	0	30	60	90	120	150	180
Degenerated	54	54	53	50	173	319	433
Normal	946 (10)	946 (4)	947 (4)	950 (4)	827 (4)	681 (4)	567 (4)
Percent	5.4	5.4	5.3	5.0	17.3	31.9	43.3

Figures in brackets indicate number of animals.

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Table 2.

Manganese content in brain of control and manganese injected rats.

	Control	Manganese injected rats		
Manganese mg/g dry weight of brain tissue	0	60 days	120 days	180 days
Mean value ± S. E. M.	2.253 ± 0.2190 (7)	3.784 ± 0.2445 (6)	4.863 ± 0.1378 (6)	6.849 ± 0.1502 (6)
Statistical significance of the difference –				
1. from control ...	–	Significant $P < 0.05$	Highly significant $P < 0.01$	Highly significant $P < 0.01$
2. from 60 days .	–	–	Not significant	Highly significant $P < 0.01$
3. from 120 days .	–	–	–	Significant $P < 0.05$

Figures in brackets indicate number of animals.

group I was 2.2530; in the animals of group II up to 60 days this was 3.7844, up to 120 days 4.8636 and up to 180 days 6.849. Statistical analysis showed that the difference in manganese content between the control and the experimental group up to 120 and 180 days is highly significant while it is just significant at 60 days.

On comparing the number of degenerated neurons and the amount of manganese content it appears that the severity of lesions is directly related to the manganese concentration in the brain. At 120 days when microscopic lesions are seen in the brain, the amount of manganese is 4.86 which is highly significant as compared to group I. At 180 days maximum lesions are observed in the brain i. e. when the amount of manganese has increased to 6.84 mg/g dry weight of brain tissue.

Discussion

The early histological changes in the human brain due to manganese toxicity are not known. The reports available are only of advanced cases where the condition resembles Parkinsonism (Canavan *et al.* 1934; Parsezki & Pfeiffer 1954). Kircher *et al.* (1960) studied the electroencephalographic pattern in patients with manganese poisoning and observed that the lesions were more numerous in the frontal lobes than in the basal ganglia or the cerebellum. Further it has also been observed that in manganese toxicity (Rodier 1955) there is a period of mental excitement in human subjects before the actual manifestations of lesions in the extrapyramidal system. How far these changes are related to early lesions is not known. In the present experiment it has been possible to produce lesions in the cerebral and the cerebellar cortex due to manganese toxicity in which the extrapyramidal system seems to escape injury.

The lesions of the basal ganglia in experimental animals produced by Mella (1924) and Pinischew *et al.* (1963) and the cortical lesions produced by Saxena (1967) in our laboratory did not demonstrate any relationship between the amount of manganese present and the severity of lesions in the brain. The present experiments however, have demonstrated that the maximum number of degenerated neurons are present when the amount of manganese in the brain is at a maximum, thus indicating that the extent of damage to the brain cells is directly related to the amount of manganese present in the brain. The period up to 120 days appears to be a threshold for the appearance of microscopic lesions. It is possible that very early pathological changes may be present which can not be detected by light microscopy, or that initially manganese produces biochemical disturbances rather than visible structural changes.

The present experiment supports the hypothesis that in experimental animals damage to the brain cells can be caused directly by soluble manganese. It remains to be determined whether manganese has a direct toxic effect on the brain tissue or whether excess manganese in the blood conjugates with protein molecules to form a toxic compound which is responsible for causing damage to brain cells. This requires further investigation. In a case of hepatolenticular degeneration, however, an abnormal protein in the liver was demonstrated which had an abnormally high copper binding property (Cumings 1959). The possibility of a vascular pathogenesis (Stadler 1936) is also ruled out as the blood vessels in our experiment did not show any abnormality.

Acknowledgement

Thanks are due to Dr. S. H. Zaidi, Director, Industrial Toxicology Research Centre, for his valuable suggestions and guidance throughout the course of the experiments. The technical assistance of Shri R. S. Srivastava and Shri Mulkraj is gratefully acknowledged. We would like to thank Mr. George for the statistical analysis and Mr. Musleh Ahmad for the microphotography.

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Internationale Zeitschrift für Klinische Pharmakologie, Therapie und Toxikologie, 3 (4): 351-357, 1970
Metals — Resorption — Intestinal tract

M. Cikrt[1]

The Uptake of Hg^{203}, Cu^{64}, Mn^{52} and Pb^{212} by the Intestinal Wall of the Duodenal and Ileal Segment in vitro

Manuskript eingegangen am 4. 2. 1970 (Nr. 233)

Summary (Übersetzung siehe Karteikarte)

For all metals studied (except Mn^{52}) there was proved a significantly higher uptake by the wall of the ileal segment in comparison to the duodenum. Also values in the serosal medium (except Pb^{212}) were significantly higher in the ileum.
Mn^{52} disclosed the lowest decrease in the incubation medium during incubation, the lowest uptake by the intestinal wall, and yet the highest values in the serosal medium of all metals studied. These results together with a significant decrease in the uptake of Mn^{52} by the intestinal wall at the anaerobic incubation support the previously expressed assumption of an active transport of manganese through the intestinal wall in vitro.
Pb^{212} administered in form of citrate salt is resorbed from the incubation medium fastest of all studied metals. Concerning Pb^{212} there is also no significant difference between the ileum and the duodenum in the serosal medium. We believe that these differences are caused by lead binding to the chelating agent.

The importance of the task of the intestinal wall in the transport of metals into the organism is well known. The principles and mechanisms of these phenomena are less lucid. As far as attention has been paid to these questions, the studies concerned mainly metals essential for the organisms or those with which the organism comes into contact frequently and in a relatively large quantity. As examples there should be mentioned the reports concerning iron and calcium (Schachter et al. 1959, 1966; Brown and Justus 1958; Dowdle et al 1960; Saltman and Helbock 1965). Clarkson and Cross (1961) studied the kinetics of mercury uptake by the intestinal wall using the method of everted intestinal sacs.
We repeated the results of Clarkson and Cross (1961) with $Hg^{203}Cl_2$ and evaluated critically the whole method and the results (in press). The results were corrected with regard to the found losses of Hg^{203} from the incubation solution during incubation. In the present work we used again the modified method of everted intestinal sacs for the study of the uptake of Cu^{64}, Mn^{52}, and Pb^{212} in the duodenal and ileal segments of the small intestine in vitro. In the second part of the paper, the results of the three metals studied were compared to results with Hg^{203} dealt in our previous paper (in press).

Material and Methods

Female Wistar rats weighing 180—200 g, fed a pellet diet, were used in the experiment. The pellets contained, besides the basic components, 2 % of mineral mixture: 80 % NaCl and 20 % trace elements (52 % $Fe_2/SO_4/_3$, 20 % MnO, 9 % $CuSO_4$, 0.1 % $Ca/CO_3/_2$). The rats were starved during 24 hours before the experiment.
The modified method of everted intestinal sacs was used for incubation according to Clarkson and Cross (1961). This method was described in detail in our previous paper (in press).
Radioisotopes of the studied metals Cu^{64}, Mn^{52} (in form of chloride), and Pb^{212} (in form of citrate salt) were added to the mucosal medium. Cu^{64} was a product of the Zentralinstitut für Kernforschung, Dresden, Germany, Mn^{52} a product of the Institute of Nuclear Research, Řež, Czechoslovakia, and Pb^{212} was produced in our laboratory by electrodeposition in the radio-

[1] Institute of Industrial Hygiene and Occupational Diseases, Prague, Czechoslovakia (Director: Prof. J. Teisinger, M.D.Dr.Sc.).

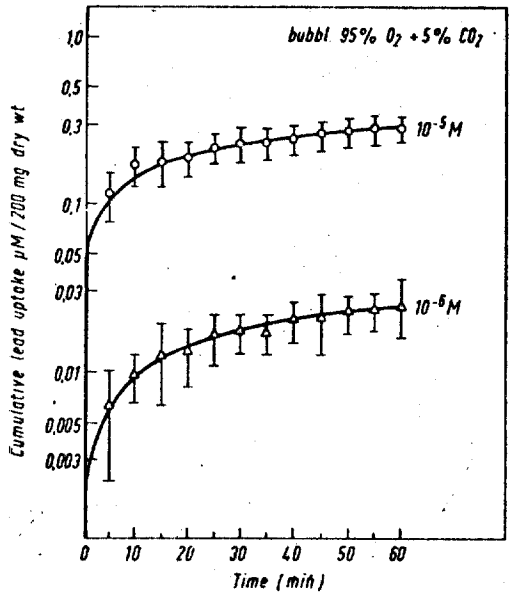

Fig. 1. Duodenum (initial Pb^{++} conc. 10^{-5} and 10^{-6} M).

emanator containing radiothorium ($RdTh^{228}$). Hg^{203} which we followed up previously was administered in the form of chloride (product of the Zentralinstitut für Kernforschung, Dresden, Germany). All metals studied were administered in two concentrations of 10^{-6} M and 10^{-5} M except Cu^{64} where a concentration of 10^{-5} M only was used.

The incubation medium was bubbled with a mixture of 95 % O_2 + 5 % CO_2 (pneumoxide) and in some cases with nitrogen. The samples were measured in a well-type scintillation counter (Frieseke & Hoepfner). The decrease in the concentration of the studied metal in the mucosal incubation medium was measured during incubation, and the amount of metal trapped by the wall of the intestinal segment — as well as the amount of metal which passed through the whole intestinal wall up to the serosal medium — was measured at the end of the incubation period. The results were expressed in µMol/200 Mg dry weight of the segment. The tissue dry weight was obtained by heating in air at 110° C for 48 hours. All necessary calculations and schemes were carried out in the same manner as in the study by Clarkson and Cross (1961). The values indicated in the schemes represent the means ± of the standard deviation ($\bar{X} \pm 2\,\sigma$) of 10—12 values measured.

Results

The uptake of Pb^{212}, Cu^{64}, Mn^{52} by ileal and duodenal segments

Pb^{212} was added to the mucosal medium in the form of citrate salt, in a concentration of 10^{-5} and 10^{-6} M. The medium was aerated by a mixture of 95 % O_2 + 5 % CO_2. At the end of the incubation period, there was a statistically significant ($p < 0.05$) higher decrease in the concentration of the incubation medium in the ileal segment than in the duodenum (Figs. 1 and 2). Results after 60 minutes of incubation:

Initial concentr.	Bubbled with	Duodenum	Ileum
10^{-5} M	pneumoxide	0.285 ± 0.051	0.413 ± 0.1
10^{-6} M	pneumoxide	0.024 ± 0.003	0.034 ± 0.01

The values are expressed as µMol/200 mg DW. Also in the intestinal wall there was in both concentrations a significant difference ($p<0.05$) in favour of the ileum:

Initial concentr.	Bubbled with	Duodenum	Ileum
10^{-5} M	pneumoxide	0.043 ± 0.011	0.104 ± 0.024
10^{-6} M	pneumoxide	0.010 ± 0.002	0.019 ± 0.004

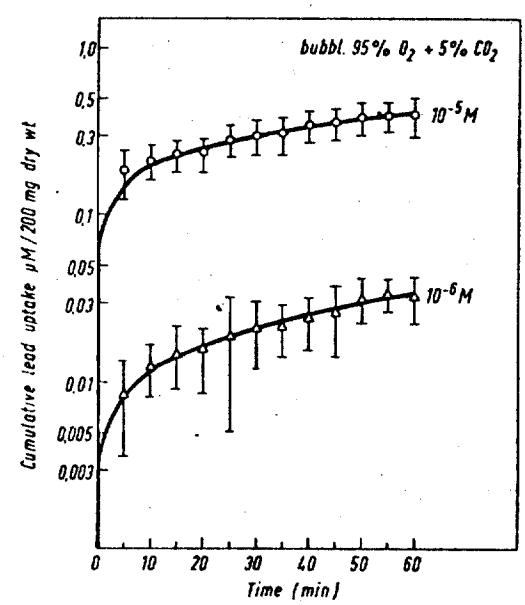

Fig. 2. Ileum (initial Pb^{++} conc. 10^{-5} and 10^{-6} M).

The values are expressed as μMol/200 mg DW. The values measured in the serosal medium do not differ significantly in both concentrations.

Initial concentr.	Bubbled with	Duodenum	Ileum
10^{-5} M	pneumoxide	0.00036 ± 0.00014	0.00038 ± 0.00012
10^{-4} M	pneumoxide	0.00008 ± 0.00003	0.00008 ± 0.00002

The values are expressed as μMol/200 mg DW.

Cu^{64} was added to the mucosal medium in the form of chloride only in a concentration of 10^{-5} M. The medium was bubbled with pneumoxide. After 60 minutes of incubation, there was no statistically significant difference in the decrease of the concentration in the mucosal medium between both incubated segments (Fig. 3).

Duodenal segment: 0.119 ± 0.041 and
Ileal segment: 0.170 ± 0.055

In the intestinal wall (duodenum 0.051 ± 0.017 and ileum 0.109 ± 0.027) and also in the serosal medium (duodenum 0.00004 ± 0.00002 and ileum 0.00038 ± 0.00017) there was a significant difference ($p < 0.01$) in favour of the ileum.
All values are expressed in μMol/200 mg DW.

Mn^{52} was added to the mucosal medium in the form of chloride, in concentrations of 10^{-5} and

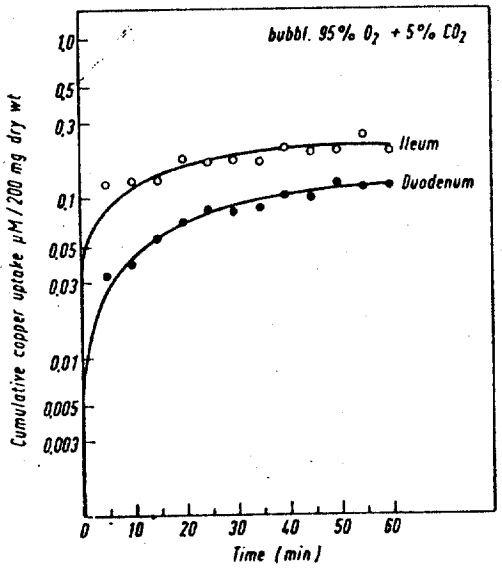

Fig. 3. Initial Cu^{++} Concentration 10^{-5} M.

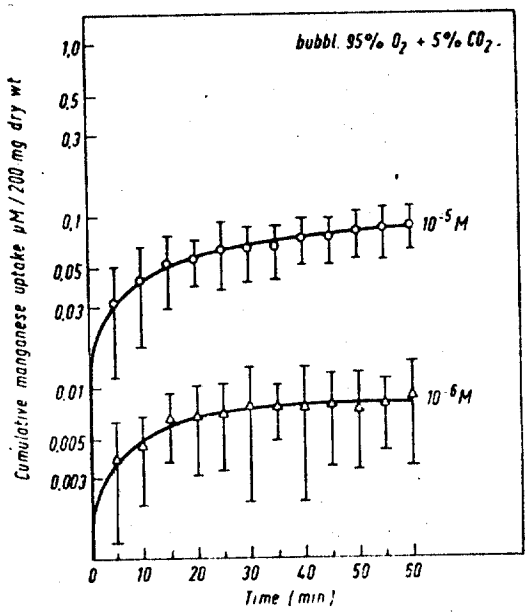

Fig. 4. Duodenum (initial Mn^{++} conc. 10^{-5} and 10^{-6} M).

10^{-6} M. The medium was bubbled in both concentrations with pneumoxide and in concentration of 10^{-5} M also with nitrogen. When bubbling with pneumoxide, there was in the concentration of 10^{-5} M, at the end of experiment, a significantly ($p < 0.05$) higher decrease in the mucosal medium in the ileal segment (Figs. 4 and 5). When bubbling with nitrogen, this difference was even more significant ($p < 0.01$).

Initial concentr.	Bubbled with	Duodenum	Ileum
10^{-5} M	pneumoxide	0.088 ± 0.024	0.142 ± 0.040
10^{-5} M	nitrogen	0.110 ± 0.033	0.157 ± 0.030
10^{-6} M	pneumoxide	0.009 ± 0.005	0.010 ± 0.005

In the intestinal wall there were no significant differences between the duodenal and ileal segments in the uptake of manganese ions.

Results after 60 minutes of incubation:

Initial concentr.	Bubbled with	Duodenum	Ileum
10^{-5} M	pneumoxide	0.021 ± 0.004	0.017 ± 0.002
10^{-5} M	nitrogen	0.010 ± 0.002	0.010 ± 0.003
10^{-6} M	pneumoxide	0.0034 ± 0.0006	0.0014 ± 0.0002

In the serosal medium a significant difference was detected in favour of the ileum only in

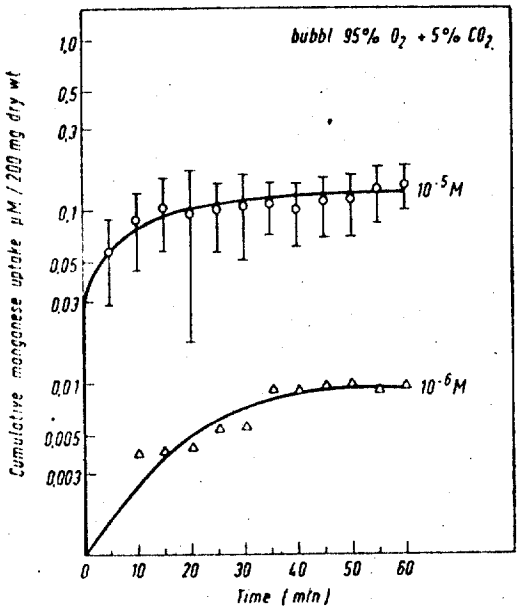

Fig. 5. Ileum (initial Mn^{++} conc. 10^{-5} and 10^{-6} M).

the experiments bubbled with pneumoxide ($p < 0.05$).

Initial concentr.	Bubbled with	Duodenum	Ileum
10^{-5} M	pneumoxide	0.0013 ± 0.0003	0.002 ± 0.0005
10^{-5} M	nitrogen	0.0014 ± 0.0006	0.0017 ± 0.0007
10^{-6} M	pneumoxide	0.00003 ± 0.00002	0.00007 ± 0.00003

All values are expressed in µMol/200 mg DW. When comparing experiments with the initial concentration of $Mn^{52}Cl_2$ of 10^{-5} M saturated with nitrogen and pneumoxide, a significant difference ($p < 0.01$) in the intestinal wall in favour of experiments bubbled with pneumoxide was determined both in the duodenum and in the ileum. In the serosal medium, on the other hand, such a difference was not found.

Comparison of Hg^{203}, Pb^{212}, Cu^{64}, and Mn^{52} uptake by the duodenal and ileal segments

We compared the results with Pb^{212}, Cu^{64}, and Mn^{52} with those with Hg^{203} obtained in our previous work (page 344).

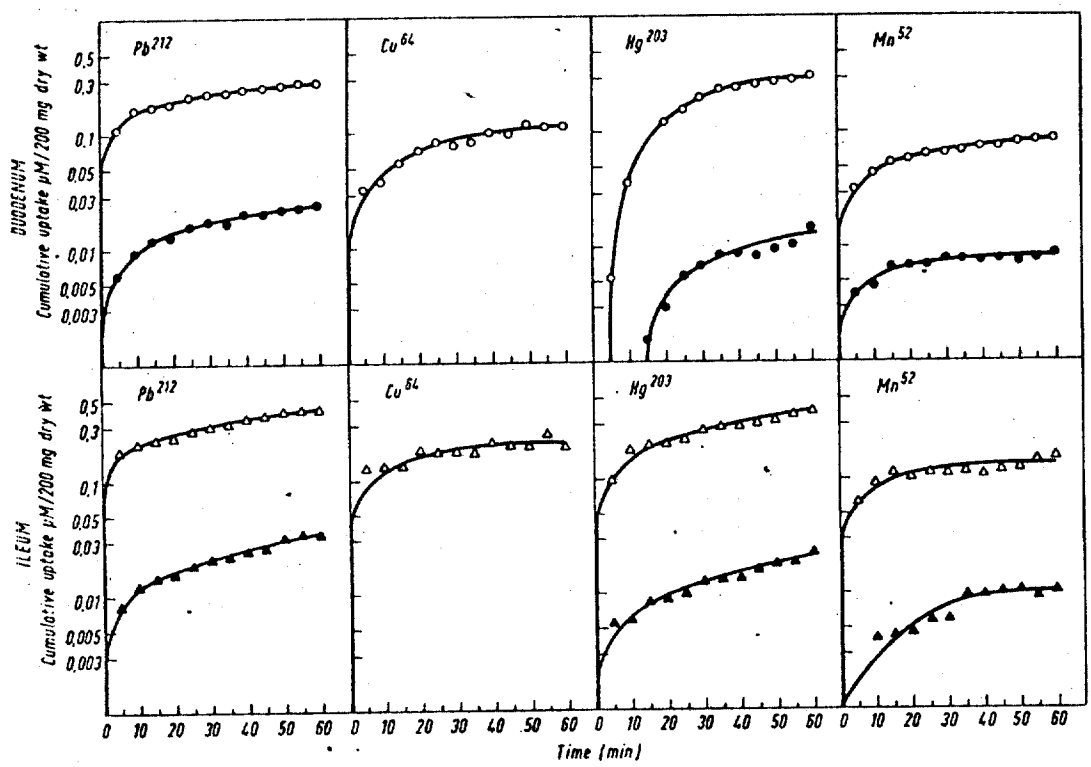

Fig. 6. ○ △ Initial Concentration 10^{-5} Mol.
● ▲ Initial Concentration 10^{-6} Mol.

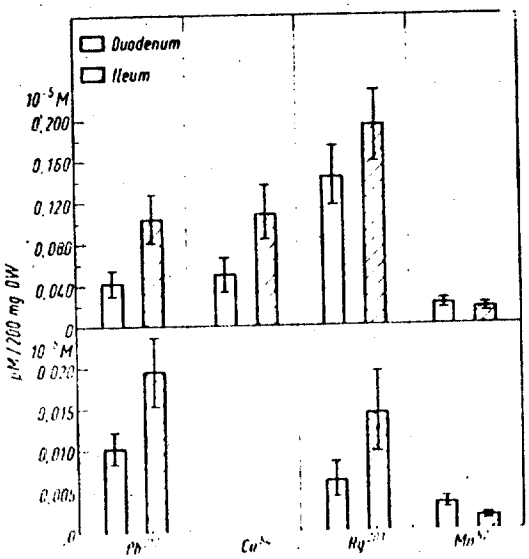

Fig. 7. Intestinal Wall (after 60 minutes-incubation). Initial Concentration of Incubation Medium 10^{-5} and 10^{-6} M.

At the initial concentration of 10^{-5} M the highest decrease of the concentration in the mucosal medium occurs with Pb^{212} and Hg^{203}, whereas the decrease of Cu^{64} and Mn^{52} is significantly ($p < 0.05$) lower. This finding counts for the duodenum as well as for the ileum (in the ileum the differences between the individual metals, however, are smaller). In a concentration of 10^{-6} M there is again the highest decrease of Pb^{212} and Hg^{203}, whereas with Mn^{52} it is significantly ($p < 0.05$) lower. This is valid for the duodenal as well as for the ileal segment (Fig. 6). The uptake by the intestinal wall in the initial concentration of 10^{-5} M in the duodenal segment was highest with Hg^{203}, which is significantly ($p < 0.05$) higher than the remaining three metals. There is no significant difference between Cu^{64} and Pb^{212}, but both metals are significantly ($p < 0.05$) higher than Mn^{52} (see Fig. 7). In the ileal segment the relation between the individual metals is similar, but the values are higher.

The uptake by the intestinal wall in a concentration of 10^{-6} M in the duodenal segment is highest with Pb^{212}, and significantly ($p < 0.05$) lower are Hg^{203} and Mn^{52}, with no significant difference between them. In the ileum the highest uptake by the intestinal segment is with Pb^{212}, lower with Hg^{203} and lowest with Mn^{52}. All differences are significant ($p < 0.05$).

in the serosal medium in the initial concentration of 10^{-5} M in duodenal segments the values were highest with Mn^{52} and significantly ($p < 0.05$) higher than with the other three metals (there were no significant differences between them). In ileal segments the highest values were again found with Mn^{52}; they were significantly ($p < 0.05$) higher than with the other three metals. There were lower values with Hg^{203} and the lowest with Cu^{64} and Pb^{212}. The difference between Hg^{203} and the two metals mentioned last was significant ($p < 0.05$) (see Fig. 8).

Discussion

We compared the uptake of 4 metals (Pb^{212}, Mn^{52}, Cu^{64}, and Hg^{203}) by the duodenal and ileal segment in vitro. With regard to Hg^{203} we made use of our previous results (in press). These results with Hg^{203} were corrected by substraction of losses of Hg^{203} from the incubation solution during incubation (loss caused by the escape of mercury from the incubation medium with steam and trapping of mercury on the walls of the glass incubation vessel).

In all metals studied there was a characteristic curve of metal decrease in the incubation medium during incubation. This curve can be divided into two phases which differ by their velocity constants. The question is, of course, what these two phases mean. It seems that the

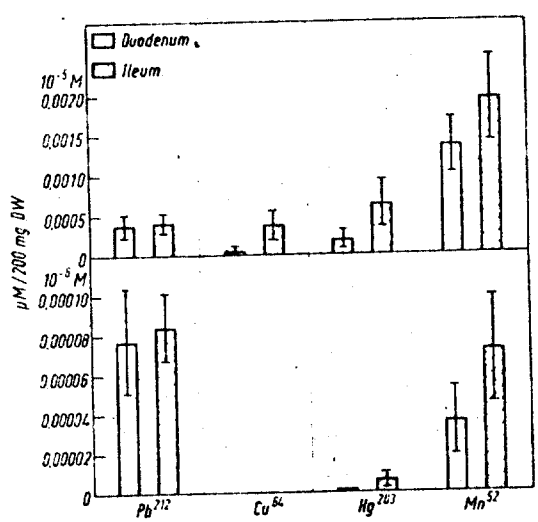

Fig. 8. Serosal Medium (after 60-minutes-incubation). Initial Concentration of Mucosal Medium 10^{-5} and 10^{-6} M.

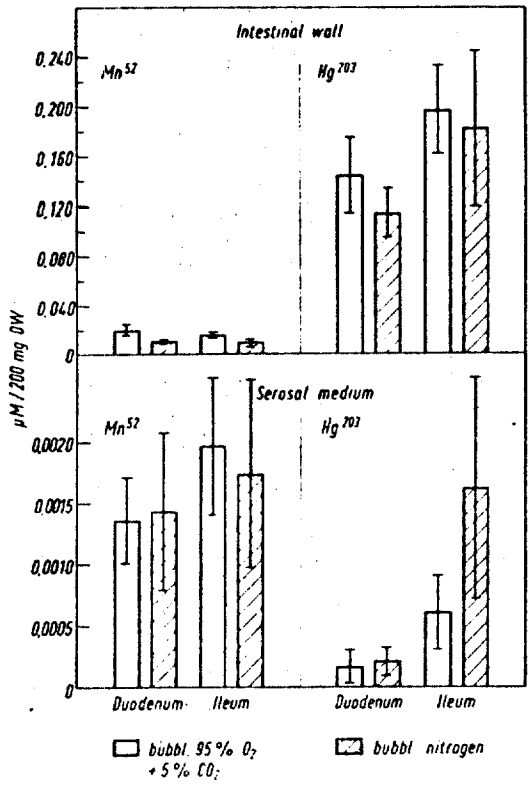

Fig. 9.

first phase, which is more rapid, represents a rapid sorption on the epithelial surface cells of the intestinal mucosa and simultaneously a gradual penetration of the metal through the intestinal wall towards the serosa. The second, slower phase expresses probably the gradual flow along the concentration gradient across the intestinal wall, possibly into the serosal medium. We may meet a similar division in the paper by SCHACHTER et al. (1966) regarding the transport of Ca on everted sacs, who proved two phases: 1. Uptake by the mucosal surface; 2. Transfer to (or in direction of) the serosal surface. SCHACHTER et al. (1966) determined that there exist two increasing differences of concentration during the transport: from the mucosal medium to the mucosa and from the mucosa to the lower layers; and these gradients correspond to the two mentioned degrees of mere transport.

For all metals except manganese there was proved a significantly higher uptake by the ileal segment in comparison to the duodenal segment. Also in the serosal medium, except Pb^{212}, there was found a significant difference between the duodenal and the ileal segments in favour of the ileum. In the case of lead we explain this exception by the fact that lead is applied in form of citrate salt (SALTMAN and HELBOCK — 1965 — mention the dependence of the transport of Fe on the chelating agent, i.e. the citrate increases the in vitro transport approximately 10 times in comparison to Fe^{2+}).

Very interesting are the results of Mn^{52} bubbling with nitrogen and pneumoxide (Fig. 9). The significant decrease in the uptake of Mn^{52} by the intestinal wall, when bubbling with nitrogen in the duodenum and in the ileum, confirms our previous results of inhibition of Mn transport (CIKRT and VOSTÁL, 1969). The fact that no difference was detected in the serosal medium may be explained by the incubation of only 60 minutes. The behaviour of Mn^{52}, when compared to the other metals studied, differs in principle. Mn^{52} showed the lowest decrease in the mucosal medium during the incubation, the lowest uptake by the intestinal wall in both segments; and yet significantly more Mn^{52} penetrates into the serosal medium than other metals during the same period of time. The results — together with the above mentioned inhibition of resorption by nitrogen saturation — tend to prove a more active transport.

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Author: Dr. M. Cikrt, Institute of Industrial Hygiene and Occupational Diseases, Prague, ČSSR.

Am. J. Med. 20: 944-945, 1956

MANGANESE DEPLETION AS AN ETIOLOGICAL FACTOR IN HYDRALAZINE DISEASE. *P. Comens* (introduced by H. A. Schroeder*). Hypertension Division, Dept. Internal Medicine, Washington University School of Medicine, St. Louis, Mo.

The induction of "hydralazine disease" in dogs with lupus erythematosus cells in the blood and pathologic changes in the kidneys consistent with disseminated lupus erythematosus was reported. Hydralazine chelates several trace metals: (1) This drug was administered to ten-day old cockerels, 10 mg. per day. In all perosis developed within six weeks, a disease considered to result from manganese deficiency. Another group was simultaneously fed hydralazine and

manganese citrate, 5 mg. per day, and in all development was normal. (2) Manganese inhibited the *in vitro* formation of the lupus erythematosus cell in human subjects in concentrations of 1×10^{-2} molar; copper, cobalt, zinc and iron caused no inhibition. (3) Rats convulse in about an hour when injected with hydralazine. Manganous glycerophosphate or pantothenic acid injected five to thirty minutes later prevented convulsions. Five other trace metals and pyridoxine had no effect. (4) While in all dogs fed hydralazine alone the lupus-like syndrome developed, no glomerular wire loops appeared in two dogs given parenteral manganous citrate concomitantly. Studies are underway in dogs to ascertain whether or not the lesions of the kidney are reversible. (5) Three patients with hydralazine disease and two with disseminated lupus have apparently improved symptomatically when manganous ion was administered; in the former hydralazine was continued. The evidence suggests that hydralazine may produce "hydralazine disease" by binding manganous ion, possibly blocking dependent enzyme systems.

MANGANESE CHLORIDE

$MnCl_2.4H_2O$ Mol. wt. 197.91

DESCRIPTION

Large, irregular, pink, translucent crystals. It is freely soluble in water at room temperature and very soluble in hot water.

IDENTIFICATION

A 1 in 20 solution gives positive tests for *Manganese*, page 927, and for *Chloride*, page 926.

Food Chemicals Codex, 1972

SPECIFICATIONS

Assay. Not less than 98.0 percent and not more than the equivalent of 102.0 percent of $MnCl_2.4H_2O$.

pH of a 5 percent solution. Between 4.0 and 6.0.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 10 parts per million (0.001 percent).

Insoluble matter. Not more than 50 parts per million (0.005 percent).

Iron. Not more than 5 parts per million (0.0005 percent).

Substances not precipitated by sulfide. Not more than 0.2 percent, after ignition.

Sulfate. Not more than 50 parts per million (0.005 percent).

TESTS

Assay. Transfer about 4 grams, accurately weighed, into a 250-ml. volumetric flask, dissolve in water, dilute to volume with water, and mix. Transfer 25.0 ml. of this solution into a 400-ml. beaker, and add 10 ml. of a 1 in 10 solution of hydroxylamine hydrochloride, 25 ml. of 0.05 *M* disodium ethylenediaminetetraacetate measured from a buret, 25 ml. of ammonia-ammonium chloride buffer T.S., and 5 drops of eriochrome black T.S. Heat the solution to between 55° and 65°, and titrate from the buret to a blue end-point. Each ml. of 0.05 *M* disodium ethylenediaminetetraacetate is equivalent to 9.896 mg. of $MnCl_2.4H_2O$.

pH of a 5 percent solution. Determine by the *Potentiometric Method*, page 941.

Arsenic. A solution of 1 gram in 35 ml. of water meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. A solution of 2 grams in 25 ml. of water meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Insoluble matter. Dissolve about 20 grams, accurately weighed, in 200 ml. of water, and allow to stand on a steam bath for 1 hour. Filter through a tared sintered glass crucible, wash thoroughly with hot water, dry at 105° for 1 hour, cool, and weigh.

Iron. Dissolve 2.0 grams in 20 ml. of water, add 1 ml. of hydrochloric acid, and dilute to 50 ml. with water. Add about 40 mg. of ammonium persulfate crystals and 3 ml. of ammonium thiocyanate T.S. Any red or pink color does not exceed that produced by 1.0 ml. of *Iron Standard Solution* (10 mcg. Fe) in an equal volume of a solution containing the quantities of the reagents used in the test.

Substances not precipitated by sulfide. Dissolve 2.0 grams in about 90 ml. of water, add 4 ml. of ammonium hydroxide, heat to 80°,

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and pass hydrogen sulfide through the solution to completely precipitate the manganese. Dilute to 100 ml., mix, and allow the precipitate to settle. Decant the supernatant liquid through a filter, and evaporate 50 ml. of the filtrate to dryness in a tared dish. Add 0.5 ml. of sulfuric acid, ignite to constant weight, cool, and weigh.

Sulfate. Dissolve 10.0 grams in 100 ml. of water, add 1 ml. of diluted hydrochloric acid T.S., mix, and filter. Heat to boiling, then add 10 ml. of barium chloride T.S., and allow to stand overnight. Filter out any precipitate in a tared crucible, wash, ignite gently, cool, and weigh. The weight of the ignited precipitate should not be more than 1.2 mg. greater than the weight obtained in a complete blank test.

Packaging and storage. Store in well-closed containers.

Functional use in foods. Nutrient; dietary supplement.

MANGANESE GLUCONATE

$[CH_2OH(CHOH)_4COO]_2Mn.3H_2O$

$C_{12}H_{22}MnO_{14}.2H_2O$ Mol. wt. 481.27

DESCRIPTION

A slightly pink colored powder. It is very soluble in hot water and is very slightly soluble in alcohol.

IDENTIFICATION

A. A 1 in 20 solution gives positive tests for *Manganese*, page 927.

B. It meets the requirements of *Identification test B* under *Copper Gluconate*, page 219.

SPECIFICATIONS

Assay. Not less than 98.0 percent of $C_{12}H_{22}MnO_{14}.2H_2O$.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 40 parts per million (0.004 percent).

Lead. Not more than 10 parts per million (0.001 percent).

Reducing substances. Not more than 0.5 percent.

TESTS

Assay. Dissolve about 600 mg., accurately weighed, in 50 ml. of water in a 250-ml. porcelain casserole, add 1 gram of hydroxylamine hydrochloride, 10 ml. of ammonia-ammonium chloride buffer T.S., and 5 drops of eriochrome black T.S., and titrate with 0.05 *M* disodium

ethylenediaminetetraacetate to a deep blue color. Each ml. of 0.05 *M* disodium ethylenediaminetetraacetate is equivalent to 24.06 mg. of $C_{12}H_{22}MnO_{14}.2H_2O$.

Arsenic. A solution of 1 gram in 35 ml. of water meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. A solution of 500 mg. in 25 ml. of water meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. A solution of 1 gram in 25 ml. of water meets the requirements of the *Lead Limit Test*, page 929, using 10 mcg. of lead ion (Pb) in the control.

Reducing substances. Determine as directed in the test for *Reducing Substances* under *Copper Gluconate*, page 219.

Packaging and storage. Store in well-closed containers.

Functional use in foods. Nutrient; dietary supplement.

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MANGANESE SULFATE

$MnSO_4.H_2O$ Mol. wt. 169.01

DESCRIPTION

A pale pink, granular, odorless powder. It is freely soluble in water and is insoluble in alcohol.

IDENTIFICATION

A 1 in 10 solution gives positive tests for *Manganese*, page 927, and for *Sulfate*, page 928.

SPECIFICATIONS

Assay. Not less than 98.0 percent and not more than the equivalent of 102.0 percent of $MnSO_4.H_2O$.

Loss on heating. Between 10 and 13 percent.

Limits of Impurities

Arsenic (as As). Not more than 3 parts per million (0.0003 percent).

Heavy metals (as Pb). Not more than 40 parts per million (0.004 percent).

Lead. Not more than 10 parts per million (0.001 percent).

TESTS

Assay. Transfer about 4 grams, accurately weighed, into a 250-ml. volumetric flask, dissolve in water, dilute to volume with water, and mix. Transfer a 25.0-ml. portion of this solution into a 400-ml. beaker, and add 10 ml. of a 1 in 10 solution of hydroxylamine hydrochloride, 25 ml. of 0.05 *M* disodium ethylenediaminetetraacetate measured from a buret, 25 ml. of ammonia-ammonium chloride buffer T.S., and 5 drops of eriochrome black T.S. Heat the solution to between 55° and 65°, and titrate from the buret to a blue end-point. Each ml. of 0.05 *M* disodium ethylenediaminetetraacetate is equivalent to 8.450 mg. of $MnSO_4.H_2O$.

Loss on heating. Heat about 1 gram, accurately weighed, in a crucible tared in a stoppered weighing bottle, to constant weight at 400°–500°. Cool in a desiccator, transfer to the stoppered weighing bottle, and weigh.

Arsenic. A solution of 1 gram in 35 ml. of water meets the requirements of the *Arsenic Test*, page 865.

Heavy metals. A solution of 500 mg. in 25 ml. of water meets the requirements of the *Heavy Metals Test*, page 920, using 20 mcg. of lead ion (Pb) in the control (*Solution A*).

Lead. Dissolve 1 gram in 3 ml. of dilute nitric acid (1 in 2) and 10 ml. of water, and boil for 2 minutes. Cool, and dilute to 100 ml. with

water. A 25-ml. portion of this solution meets the requirements of the *Lead Limit Test*, page 929, using 25 ml. of *Ammonium Citrate Solution*, 1 ml. of *Potassium Cyanide Solution*, 0.5 ml. of *Hydroxylamine Hydrochloride Solution*, and 2.5 mcg. of lead ion (Pb).

Packaging and storage. Store in well-closed containers.

Functional use in foods. Nutrient; dietary supplement.

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J. Clin. Invest 37; 1298-1305, 1958

THE HIGH SPECIFICITY OF THE MANGANESE PATHWAY THROUGH THE BODY [1, 2]

By GEORGE C. COTZIAS AND JAMES J. GREENOUGH

(*From the Medical Department, Brookhaven National Laboratory, Upton, N. Y.*)

(Submitted for publication March 13, 1958; accepted May 2, 1958)

The accepted ideas about the physiological role of manganese have been derived predominantly from *in vitro* experiments. However, these are characterized by lack of specificity: Only a limited discrimination between manganese and some other metals is shown, for instance, in metal transport systems (1, 2), in enzyme activation (2–4) and in the preservation of mitochondria (5). The conclusions from these experiments were of interest because they threw new doubt on the functional specificity and even on the essentiality of manganese in the living organism. Thus it became necessary to ascertain whether manganese may be replaced in the intact animal as well. To investigate this, we have used a variation of the classical technique of flooding the organism with metals which we were led to believe would substitute for manganese. Surprisingly, these kindred metals were ineffective in eluting radiomanganese from the body: Only manganese compounds proved effective in that regard.

The results suggest that there exists a segment in the pathway of manganese through the body, the properties of which permit the passage of that metal only. This paper deals with the implications of this finding both in reference to manganese as well as to the elements which have been thought not to possess such an *in vivo* specificity.

MATERIALS AND METHODS

Experiments with intact animals

Animals. These were seven week old Swiss albino male mice, weighing 16 to 18 grams. They were housed in cages, the bedding of which consisted of shredded corn cobs. When dietary restrictions were imposed, metal screening was used to prevent the animals from eating the bedding. Regardless of the type of bedding, the furs remained radiologically clean, as shown by the failure of Versene[39] and detergent baths to lower the total body radioactivity.

Diets. Since the manganese turnover depends upon dietary intake, an effort was made to keep the manganese intake constant. In the bulk of the experiments, the same lot of Purina Fox chow was used ($MnSO_4$ reported by manufacturer as 0.02 per cent). As a "manganese free" diet, a vitamin B_{12} deficient rat diet was used.[3] To this a salt-vitamin fortification mixture[4] was added without supplementary manganese, unless otherwise stated.

Stable metals and their salts. Rhenium dioxide was prepared by dissolving the metal in nitric acid and heating to dryness with anhydrous hydrazine HCl. The procedure was repeated twice with the precipitate. This was finally washed with water, ground into a fine suspension in normal saline and injected into the animals intraperitoneally. All the metals, oxides and salts[5] used in these experiments to challenge the isotope were injected with normal saline as the vehicle. In some experiments (see Table I) the sulfates or chlorides of the metals were dissolved with added equimolar amounts of sodium citrate (hereafter referred to simply as "citrate"). In those latter experiments the controls received equimoler sodium citrate instead of the routinely used saline injections. The doses of the challenging materials ranged from 1×10^{-8} to 1×10^{-5} mole of metal. These were given either as single or as daily injections for one week.

[1] This work was supported by the United States Atomic Energy Commission. It was accorded an Honorable Mention in the A. Cressy Morrison Award in Natural Science in 1957, by the New York Academy of Sciences.

[2] A preliminary report of this paper was presented before the Federation of American Societies for Experimental Biology in Chicago, March, 1957.

[3] Nutritional Biochemicals Corporation. The complete diet contained 2.5γ of manganese per 100 gram diet (or 1γ of manganese per gram ash).

[4] Nutritional Biochemicals Corporation, "Vitamin Diet Fortification Mixture."

[5] Spectrographic tests of the $MnSO_4$ powder showed it to be free from contaminating metals, while both the $FeSO_4$ and the $CrCl_3$ powders contained 0.01 per cent manganese. The utilized chemical agents and their sources of purchase are as follows: cobaltous sulfate, ferrous chloride, manganese dioxide, nickelous sulfate and sodium citrate from Baker and Adams Co.; copper sulfate, magnesium sulfate, potassium permanganate and zinc sulfate from Baker's; ferric chloride from Merck & Co.; manganous chloride from Mallinkrodt; manganese metal from A. D. Mackay; vanadyl sulfate from Fisher; and vanadium metal from Amend Drug. The rhenium compounds were obtained from the University of Tennessee except for the rhenium pentachloride (K & K Labs.).

Isotopes. Mn^{52} (T/2 = 5.7 days) was prepared by the Cr^{52} (d, 2n) Mn^{52} reaction in a 60 inch cyclotron by one of us (J. J. G.). Separation of the tracer Mn^{52} from the chromium necessitated a precipitation with manganese carrier. When the latter was found to have a marked influence on the phenomena under study, this preparation was abandoned in favor of Mn^{54}. The latter isotope was prepared carrier free by the Nuclear Science and Engineering Corporation. It was received as $Mn^{54}Cl_2$ in hydrochloric acid (200 μc. per ml.). Neutral dilutions (0.8 and 1.6 μc.) were given intraperitoneally to the animals in 0.1 or 0.2 ml. of saline. While with the Mn^{52} variations even from 0.1 to 0.2 μc. brought upon concordant variations of the turnover rate, the Mn^{54} preparation was free from such complicating effects. Accidental injections into a hollow viscus were soon followed by almost complete loss of the animal's radioactivity.

Measurements of the radioactivity. Each tagged animal was placed in a 50 ml. plastic centrifuge tube with its mouth opposite to the outlet of an oxygen line. After plugging with cotton, the tube was inserted into the horizontal well of a Texaco® counter. The same procedure (minus the ventilation) was used for pooled organs, to permit the direct comparison between animals and their organs. As a rule counting was accurate to within 1 per cent in both cases, and its geometry was constant between 3 and 30 ml.

Calculations of turnover rates. The animal's total body radioactivity was counted within one hour after isotope injection. This was repeated daily, in some cases for as long as 20 to 30 days. The subsequent counts were expressed as percentages of the first, after being corrected for background and, if needed, for isotopic decay. The data were then plotted on semilogarithmic paper as a function of time and the curves were drawn (Figure 1). When necessary, these were analyzed graphically for their constituent components (Figure 2) by subtracting the straight end from the remainder of each curve (6). In the experiments in which challenging injections of carrier metals were given, the following controls were used: *1)* saline- or sodium citrate-challenged animals; *2)* each animal's own prechallenge turnover rate; *3)* animals that had received $MnCl_2$ in amounts often less than equimolar to that of the challenging metal. At the end of some experiments, in which the challenge did not effect the turnover rate, the isotope's continued availability for exchange was demonstrated by injecting $MnCl_2$ solutions.

Summary of typical protocol. Thirteen mice were injected intraperitoneally with 1.0 μc. of $Mn^{54}Cl_2$ in 0.2 ml. saline, and their total body radioactivity determined. They were then divided into four groups. The retained radioactivity was measured at daily intervals. The corrected counts were plotted semilogarithmically against time as per cents of the first count. On the third day, Group I received 1×10^{-4} mole of $CrCl_2$; Group II, 0.2 ml. of saline; Group III, 2×10^{-4} $MnCl_2$; Group IV, 1×10^{-4} $FeCl_2$. The day following the injection the phenomenon illustrated in Figure 4 was seen. It was followed for five days and then the 2×10^{-4} mole of $MnCl_2$ were given to the animals that had received $CrCl_2$ and $FeCl_2$. This was done in order to test whether their tracer had remained exchangeable. The animals were then sacrificed, and the peritoneal cavities were found to be free of metallic deposits.

OBSERVATIONS

Turnover of body manganese. The results shown in Figure 1 were obtained with a group of nine Mn^{54} tagged animals. These animals were maintained on Purina Fox chow. The individual data fell into exceedingly smooth curves so that, subsequently, breaks in the curves which were experimentally induced could be used as additional controls. When these curves were graphically analyzed, at least two components were found (T/2 = 48 to 68 hours and T/2 = 230 to 300 hours).

Two groups of three mice each were given 1 per cent ammonium chloride and 1 per cent sodium bicarbonate instead of drinking water. After no effect on the turnover was seen, their bottles were switched. This again failed to produce any change in the turnover rates.

Effect of diet on manganese turnover. The feeding of the "manganese-free" diet to a group of three mice a few days prior to and during the experiment did not effect the rate of the first component (T/2 = 54 hours), while that of the second increased to T/2 = 860 hours. The diet was enriched with carrier manganese sulfate (0.01 per cent of Mn^{++} w/w), and the procedure was repeated in three other mice. This showed turnover

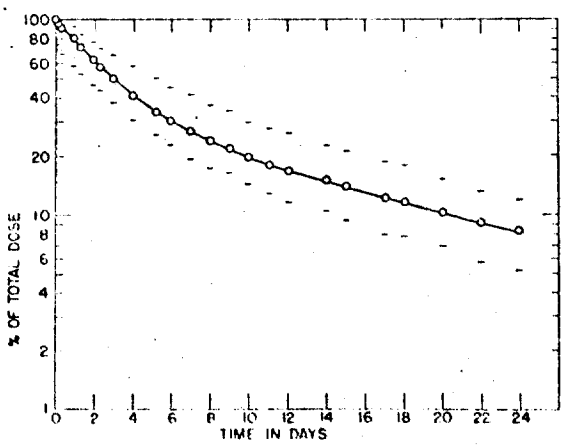

FIG. 1. TOTAL BODY TURNOVER OF Mn^{54} IN MICE (SEE TEXT)

The dashed lines define the envelope of all the data.

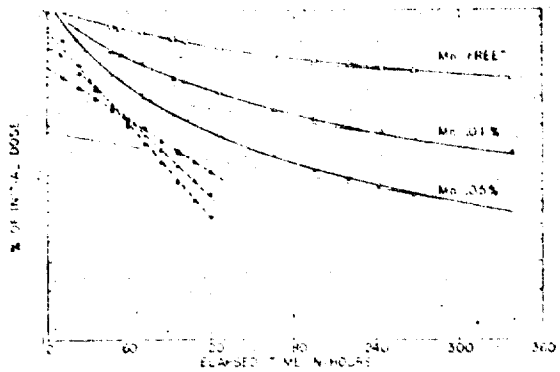

FIG. 2. TOTAL BODY TURNOVER OF Mn^{54} AS INFLUENCED BY DIETARY MANGANESE

rates of T/2 = 38 and T/2 = 238 hours for each of the two components. Increase of the manganese supplement to 0.05 per cent caused further decreases of these numbers to T/2 = 31 and T/2 = 208 hours in another group of three mice. Starvation of three animals markedly decelerated the turnover. Obstruction of the intestine at the anus in another three mice stopped the turnover entirely.

These experiments show that the rate of radiomanganese loss from the body is sensitive to the level of dietary manganese and to the state of gastrointestinal function. They are compatible with intestinal excretion of this metal (*7*). Concordantly, the acceleration of the observed turnover by added dietary manganese might have had the following implications: *1*) that more carrier manganese is absorbed from the gut than hitherto suspected and elutes the radioisotope from the tissues; or *2*) that on elimination from the gut, the stable metal carries with it a significant fraction of the bile-bound tracer (*6, 7*) which normally might have been reabsorbed (*8*). Regardless of which explanation might be correct, this uncertainty would throw doubt on experiments with any stable metal, if these were fed. Thus the oral route was abandoned in favor of the intraperitoneal.

Accelerated elimination of radiomanganese following challenge with manganese carrier. Both Mn^{54} and Mn^{52} injected animals were used in these experiments.

As can be seen in Table I, various forms of the stable metal were given at various times. The valence state of the carrier ranged from 0 in the metal powder to 7 in the permanganate. Injections of all of these compounds were followed by acceleration of the rate of turnover, which resulted in losses ranging from 15 per cent to 80 per cent within twenty four hours. Figure 3 illustrates two characteristic experiments at two different postinjection times with two different isotopes. By varying the time at which the

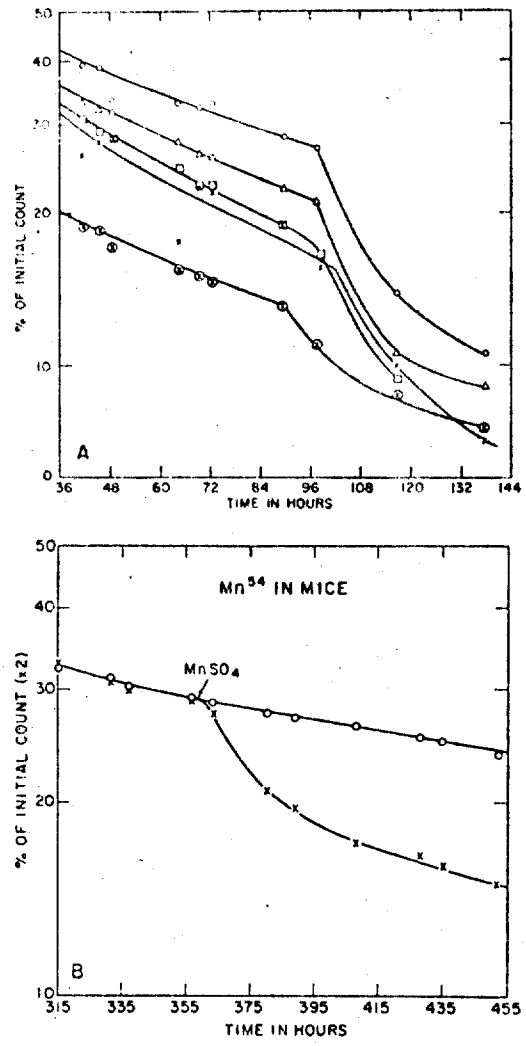

FIG. 3. EFFECT OF ADDING CARRIER MANGANESE ON RADIOACTIVE MANGANESE TURNOVER

A = Total body turnover of Mn^{54} in mice receiving 8×10^{-6} mole of carrier manganese as $MnSO_4$ at the 86th hour of the experiment.

B = Averages of total body turnovers of Mn^{54} in three mice compared with those of three mice receiving 4×10^{-6} mole of carrier manganese as $MnSO_4$ at the arrow.

challenge was made, it was found that an appreciable fraction of the tracer had remained exchangeable, even three weeks after tagging the animals. The exchange has not been quantitated as yet. The impression was gained, however, that elution of the tracer manganese was more obvious when the challenge was made early or with high doses of carrier. An easily observed acceleration of the turnover was evident following injections of 9×10^{-8} mole of $MnSO_4$ as late as the sixth day. The extreme valence states (Mn^0 and Mn^{7+}) seemed less effective than Mn^{2+}.

These experiments proved that the radioisotope remained largely and continuously available for elution by carrier. The elution was obvious after challenges with $MnSO_4$ amounting to 30 per cent of the estimated manganese content of the body.

Failure of some other stable metals to affect Mn^{54} turnover. Manganese is a member of both the first transition group and of group VIIB of the elements. Many of the members of the transition group (in periods four and five) have been known to substitute for manganese *in vitro* (1–5, 9). Manganese and rhenium are the only stable members of group VIIB. Rhenium is Mendeleef's celebrated dvi-manganese (10). His eka-manganese (technetium), which would have been very crucial in these studies, has only radioactive isotopes.

These metals were used as eluting agents in experiments identical with those described in the case of carrier manganese. Magnesium, the element most widely used *in vitro* instead of manganese, was also included. In another experiment, a member of group VIIA was tested because of its possible interchange with manganese in the thyroid (11).

The upper limit of the concentration in the challenging injections was set by the toxicity of the individual compounds. Safe doses were chosen after preliminary experimentation with groups of mice. Care had to be taken to avoid interference with the animals' eating and bowel habits which were known to affect the end point.

The lower limit was set at 30 per cent of the mouse's total body content of each of the stable metals used as eluting agents. This value was chosen because of the effect of carrier manganese described above. Furthermore, 30 per cent of anything appeared to be a large amount. Table I shows both the metals' estimated body content and the challenging doses given. High values were intentionally chosen in making these estimates. A mouse weighing 20 grams was chosen as a prototype in spite of the fact that the experimental animals weighed 16 to 18 grams. Even so, the desired value of 30 per cent was reached or exceeded by the individual injections. It was markedly surpassed in the experiments in which the injections were given daily for one week.

Every effort was made to compare similar valence states with each other. Some animals which had received toxic doses of ferrous and chromous chlorides had small peritoneal metallic desposits at autopsy. The mouse peritoneum had shown a high absorptive capacity in the experiments with manganese metal and dioxide powders. In spite of this the precaution was taken of including citrate in many of these injections, since this markedly facilitates absorption by forming chelates with these metals (12). The metals' release in the body was expected because citrate is rapidly metabolized.

These stable metals were incapable of affecting in any way the rate of elimination of radiomanganese from the body, in spite of all the specifications listed above. Figure 4 illustrates the failure of ferrous and chromous citrates to bring upon any change of the radiomanganese turnover in concentrations 50 times higher than an effective amount of manganous sulfate. That the isotope was available for exchange in these animals also was shown by the effect of the manganous salt at the end of that observation.

Experiments with organs

Following the observation that challenges with carrier manganese had caused elution of radiomanganese readily, reproducibly and regardless of the state of the carrier, the failure of any of the other stable metals to do so seemed incongruous. The following explanation for the apparent incongruity was considered and tested: It was thought that the nonmanganese metals might have caused elution of the tracer from one organ into another instead of elimination out of the body. This would not have been detected by our method, since it was designed to quantitate the radioactivity of the entire body and not to differentiate be-

TABLE I

Summary of metals administered to manganese[54] injected mice

No. of mice	Metal or salt	Atomic number	Moles per 20 Gm. mouse*	Moles per challenge	Mn^{54} isotope
7	$MgSO_4$	12	4.2×10^{-4}†	$5 \times 10^{-4} - 5 \times 10^{-4}$	146
3	$VOSO_4$	23	1.3×10^{-7}‡	1.0×10^{-6}	310-505§
9	Cr Cit	24	3.1×10^{-7}‡	$2 \times 10^{-6} - 1 \times 10^{-5}$	(−)6-505§
13	$CrCl_2$	24		$4 \times 10^{-6} - 1 \times 10^{-5}$	50-650
3	Mn^0	25	2.8×10^{-7}‡	$9 \times 10^{-5} - 4.5 \times 10^{-4}$	6-146
11	Mn Cit	25		$1.8 \times 10^{-6} - 9 \times 10^{-6}$	(−)6-674§
28	$MnSO_4$	25		$9 \times 10^{-5} - 1 \times 10^{-5}$	50-650
3	MnO_2	25		1.1×10^{-4}	(−)24
3	$KMnO_4$	25		2.5×10^{-6}	354
6	$FeCl_2$	26	2.1×10^{-6}‖	$9 \times 10^{-6} - 1 \times 10^{-5}$	50-650
11	Fe^{++} Cit	26		1.8×10^{-6}	(−)6-480§
3	Fe^{+++} Cit	26		1×10^{-6}	1
3	$FeCl_3$	26		3×10^{-6}	48
6	Co Cit	27	9×10^{-8}‡	$1.7 \times 10^{-6} - 8.5 \times 10^{-6}$	311-480§
3	$CoSO_4$	27		3.5×10^{-7}	266
3	Ni Cit	28	6.1×10^{-7}‡	$1.7 \times 10^{-6} - 8.5 \times 10^{-6}$	311-480§
7	Cu Cit	29	6.3×10^{-7}‖	$1 \times 10^{-7} - 3 \times 10^{-7}$	(−)6-364§
10	$CuSO_4$	29		$1 \times 10^{-7} - 6 \times 10^{-7}$	50-318§
3	Zn Cit	30	1.6×10^{-5}‡	$1.5 \times 10^{-6} - 7.6 \times 10^{-6}$	311-480§
3	$ZnSO_4$	30		3.5×10^{-7}	266
3	KIO_3	53	7.1×10^{-5}†	4.6×10^{-6}	354
7	Re^0	75		5.5×10^{-5}	6-50
7	ReO_2	75		4.6×10^{-6}	(−)24-270§
3	Re_2O_7	75		9.7×10^{-6}	1
3	$NaReO_4$	75		2.7×10^{-5}	354

* These are estimates of the mouse's total body content prior to challenge. All estimates pertain to the individual metal involved in the test and not to the salt listed.
† Estimated from Hawk, Oser and Summerson (14).
‡ Estimated from Tipton and co-workers (18).
§ Include daily injections for one week.
‖ Estimated from Underwood (19).

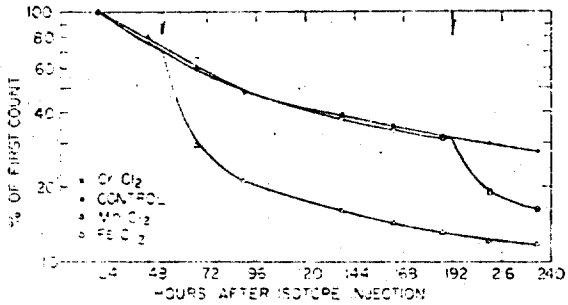

FIG. 4. TOTAL BODY TURNOVER OF Mn^{54} IN MICE AS INFLUENCED BY SOME CARRIER METALS

At first arrow 0.2 ml. of saline, 1×10^{-5} mole of either $FeCl_2$ or of $CrCl_2$, or 2×10^{-7} mole of $MnCl_2$ were injected in each of the groups. The 2×10^{-7} mole of $MnCl_2$ were injected, at the second arrow, to the $FeCl_2$ and $CrCl_2$ treated animals.

tween its parts. Thus we investigated the effects of some of these stable metals on the partition of the tracer amongst the organs of the body. The intracellular organelles could be also tested from that standpoint, particularly since it was observed earlier (13) that the mitochondria concentrate manganese isotopes. For this, one might have chosen an organ on the basis of some gross observation: As will be seen below, no such lead was forthcoming.

In view of the size of the enterprise involved in using all of the previously tested metals in this study as well, we had to arrive at a compromise: We chose elements 24, 25 and 26 (Cr^{++}, Mn^{++} and Fe^{++}).

For brevity, only Experiments A and B (Fig-

ure 5) are described out of four concordant ones (the others differed in several experimental details). In both of these the animals were divided into groups of three and eight, respectively, injected with radiomanganese tracer and tested for radioactivity on the first day. In Experiment A these groups were injected with 1×10^{-5} mole of sodium citrate or manganous, ferrous or chromous citrates per animals, respectively. The injections were made both in the morning and the evening of the second day. On the third, they were sacrificed within the hour following the test of their radioactivity. The ferrous citrate animals had given the impression of toxicity by displaying lesser physical activity and rougher furs than the controls. Thus, in Experiment B, the number of mice per group was increased, the manganous citrate group was omitted, the challenging dose of ferrous citrate was reduced to 2×10^{-6} mole and all the carriers were injected on the second and third days. The animals were sacrificed on the fourth day and were handled as in Experiment A.

The bodies of the animals were dissected and their organs divided as shown in Figure 5. The organs of each group were pooled and tested for radioactivity. Careful inspection of the peritoneal surfaces showed no metallic deposits.

As is shown in Figure 5, the only appreciable effect on the distribution of the tracer amongst the organs tested was again brought about by the manganese preparation. The others yielded a distribution comparable to that of the controls.

This phenomenon had the characteristics of the effect we had planned to show with the other stable metals, namely elution from one to another part of the body. It occurred above and beyond the accelerated elimination induced by manganese: In the experiments with the dissected organs, we have reported only the radioactivity retained in the body.

DISCUSSION

The uncertainty of projecting facts from the test tube into the realities of integrated life is clearly illustrated by this study. At the outset one was led to expect that several metals might elute radiomanganese from the body. Such expectation was based on the following facts: *1*) Many of the *in vitro* substitutes for manganese

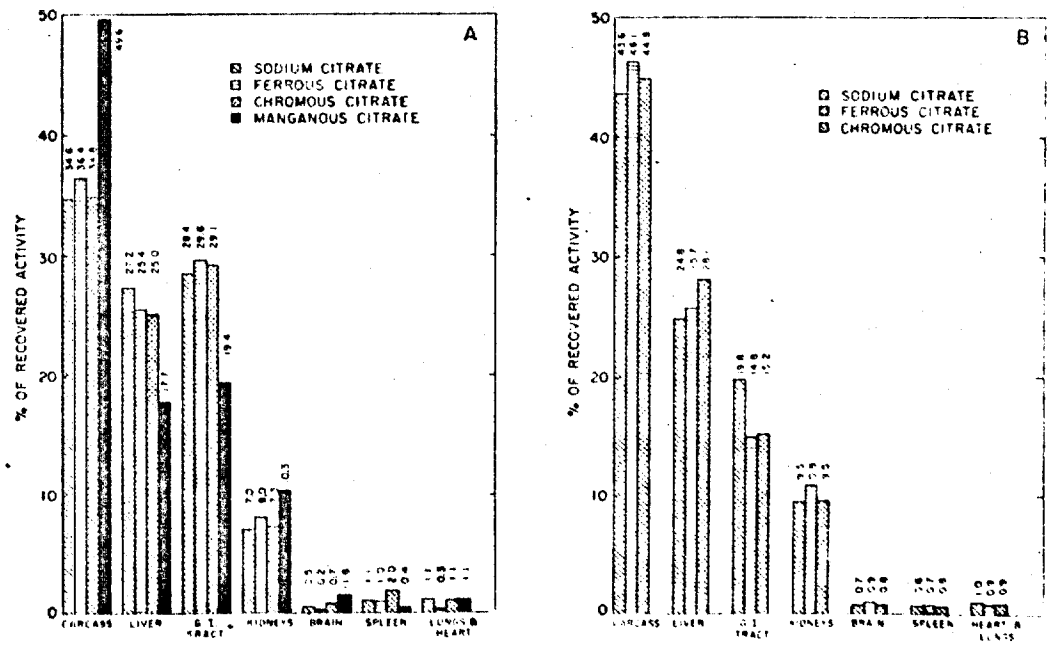

FIG. 5. PARTITION OF RADIOMANGANESE AMONG TISSUES OF MICE FOLLOWING INJECTION OF STABLE METAL COMPOUNDS

A and B summarize the results of Experiments A and B, respectively, described in the text

bind more strongly with tissue constituents (2). 2) Some are abundant, like magnesium, while the trace concentrations of manganese might not suffice to satisfy all its postulated reactions (14). 3) The known *in vitro* transport mechanism does not guard against competitors (11).

A review of the coordination chemistry of manganese (2, 12) did not reveal any one single property which alone could explain these results. If all the known coordination properties are considered, however, this specificity can indeed be easily explained. Therefore, one is justified to assume that there exist in the body micro-compartments possessing many properties, the sum total of which determine the entrance of manganese and manganese only. These might include: a fairly resilient octahedral arrangement of six charges; oxygen as the predominating donor atom; a space with a radius of 0.5 to 0.9 A. Such an environment would discriminate effectively against the entrance of other metals. If one also assumes that a given redox potential prevails at such a site, then specific reactions would be favored. A redox potential of minus 1.33 volts, for instance, would favor the reaction $Mn^{++} + H_2O = MnO_2 + 4H^+ + 2E^-$, while it would discriminate against other reactions.

The sum of such factors would constitute a specific segment in the anatomical pathway of manganese through the tissues of the body. The existence of specific atom configurations would not preclude binding of manganese by other less specific sites (2, 12). If this hypothesis were granted, one could ascribe many of the *in vitro* results to oversaturation of the few specific manganese sites with excess of metal. Such oversaturation would bring into evidence the properties of the numerous nonspecific sites. However, regardless of whether one favors this explanation, the observed irreplaceability of manganese in the body supports the concept of its performing specific tasks, rather than functions which might be taken over by other metals whenever the randomness inherent in the mass-law so dictates.

There is another extrapolation which one might have made *a priori* which also was shown by this work to be incorrect. One might have expected that manganese would be displaced by some metal (other than manganese) because of such well-known precedents as the displacement in the body of bromide by chloride (15), of molybdenum by tungsten (16) and of strontium by calcium (17). There exists, however, at least one metabolic difference between manganese and the elements listed above: the route of excretion. Manganese is excreted in the feces, while the others (Br, Mo, Sr, and so forth) are excreted primarily in the urine. This suggests that a search for other common differences might be rewarding. Furthermore it is obviously doubtful that manganese is the only substance whose pathway is strewn with specific segments, since one would expect similar behavior from iron also. With this in mind, an investigation of the pathways of other elements is now in progress.

SUMMARY

1. A procedure is described which permits observation of the elution of radiomanganese by injected stable metal compounds in intact mice and their tissues. This delineates an area for direct correlations between *in vivo* and *in vitro* phenomena.

2. Stable manganese compounds ranging from powdered metal to permanganate were effective eluting agents of the body's radiomanganese.

3. Injections of stable manganous citrate caused a pronounced change in the isotope's partition amongst the organs of these mice.

4. Stable members of the first transition group and of group VII of the elements as well as magnesium were tested for their capacity to elute the manganese tracer. In spite of injections of large doses, none of these affected the animal's normal rate of elimination of radiomanganese.

5. Chromous and ferrous citrates failed to cause deviations from the normal partition of Mn^{54} in the body.

6. The hypothesis is proposed that there exists a specific segment in the pathway of manganese through the body.

7. The specificity displayed by the manganese pathway is compared with the apparent lack of specificity illustrated by the *in vivo* displacement of bromide by chloride, of molybdenum by tungsten, of strontium by calcium and others. One common metabolic difference between these elements and manganese is noted: the route of excretion. This is entirely fecal for manganese and primarily renal for the other elements cited.

ACKNOWLEDGMENTS

The spectrographic analyses reported here were done by courtesy of Morris Slavin, Chemistry Department, Brookhaven National Laboratory. The authors wish to thank Mrs. Helen Hamel for assistance. Dr. Lee E. Farr's continued encouragement is gratefully recognized.

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Chronic manganese poisoning

Neurology 18:376-382, 1968

Clearance of tissue manganese concentrations with persistence of the neurological picture

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Sergio Fuenzalida, M.D., and Ismael Mena, M.D.

INTEREST IN chronic manganese poisoning was evoked by some clinical similarities to parkinsonism and to Wilson's disease.[1] An earlier study of Chilean ex-miners with neurological consequences of this poisoning showed that the clinical picture prevailing in Chile resembled that reported from other parts of the world.[1] Furthermore, it was shown that the rate of loss of artificial radiomanganese (^{54}Mn) from the whole body of healthy, working manganese miners was accelerated, whereas it was normal in patients with chronic manganese poisoning.

By examining the blood and plasma clearances as well as the disappearance of ^{54}Mn from individual segments of the body, the present study investigated whether the rapidly excreted metal was located in a single major pool or in a system if interconnecting pools. The data favored the latter alternative: healthy, working miners had accelerated tissue turnovers of radiomanganese in contrast to the poisoned ex-miners who had normal turnovers. Earlier work on animals had shown that accelerated turnovers are sometimes due to elevated tissue concentrations of natural manganese (^{55}Mn).[2-4] Therefore, it was suspected that the healthy miners conformed to this laboratory precedent, whereas the diseased ex-miners did not. Direct evidence was needed to decide whether the treatment of Wilson's disease with metal binding agents[5] constitutes a model for treating chronic manganese poisoning. Thus, the concentrations of ^{55}Mn were also determined in selected tissues from healthy miners and patients with chronic poisoning.

MATERIALS AND METHODS

A. *Radioisotopic studies. [1] Subjects.* All individuals were studied at the Department of Nuclear Medicine, Catholic University, Santiago, Chile. They were divided into the following 3 groups: (a) 19 normal controls who were men and women of the hospital staff between 20 and 30 years of age and who continued working, (b) 20 healthy, working miners 23 to 60 years of age who had worked in the mines for one to twenty-seven years and who were studied as inpatients for thirty days in Santiago (their state of health was documented earlier[1]), and (c) 18 patients with chronic manganese poisoning, ranging in age from 18 to 56 years, who had stopped working in the mines two to twenty-five years ago.

2] Isotope. Carrier-free ^{54}Mn was obtained from the Nuclear Science and Engineering Corporation in 20% hydrochloric acid (HCl) (0.5 mc. per milliliter). Single calibrated doses of 40 μc. in 0.9% sodium chloride (NaCl) solution were injected into an antecubital vein.

3] Blood and plasma sampling. The opposite antecubital vein was entered and 2-ml. heparinized samples were drawn every twenty sec-

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Supported by NIH grant OH 00159-03 and by the U.S. Atomic Energy Commission. Coordinated by PAHO/WHO Project AMRO 4609

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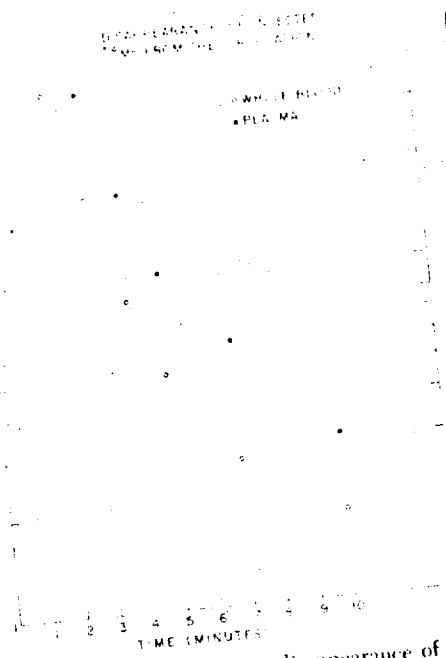

Fig. 1. Early phases of the disappearance of injected ^{54}Mn from the circulation of a normal subject. The data from all subjects are summarized in Table 1.

chemically separated from the mixture of co-activated radionuclides.[7,8] A second purification of the ^{56}Mn was performed when necessary, but even so, the lowest counting rates obtained were commensurate with those presented earlier.[7,8] Manganese standards were treated identically.

5] Statistical analysis. Fisher's *t* test and p values were generously computed by the Mathematics Department at Brookhaven.

RESULTS

1] Clearance of the tracer from whole blood and plasma. Typical examples of the early phases of the isotope's disappearance from the circulation are shown in Figure 1. In all cases, the initial clearance was exceedingly fast and only about 1% of the radioactivity initially recorded remained in the whole blood or in the plasma† after the elapse of about ten minutes. The mean half-life and standard deviation of the first ten minutes of these clearances are shown in Table 1 for each group. The group of healthy miners showed significantly slower ^{54}Mn clearance rates from both blood and plasma than did the control group. Statistical significance was not shown by any other permutation of these data.

The rapid initial phase of the clearance was followed by a slower component which remained in evidence for the subsequent thirty to sixty minutes (Fig. 2). The radioactivity remaining in plasma† did not show measurable changes during the ensuing thirty days. By contrast, all records of the whole blood showed a definite rise in the level of ^{54}Mn. The rise began about five to seven days after the injection of the tracer, lasted for the entire period of observation, and reached a height approximately double that of the base line.

Statistically significant differences were not encountered between the groups studied during any of the stages which followed the initial rapid phase of the clearance of the tracer.

2] Whole body turnovers. These were computed over thirty days. The mean half-life, standard deviation of the longest term exponential function, and their statistical significance in the three groups studied are presented in Table 2.

3] Regional turnovers. The activity over a thirty-day period in 3 normal subjects as measured over the right hypochondrium, the head, and the midthigh is presented in Figure 3. In all cases, the largest uptake of isotope was re-

TABLE 1

HALF TIMES OF CLEARANCE OF INJECTED ^{54}Mn FROM THE PLASMA AND WHOLE BLOOD

Group*	No.	Plasma biological half-life (minutes)	Whole blood biological half-life (minutes)
A	19	1.34 ± 0.3	1.28 ± 0.2
B	20	2.03 ± 0.7	2.19 ± 0.4
C†	18	1.44 ± 0.2	1.33 ± 0.2
Significant differences		A versus B: $0.01<p<0.02$	A versus B: $0.001<p<p\ 0.01$

*A = normal individuals; B = healthy manganese miners; C = patients with chronic manganese poisoning (abbreviations apply for all tables)

†See Figs. 1 and 2

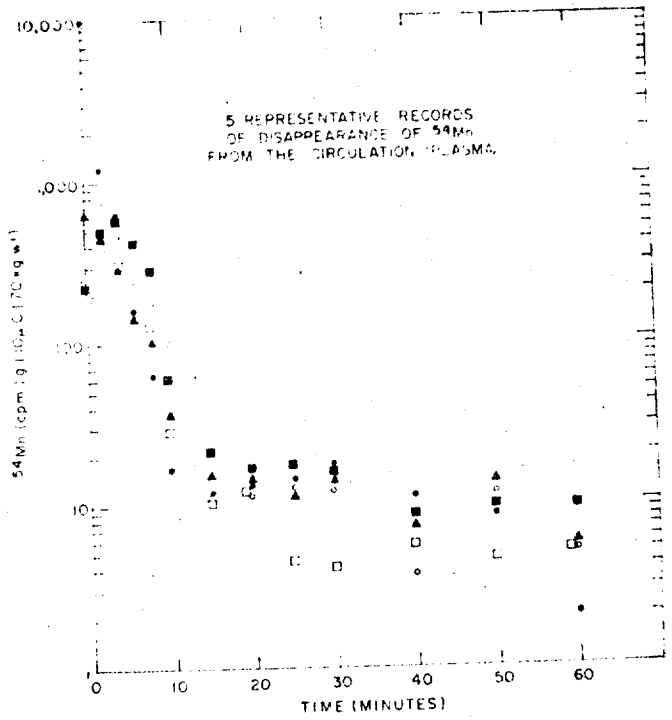

Fig. 2. Disappearance of injected ^{54}Mn from the plasma in 5 normal subjects (see Table 1)

corded by the measurement over the liver,[6] while the uptake of the other 2 regions was lower by at least one order of magnitude. However, the uptake of the head was about double that of the thigh. Furthermore, the rate of decline of the radioactivity was much faster over the liver than over the other two regions monitored. The mean half-lives; the standard deviation of the single exponential function over twenty-eight days in the whole body, liver, head, and thigh; and the statistical significance of the differences are shown in Table 2 for each of the groups studied. The group of healthy miners showed faster mean rates of loss for ^{54}Mn over all areas, but only the measurements of the whole body and the liver showed statistically significant differences from the normals.

4] Concentration of ^{55}Mn in tissues. As illustrated in Table 3, a high gradient of manganese concentrations was maintained between liquid and solid tissues even when the concentration of the metal was elevated. Similarly, the whole blood always maintained much higher concentrations than did either serum or cerebrospinal fluid.

The group of healthy miners showed higher mean values in all tissues than did the patients with chronic manganese poisoning except for the muscle biopsies. This single exception seems accidental; two of the muscle biopsies from the patients with chronic poisoning showed much higher amounts of metal than did all other samples analyzed here or re-

TABLE 2

HALF TIMES OF DISAPPEARANCE OF INJECTED ^{54}Mn FROM THE WHOLE BODY, LIVER, HEAD, AND THIGH IN THE 3 GROUPS LISTED UNDER TABLE 1 (See Fig. 2)

Group	No.	Whole body biological half-life (days)	Liver biological half-life (days)	Head biological half-life (days)	Thigh biological half-life (days)
A	19	37.5 ± 7.5	24.6 ± 7	54 ± 5	57 ± 11
B	20	15 ± 2	13 ± 3	37 ± 14	39 ± 19
C	18	28.3 ± 8	26 ± 7	62 ± 6	48 ± 16
Significant differences		† ‡	‡ §	N.S.*	N.S.

*Not significant
†A versus B: $P < 0.001$
‡B versus C: $0.001 < P < 0.01$
§B versus C: $P < 0.01$

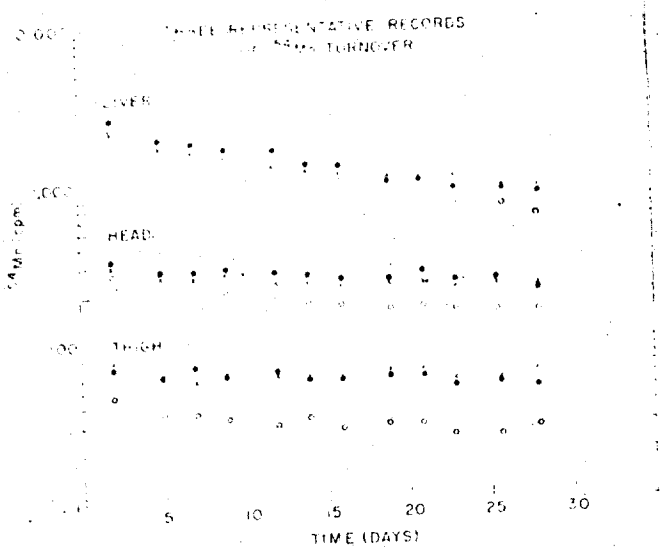

Fig. 3. Disappearance of the injected ^{54}Mn from liver, head, and thigh in 3 normal subjects (see Table 2)

ported in the literature, indicating probable contamination with manganese. Statistically significant differences between these 2 groups were reflected in the manganese concentration of 3 tissues, most pronounced in whole blood (Table 3).

The patient studied while at work in the mines (J.V.) was not included in Table 3. His data, in Figure 4, illustrate a decline in the manganese concentrations of his whole blood, serum, and urine. A similar decrease in manganese concentration was also found in his hair. Upon hospitalization 53 μg. of manganese per gram of hair were found as opposed to 21.6 μg. per gram three months later. In contrast, no decrease in manganese concentration was evident during the hospitalization of one patient with chronic manganese poisoning, who had retired from the mines ten years before this study was initiated. In the latter subject, the initial whole blood and serum values were 13.1 and 1.5 μg. per liter, respectively, whereas eleven months later they were 13 and 2.57, respectively.

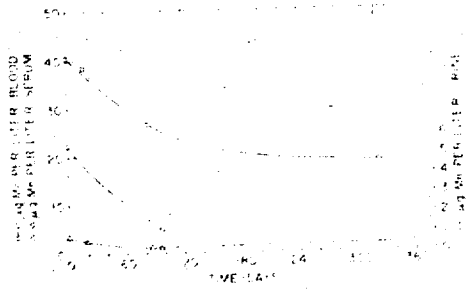

Fig. 4. Decrease in the ^{54}Mn concentration in the blood, serum, and urine of subject J.V. during eleven months following exposure

DISCUSSION

The artificial tracer, ^{54}Mn, disappeared initially from the bloodstream with a rapidity

TABLE 3

MEASUREMENTS OF ^{54}Mn CONCENTRATIONS IN THE TISSUES INDICATED

Tissue	*Group*	*Samples analyzed*	*Mean*	*±S.D.*	*P*
Whole blood	A	23	25.6	1.64	0.0000
	B	15	11.7	1.11	
Serum	A	23	2.17	0.51	0.2 >p>0.17
	B	15	1.29	0.11	
Spinal fluid	A	12	2.72	0.62	0.1 >p>0.08
	B	6	1.04	0.19	
Urine	A	15	3.49	0.92	0.4 >p>0.33
	B	7	2.08	0.61	
Hair	A	17	70.8	22.75	0.04>p>0.03
	B	13	11.7	3.56	
Skin	A	17	3.21	0.92	0.05>p>0.04
	B	14	1.01	0.27	
Muscle	A	6	1.09	0.01	0.40>p>0.33
	B	11	1.78	0.49	

suggestive of clearance by a single passage. The components which followed this early rapid phase have been discussed earlier.[6] The only difference between the sum of the earlier and the sum of the present data is that the relative amount of isotope remaining ten minutes after its injection was now much smaller. It is possible to ascribe this difference to the fact that, in the present study, carrier-free $^{54}Mn^{++}$ was used, whereas the $^{56}Mn^{++}$ used earlier contained stable manganese ($^{55}Mn^{++}$).[6] A "carrier effect" also might explain the differences in half-life shown by the healthy miners in relation to the controls (Table 1). These men had a higher concentration of ^{55}Mn in their blood than did the other two groups.

The radioisotope remaining in the circulation after the initial, rapid phases of its clearance behaved differently in whole blood as compared with plasma. In blood, there was a rise in the tracer's concentration, probably reflecting the incorporation of ^{54}Mn into the heme of the erythrocytes.[8,10] In plasma, the concentration of the tracer did not change over the thirty days of observation from the levels shown in Figure 2. The explanation for this stable plasma concentration is not obvious. Hence, calculated values of "exchangeable" manganese were meaningless and have not been included here.

Among the three groups studied, the healthy miners showed the slowest clearances of the injected radioisotope from the bloodstream. All regions monitored in the healthy miners showed a higher mean rate of manganese turnover than in the patients with chronic manganese poisoning, although these differences were statistically significant only in the cases of the whole body and the liver turnover. These findings are compatible with manganese overload in the healthy, working miners in contrast to the patients with chronic manganese poisoning. This assumption was tested by the analyses for ^{55}Mn.

The blood and serum manganese values of the patients with chronic manganese poisoning were quite similar to normal values determined on North Americans.[8] The corresponding elevated levels among the healthy miners were comparable with those found in a patient after chronic feeding of manganous sulfate.[8]

The most striking difference between the three groups was encountered in the ^{55}Mn analyses of whole blood, which, even if taken by themselves, prove that chronic exposure to manganese may induce an increase in tissue concentration of this metal without necessarily inducing evidence of neurological damage. Furthermore, following termination of the exposure, the excess manganese was spontaneously cleared, whereas the neurological symptom, if present, remained unchanged. Both points are further illustrated by patient J.V. After chronic poisoning was diagnosed he was removed from the mines. Discontinuation of exposure to manganese ores failed to restore his neurological status, although it eventually restored his tissue manganese concentrations. By contrast, one individual with chronic poisoning long removed from the mines showed steady blood and serum concentrations during eleven months of observation, in keeping with evidence obtained earlier on North Americans.[8]

The above findings link high tissue concentrations to exposure but certainly not to neurological disease. Therefore, one must consider the urinary excretion of this metal. Elevated urinary concentrations of ^{55}Mn had been accepted as constituting proof of chronic manganese poisoning.[11] The present data show that, although changes in urine concentration might reflect changes in tissue concentration, they do not reflect changes in the neurological status of the subject.

The present findings suggest that the use of metal binding agents will be ineffective in treating the neurological picture and that alternate solutions for treating the neurological manifestations are required.[12] The possibility of effective chelation therapy during the early, psychiatric phase remains to be studied.[3]

SUMMARY

The rate of disappearance of ^{54}Mn from the blood and serum, from the whole body, from an area representing the liver, and from the head and from the midthigh was measured in normal Chileans, working manganese miners, and ex-miners with chronic manganese poisoning. The bulk of the tracer was cleared from the bloodstream with a mean half time ranging between 1.3 and 2.2 minutes, being slower in

the healthy miners. By contrast, these healthy miners showed, on the average, faster losses of the isotope from the areas studied than did the other two groups, suggesting that they had elevated tissue concentrations of ^{55}Mn. Analyses for manganese performed on whole blood, serum, cerebrospinal fluid, urine, hair, skin, and muscle samples confirmed that healthy manganese miners carried higher tissue concentrations of metal than did the group of ex-miners with chronic manganese poisoning. These findings indicate that the presence of elevated tissue concentrations is not necessary for the continuance of the neurological manifestations of chronic manganese poisoning. Hence, it must be assumed that metal chelation therapy would be ineffective in reversing the neurological manifestations. The possible effectiveness of chelation therapy during the early, psychiatric phases of chronic manganese poisoning remains to be investigated.

The authors thank the Corral Quemado Company for keeping the miners on its payroll during hospitalization; Miss Judith Edwards for expert assistance with the analyses; Dr. Irwin Lourie, Panamerican Health Organization, for coordinating this international project; and Dr. Stanton Cohn of Brookhaven National Laboratory for his generous help with the manuscript.

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Cold Spring Harbor Symp. Quant. Biol., 16: 215-228 1951

MUTAGENIC ACTION OF MANGANOUS CHLORIDE[1]

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For several years we have been conducting a survey of the mutagenic capacities of chemicals by testing various organic and inorganic compounds. This survey has shown that mutagenicity is not a specific property of any one group of chemicals, but appears among widely different groups (Demerec, Bertani, and Flint, 1951). In the course of the study we discovered that certain manganous and ferrous compounds are very potent mutagens, inducing mutations under conditions that produce very little or no killing (Demerec *et al.*, 1950). Extensive studies made with $MnCl_2$ then revealed an unusual situation. We found that the genetic effectiveness of this compound could be made to vary by as much as a factor of 2.5×10^4 by changing the physiological state of the organisms treated. Details of this work will be reported here.

Material and Methods

In all the experiments to be discussed, the B/r strain of *Escherichia coli* was used; and the major part of the study dealt with the induction of mutations from streptomycin dependence (B/r/Sd-4) to nondependence. The properties of this strain have been analyzed in detail by Bertani (1951).

Sd-4 bacteria, when plated on broth-agar medium containing no streptomycin, divide normally several times (residual growth), and then the division process becomes abnormal; that is, the nuclei continue to multiply but the cells fail to separate, giving rise to filaments. The extent of residual growth depends on the concentration of streptomycin in the culture from which the plated sample is taken, and somewhat on the size of the sample When 5×10^7 or fewer bacteria, which have been raised in broth containing 10 micrograms of streptomycin per milliliter, are plated on medium containing no streptomycin, they will pass through three or four divisions—that is, will multiply by a factor of about 12. If any nondependent (background) mutants were present in the plated sample, or if any occur during residual growth, these will continue to divide and will form colonies. Experimental evidence indicates that most of the spontaneous mutants found on such plates have originated during residual growth and that only a small fraction are background mutants. The rate of spontaneous mutation in Sd-4 is low, about 1.4×10^{-8} per bacterial generation. By observing the frequency of mutants in this strain, it is possible to obtain dependable quantitative measurements of rates of induced mutation. The main advantages of this Sd-4 method can be summarized as follows: (1) residual growth is uniform if culture conditions are uniform; (2) relatively few factors influence the residual growth, and these can be controlled easily; (3) background mutants are few; (4) the induced mutations are expressed within the first few divisions, and the residual growth is therefore sufficient for expression of a considerable part of them.

In order to avoid repetition, we shall outline here in detail the procedure followed in the Sd-4 experiments. When certain steps were modified or omitted, this will be mentioned in presenting the results of specific experiments. The general pattern of experimental procedure was as follows:

1) Sd-4 bacteria in the resting stage were cultured in aerated broth containing 10 micrograms of streptomycin per milliliter in a water bath at 37° Centigrade. A fully grown culture—about 24 hours old—was used.

2) The number of bacteria in the culture was determined by diluting and plating on broth-agar medium containing 50 micrograms of streptomycin per ml.

3) Samples of 0.1 ml of a 1/10 dilution of the culture were plated on broth-agar medium containing no streptomycin, and the plates were incubated at 37° C. Usually six to ten plates were used. These served as controls when it was desirable to have data on spontaneously occurring mutants.

4) Samples of five ml, or sufficient for different parts of the experiment of the day, were centrifuged. Centrifuging was done in a room whose temperature was kept constant at 16° C. It took 30 minutes.

5) The supernatant was discarded.

6) If the bacteria were not to be washed, an aqueous solution of $MnCl_2$ (.04% = 0.002 M) was added directly to the pellet and the suspension was kept at 37° C for one hour.

[1]This study was conducted in part under a grant received from the American Cancer Society through the Committee on Growth of the National Research Council.

7) If the bacteria were to be washed, the pellet was resuspended in the solution to be used in washing, the suspension was centrifuged again, and the supernatant was discarded. An aqueous solution of $MnCl_2$ (.01%) was then added to the pellet and the suspension was kept at 37° C for one hour.

8) In a few experiments, instead of being resuspended directly in .01 per cent $MnCl_2$, the pellet was resuspended in distilled water. After a certain length of time .03 per cent $MnCl_2$ was added to make the final concentration .01 per cent. This suspension was kept at 37° C for one hour after the addition of the $MnCl_2$.

9) In all cases, the suspension was then assayed, as in (2), to determine the number of bacteria surviving the $MnCl_2$ treatment. From this assay and the earlier assay, the percentage of survivors was computed.

10) Samples of 0.1 ml of the suspension, diluted with broth 1/10, 1/50, or 1/100, depending on the experiment, were plated on broth agar containing no streptomycin, and the plates were incubated at 37° C. Usually six to ten plates were used for each part of an experiment.

11) After six days of incubation, the colonies on the step-(3) and step-(9) plates were counted, and the number per 10^8 plated survivors was computed. Colonies on the step-(3) plates represented spontaneous mutants, which either had been present in the plated sample (background mutants) or had occurred during the experiment. Colonies on step-(9) plates represented spontaneous mutants *plus* the mutants, if any, induced by the treatment.

In addition to the Sd-4 experiments, a few comparable studies were done with a strain sensitive to T1 phage, in which T1-resistant mutations were induced. Several tests were also made with back-mutations of five nutritionally deficient strains (involving leucine, methionine, methionine-threonine, tryptophane, and cystine deficiencies) and of a lactose-nonfermenting strain.

Results

First Experimental Results

At the beginning of this study, experiments with $MnCl_2$ were only part of a broader survey of the mutagenic potencies of a number of chemicals, and routine procedure was used in their execution. Bacteria were washed in either distilled water or saline and resuspended in water, and $MnCl_2$ was added to the suspension. These experiments showed that when bacteria were kept for one or two hours in 0.04 per cent solution of $MnCl_2$ very few, if any, were killed but many mutants were induced. A very unusual feature was the great variation in results observed in different experiments. Treatments that were identical with regard to concentration of the chemical and length of exposure induced frequencies of mutants that varied by as much as a factor of 10^4. An extensive analysis of conditions that differed from experiment to experiment revealed that the effectiveness of $MnCl_2$ in inducing mutations was greatly affected by the materials used in washing the bacteria before treatment. One of the first findings was that the mutagenic effect was considerably less if the bacteria were washed in water than if they were washed in saline or not washed at all.

Washing with Water, NaCl, KCl, $CaCl_2$, or Sugars

A series of experiments was made to find out more about the extent of this effect. In addition to water, various concentrations of NaCl, KCl, $CaCl_2$, dextrose, and sucrose were used in washing.

The experimental procedure was as outlined. After being washed in the indicated solution the bacteria were resuspended in 0.04 per cent $MnCl_2$ (step 7). The results are presented in Tables 1 and 2.

It should be noted that Table 1 gives the results of one experiment out of several that were performed. This is in general true of all tables included in this paper. There was found to be considerable variation in results obtained with similar treatments in different experiments. For example, the observed range of frequencies of induced mutants when bacteria were washed in water before treatment with $MnCl_2$ was 290 per 10^8 to 3581 per 10^8. With washing in 0.15 M NaCl, the range was 2708 per 10^8 to 8370 per 10^8; and with washing in 0.3 M NaCl it was 10,780 per 10^8 to 34,900 per 10^8. Such evidence as we have indicates that this variability is caused by differences among bacteria raised in different cultures, which may be due to slight variations in medium, aeration, or some other condition. In any one experiment using bacteria from the same culture, if the frequency of mutants was high after washing in water it was also high after washing in the various chemical solutions, and if the frequency was low in one part it was low throughout. In other words, the ratios between frequencies of induced mutants in different parts of an experiment were similar for different experiments. Because of the large variation between experiments the data could not be pooled and presented as averages; and, since to give all data would require too much space, only those from one typical experiment are included. Every ex-

TABLE 1. COMPARATIVE EFFECTS OF WASHING IN DIFFERENT CONCENTRATIONS OF NaCl, KCl, AND $CaCl_2$ ON NUMBER OF MUTANTS PER 10^8 BACTERIA SURVIVING SUBSEQUENT TREATMENT WITH 0.04% $MnCl_2$ FOR 60 MINUTES AT 37° C. RESULTS OBTAINED IN ONE EXPERIMENT.

Concentration (Molar)	NaCl			KCl			$CaCl_2$		
	Survivors		Mutants per 10^8	Survivors		Mutants per 10^8	Survivors		Mutants per 10^8
	Total	%		Total	%		Total	%	
Water (control)	1.99×10^7	79	497	1.89×10^7	75	556	1.87×10^7	74	321
0.0047	1.87×10^7	74	1,190	1.74×10^7	69	1,000	1.51×10^7	75	736
0.0094	1.48×10^7	58	2,670	1.67×10^7	66	1,790	1.73×10^7	68	1,127
0.0188	1.38×10^7	55	3,860	1.12×10^7	56	3,170	1.32×10^7	52	1,953
0.0375	1.43×10^7	56	4,040	1.79×10^7	71	2,730	1.53×10^7	60	1,902
0.075	1.26×10^7	62	3,560	1.98×10^7	78	3,240	1.45×10^7	58	2,460
0.15	1.11×10^7	55	5,240	1.01×10^7	50	6,760	1.42×10^7	56	3,790
0.3	4.82×10^6	19	22,012	4.00×10^6	15	18,500	1.4×10^7	56	3,120

TABLE 2. EFFECT OF WASHING IN DIFFERENT CONCENTRATIONS OF SUCROSE AND DEXTROSE ON THE NUMBER OF MUTANTS PER 10^8 BACTERIA SURVIVING SUBSEQUENT TREATMENT WITH 0.04% $MnCl_2$. THE RESULTS FOR DEXTROSE AND THOSE FOR SUCROSE WERE OBTAINED IN SEPARATE EXPERIMENTS.

Concentration (Molar)	Sucrose			Dextrose		
	Survivors		Mutants per 10^8	Survivors		Mutants per 10^8
	Total	%		Total	%	
Water (control)	1.19×10^7	99	210			
.04	1.37×10^7	100+	234			
.08	1.06×10^7	88	839			
.12	1.47×10^7	100+	925			
.16	1.23×10^7	100+	974			
.24	1.02×10^7	85	1,078			
.25				2.22×10^7	93	2,014
.32	1.06×10^7	89	3,625			
.5				1.64×10^7	78	3,220
1.0				1.34×10^7	56	5,455
1.5				1.15×10^7	48	9,574

periment was repeated several times, however, to make certain that the observed ratios were repeatable.

The data of Tables 1 and 2 show that washing the bacteria in solutions of different compounds before treatment altered the mutagenic action of $MnCl_2$. They also show that the mutagenic effect increased with the concentration of the compound used in washing; that washing in NaCl and in KCl gave similar results, higher than those of washing in $CaCl_2$; and that washing with nonelectrolytes produced the same general effect as washing with electrolytes.

A few experiments made with lactose showed its effect to be similar to those of sucrose and dextrose.

It should be noted that the frequencies of survivors were computed by comparing the results of assays of the numbers of living bacteria present in the cultures from which samples were taken and of similar assays after treatment of the samples. This is not an accurate indication of the amount of killing, as some of the bacteria of the treated series may have been lost during centrifugation. The data of Tables 1 and 2 indicate, therefore, that all, or at least a very high percentage, of the bacteria washed in water survived treatment with $MnCl_2$, but that survival decreased with increasing concentrations of the salts or sugars used for washing. Another set of experiments, made to determine the causes of killing, confirmed the conclusion that the low percentages of survivors when bacteria were washed in certain solutions before treatment were due to the effects of the substances and concentrations used in washing rather than to the treatment with $MnCl_2$.

Conditions during Washing

Experiments were next made to determine whether or not length of time of washing or temperature during washing in NaCl had any effect on the results. Except for these variables, the same procedure was followed as in the experiments reported in Tables 1 and 2. The data are given in Tables 3 and 4. In one experiment (Table 3) similar frequencies of mutants were observed whether bacteria were washed for 10 minutes or for two hours; and in another (Table 4) the frequency of mutants did not vary significantly whether the temperature during washing was 0° C or 25° C.

TABLE 3. EFFECT OF LENGTH OF WASHING IN 0.3 M SOLUTION OF NaCl ON THE NUMBER OF MUTANTS PER 10^8 BACTERIA SURVIVING SUBSEQUENT TREATMENT WITH 0.04% $MnCl_2$

Time washed	Survivors		Mutants per 10^8
	Total	%	
Not washed	6.72×10^6	42.4	7,020
10 minutes	1.08×10^6	10.1	38,600
20 "	7.2×10^5	5.4	44,700
30 "	5.65×10^5	4.2	54,000
45 "	3.07×10^5	2.3	64,000
1 hour	1.19×10^6	7.4	30,800
2 "	3.6×10^5	2.2	53,900

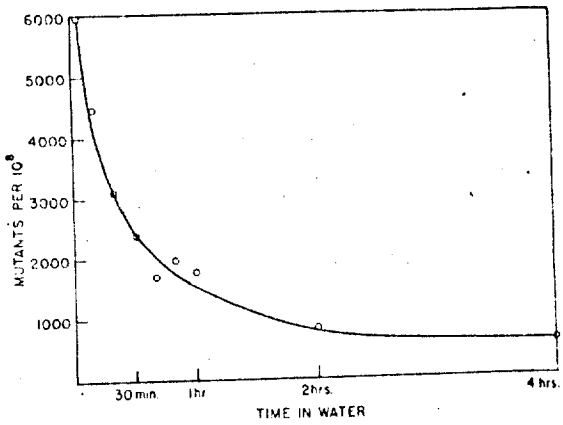

FIG. 1. Mutagenic effect of $MnCl_2$ on bacteria washed in 0.15 NaCl and kept in water for various periods before treatment.

tonic solutions of NaCl or KCl does not alter the effectiveness of $MnCl_2$, but that this effectiveness is gradually reduced as the concentrations used in washing become lower and increased as they become higher.

Thus it appears more likely that washing in water "leaches" out a certain proportion of whatever bacterial constituent is important for the action of $MnCl_2$. The experiment plotted in Figure 1 shows how this "leaching" process is related to the length of time the bacteria are kept in water. In this experiment the bacteria were first washed in 0.15 M NaCl, then centrifuged; after the supernatant was discarded they were resuspended in water and kept for the specified periods before the addition of $MnCl_2$ solution. The curve in Figure 1 is typical of a reaction in which rate of change is proportional to concentration of the chemical responsible for it.

TABLE 4. EFFECT OF TEMPERATURE DURING WASHING IN 0.15 M SOLUTION OF NaCl ON NUMBER OF MUTANTS PER 10^8 BACTERIA SURVIVING SUBSEQUENT TREATMENT WITH 0.04% $MnCl_2$

Temperature Centigrade	Survivors		Mutants per 10^8	Control	
	Total	%		Total	Mutants per 10^8
0°	5.32×10^7	82	6,418	2.59×10^8	10.1
25°	5.84×10^7	78	6,089	3.15×10^8	10.8

Resuspension in Water after Washing in Salts

It has been shown that washing bacteria in water before $MnCl_2$ treatment resulted in fewer mutants than washing in various concentrations of NaCl. This might indicate that washing in water removes some constituent from the bacterial cells that is important for the action of $MnCl_2$, and that this process is retarded by the presence of salt, or, on the other hand, that salt adds a component that stimulates the action of $MnCl_2$.

Experiments in which bacteria were not washed at all, but were treated immediately after centrifugation and discarding of the supernatant, showed that under these conditions the effect of the $MnCl_2$ was in the same range as that observed when the bacteria were washed in 0.15 M concentrations of NaCl or KCl. This suggests that washing in iso-

NaCl or Dextrose in the $MnCl_2$ Solution

In the course of our work we had observed that the mutagenic effectiveness of $MnCl_2$ was inhibited if it was dissolved in saline instead of in water. In following up this observation, several experi-

ments were performed like the two reported in Table 5. Samples of bacteria taken from the same culture—some washed in water, others not washed or washed in 0.3 M NaCl—were treated with 0.04% $MnCl_2$ dissolved in various concentrations of NaCl in one experiment and of dextrose in the other. In each experiment, a sample was treated with $MnCl_2$ dissolved in water for the purpose of comparison. The results show that the mutagenic action of $MnCl_2$ was greatly inhibited when the solution contained NaCl. This occurred regardless of how the bacteria were handled before treatment, although the degree of inhibition in the three parts of the experiment was consistently highest when the bacteria were washed in 0.3 M NaCl and lowest when they were washed in water. Since five to twenty spontaneous mutants were to be expected, it appears that suppression of mutagenic action was complete when the $MnCl_2$ was dissolved in 0.3 M NaCl, and nearly complete when it was dissolved in 0.15 M NaCl.

TABLE 5. FREQUENCIES OF MUTATIONS INDUCED IN BACTERIA THAT WERE EITHER WASHED IN DISTILLED WATER, NOT WASHED AT ALL, OR WASHED IN 0.3 M NaCl, AND THEN TREATED WITH 0.04% $MnCl_2$ DISSOLVED IN EITHER NaCl OR DEXTROSE

$MnCl_2$ dissolved in	Water			Not washed			0.3 M NaCl		
	Survivors		Mutants per 10^8	Survivors		Mutants per 10^8	Survivors		Mutants per 10^8
	Total	%		Total	%		Total	%	
Water	3.24×10^7	68	943	3.42×10^7	71	3,184	1.07×10^7	55	9,664
0.05 NaCl (M)	9.44×10^7	74	167	1.42×10^8	100+	44	7.5×10^7	78	52
0.15 NaCl	1.1×10^8	86	22	1.33×10^8	100+	13	8.46×10^7	88	20
0.3 NaCl	1.01×10^8	79	16	1.14×10^8	89	12	9.3×10^7	97	15
Water	1.05×10^7	94	552	1.97×10^7	50	3,360	6.52×10^6	53	15,200
0.05 dextrose (M)	3.32×10^6	30	21,400	2.05×10^7	52	2,250	7.08×10^6	58	10,800
0.1 dextrose	2.72×10^6	24	19,680	1.34×10^7	51	1,449	4.44×10^6	43	10,900
0.25 dextrose	8.16×10^6	74	4,510	2.51×10^7	64	934	7.44×10^6	61	5,520
0.5 dextrose	7.64×10^6	69	1,640	2.27×10^7	58	414	8.64×10^6	71	2,280
1.0 dextrose	8.96×10^6	81	268	2.68×10^7	68	37	9.36×10^6	76	1,760

A quite different pattern of results was obtained when the treatment solutions of $MnCl_2$ contained dextrose. With bacteria previously washed in water, there were striking increases in frequency of mutants in the four treatments in which the concentrations of dextrose were between 0.05 and 0.5 M. A decrease in frequency was observed only in the treatment in which the concentration of dextrose was one M; this concentration was equivalent to about 20 per cent. With unwashed bacteria or bacteria washed in 0.3 M NaCl before the treatment with $MnCl_2$ + dextrose, the frequencies of mutants were considerably higher than those obtained by treatment with $MnCl_2$ + NaCl.

These results show that the mutagenic action of $MnCl_2$ was affected differently by the presence of NaCl in the solution than by the presence of dextrose. Inhibition of the mutagenic action by NaCl may be due to competition between Mn and Na ions for certain sites, and the stimulating effect of dextrose in the solution when the bacteria are washed in water may indicate that dextrose stimulates some metabolic process which favors uptake of Mn ions.

Other Physiological Conditions

In attempting to account for the wide variation between results of different experiments in which identical concentrations of $MnCl_2$ were used in treatment, it was also logical to inquire whether the degree of the mutagenic effect was influenced by other conditions affecting the physiology of the bacteria. Experiments were therefore made to compare induction of mutations by $MnCl_2$ in resting bacteria—which we regularly used—and in growing bacteria, and also to compare results in resting bacteria grown in aerated broth with those in resting bacteria grown in unaerated broth.

Data from representative experiments are given in Tables 6 and 7. They show that such conditions had considerable influence on the mutagenic effectiveness of $MnCl_2$. The treatment had much less effect on growing bacteria than on bacteria from the same culture after it had reached saturation point (Table 6). It also produced fewer mutants in bacteria raised in nonaerated cultures than in bacteria raised under aeration (Table 7).

Culture Medium

Further experiments were made to determine the possible effect of the culture medium in which

TABLE 6. MUTANTS PER 10^8 SURVIVORS INDUCED BY 0.04% $MnCl_2$ IN GROWING AND IN RESTING BACTERIA FROM THE SAME CULTURE. BACTERIA WERE WASHED IN 0.3 M NaCl BEFORE TREATMENT.

Growing (titer 1×10^7)			Resting (titer 2×10^8)		
Survivors		Mutants per 10^8	Survivors		Mutants per 10^8
Total	%		Total	%	
7×10^6	48	718	2.2×10^8	42	6,404

in *E. coli*, suggested that these two elements are antagonistic to each other. Since the M9 medium contains an appreciable amount of $MgSO_4$, it appeared probable that this might be responsible for the reduced mutagenic effect of $MnCl_2$.

Several experiments were performed to test the possibility, and all gave positive results. In one experiment, bacteria were grown in broth to which 0.04 per cent of $MgSO_4 . 7H_2O$ had been added, and then were treated with $MnCl_2$. As can be seen from the data in Table 9, considerably fewer

TABLE 7. EFFECT OF CONDITIONS UNDER WHICH BACTERIA WERE RAISED (AERATED *VS.* NONAERATED BROTH) ON NUMBER OF MUTANTS PER 10^8 BACTERIA SURVIVING TREATMENT WITH 0.04% $MnCl_2$

Bacteria washed in	Aerated			Nonaerated		
	Survival		Mutants per 10^8	Survival		Mutants per 10^8
	Total	%		Total	%	
Water	1.82×10^7	76	483	3.18×10^7	100+	63
Not washed	1.89×10^5	94	3,343	2.64×10^7	100+	463
0.3 M NaCl	1.21×10^7	50	12,060	2.7×10^7	100+	1,745

the bacteria were grown. For comparison with broth, we used M9 synthetic medium, which is composed of potassium and sodium phosphates, magnesium sulfate, sodium and ammonium chlorides, dextrose, and water. The results are very striking (Table 8). They show definitely that treatment with $MnCl_2$ induced many fewer mutants in the bacteria grown in M9 medium than in those grown in broth.

Antagonistic Effect of Magnesium

This difference could have been caused by physiological differences between the two sets of bacteria; or on the other hand it might indicate that some chemical present in the synthetic culture medium and absorbed by the bacteria inhibited the mutagenic action of $MnCl_2$. Studies by Abelson and Aldous (1950), on interference by manganese with normal magnesium metabolism

mutants were obtained than would have been expected if the bacteria had been raised in broth without additional Mg. In another experiment (Table 10) bacteria were washed in different concentrations of $MgSO_4$ before being treated with $MnCl_2$; and, again, fewer mutants than the expected number were observed. In a third (Table 11), the $MnCl_2$ used in treatment was dissolved in various concentrations of $MgSO_4$.

These results suggest that Mg does act as an inhibitor of the mutagenic action of $MnCl_2$.

Certain Other Salts

Tests were made with several other salts containing metallic ions, to determine their effect on the mutagenic action of manganese. Table 12 gives the results of three experiments completed so far. In the first two the bacteria were washed before $MnCl_2$ treatment with 0.15 M solutions of

TABLE 8. MUTANTS PER 10^8 SURVIVORS INDUCED BY 0.04% $MnCl_2$ IN BACTERIA GROWN IN SYNTHETIC (M9) MEDIUM

Washed in	Treated			Control	
	Survivors		Mutants per 10^8	Total	Mutants per 10^8
	Total	%			
Water	2.5×10^8	91	59	2.8×10^8	6
0.15 M NaCl	2.4×10^8	94	56	2.5×10^8	5.6

TABLE 9. MUTANTS PER 10^8 SURVIVORS INDUCED BY 0.04% $MnCl_2$ IN BACTERIA GROWN IN BROTH TO WHICH 0.04% $MgSO_4.7H_2O$ WAS ADDED

Washed in	Survivors		Mutants per 10^8
	Total	%	
Water	2.84×10^7	75	239
Not washed	4.41×10^7	95	766
0.3 M NaCl	3.2×10^7	63	1,882

TABLE 10. MUTANTS PER 10^8 SURVIVORS INDUCED BY TREATMENT WITH 0.04% $MnCl_2$ PRECEDED BY WASHING IN DIFFERENT CONCENTRATIONS OF $MgSO_4.7H_2O$.

$MgSO_4$ Concentration %	Survivors		Mutants per 10^8
	Total	%	
0.01	1.46×10^8	100+	194
0.02	1.07×10^8	78	228
0.04	1.09×10^8	91	143

salts, and in the third they were washed in 0.015 M solutions because the salts used were too toxic in higher concentrations. Each experiment included NaCl as a standard for comparison.

The first experiment compared the effects of the Na, Ca, Co, Mg, and Zn ions. The results indicate that less than half as many mutants per 10^8 survivors were obtained with Ca as with Na; that Co reduced the mutagenic effect by a factor of one hundred, Mg weakened it even further, and Zn suppressed it entirely—since in this part of the experiment the frequency of mutants was not higher than that expected in untreated bacteria.

TABLE 11. MUTANTS PER 10^8 SURVIVORS INDUCED BY TREATMENT WITH 0.04% $MnCl_2$ DISSOLVED IN DIFFERENT CONCENTRATIONS OF $MgSO_4.7H_2O$.

Concentration %	Survivors		Mutants per 10^8
	Total	%	
0.01	2.08×10^8	100+	151
0.02	1.98×10^8	100+	125
0.04	1.9×10^8	100+	26

The second experiment showed that the effect of K was very similar to that of Na. With regard to Ca, it confirmed the results of a previous experiment in which bacteria were washed in 0.15 molar concentrations of NaCl or $CaCl_2$ before treatment with $MnCl_2$, and about twice as many mutants per 10^8 were induced after the NaCl washing.

The third experiment compared the effects of washing in the presence of Na, Be, U, Cd, and Cr.

The results of these three experiments indicate that, compared with Na and K, the other ions tested reduced the mutagenic effect of $MnCl_2$ in increasing degrees, approximately in the following order: Ca, Be, U, Cd, Cr, Co, Mg, and Zn.

Another set of experiments (Table 13) was carried out to test the role of the anion part of the molecule of compounds used in washing. Eighteen salts of Na were tested, in three different experiments (indicated in the last column of the table), each of which included NaCl as a standard for comparison of results. The ratio between the frequency of mutants observed with each compound and the frequency observed in the same experiment with NaCl is given in column 8

TABLE 12. EFFECTS OF WASHING BACTERIA IN VARIOUS SALTS BEFORE TREATING WITH $MnCl_2$. RESULTS OF THREE EXPERIMENTS.

Salt	Formula	Molar	Survivors		Mutants per 10^8
			Total	%	
Sodium chloride	NaCl	0.15	1.17×10^7	58	7,103
Calcium chloride	$CaCl_2.2H_2O$	0.15	1.63×10^7	81	2,890
Cobalt (ous) chloride	$CoCl_2.6H_2O$	0.15	7.3×10^7	73	63
Magnesium chloride	$MgCl_2.6H_2O$	0.15	9.1×10^7	91	18
Zinc chloride	$ZnCl_2$	0.15	4.73×10^7	47	6
Sodium chloride	NaCl	0.15	1.11×10^7	55	5,240
Potassium chloride	KCl	0.15	1.01×10^7	50	6,760
Calcium chloride	$CaCl_2.2H_2O$	0.15	1.42×10^7	56	3,790
Sodium chloride	NaCl	.015	1.8×10^7	50	1,500
Beryllium fluoride	$BeFl_2$	.015	2.32×10^8	43	190
Uranium chloride	$UO_2Cl_2.H_2O$	.015	1.05×10^7	2	113
Cadmium chloride	$CdCl_2.\frac{1}{2}H_2O$	.015	1.16×10^8	21	49
Chromium (ic) chloride	$CrCl_3.6H_2O$	.015	1.59×10^7	9	35

TABLE 13. EFFECTS OF WASHING BACTERIA IN 0.15 MOLAR SOLUTIONS OF VARIOUS SODIUM SALTS BEFORE TREATING WITH $MnCl_2$, AND THE DEGREE OF EFFECT EXPRESSED AS RATIO TO NaCl EFFECT. RESULTS OF THREE EXPERIMENTS (SPECIFIED IN LAST COLUMN).

	Chemical	Formula	pH	Survivors		Mutants per 10^8	Degree	Experiment
				Total	%			
1	Sodium salicylate	$NaC_7H_5O_3$	5.3	6.36×10^6	88	141	.07	2
2	" benzoate	$NaC_7H_5O_2$	5.9	7.12×10^6	99	211	.1	2
3	" fluoride	NaF	5.9	4.05×10^7	84	516	.15	3
4	" iodide	NaI	5	5.19×10^6	12	1,390	.39	1
5	" iodate	$NaIO_3$	5	6.48×10^6	15	2,665	.75	1
6	" nitrate	$NaNO_3$	4.8	9.76×10^6	75	3,020	.87	3
7	" chlorate	$NaClO_3$	5	4.16×10^6	53	1,875	.92	2
8	" chloride	NaCl	5	4.76×10^7	54	3,530	1.	1
	" "	"	5	4.56×10^6	63	2,030	1.	2
	" "	"	5	9.12×10^6	70	3,470	1.	3
9	" formate	$NaCHO_2$	5.9	4.28×10^6	60	2,030	1.	2
10	" acetate	$CH_3COONa.3H_2O$	6.6	4.8×10^6	67	2,040	1.	2
11	" thiosulfate	$Na_2S_2O_3.5H_2O$	5	4.2×10^6	58	2,960	1.46	2
12	" bicarbonate	$NaHCO_3$	8.2	2.37×10^6	5	6,340	1.8	1
13	" bromide	NaBr	5	3.45×10^6	64	4,270	2.1	2
14	" sulfate	Na_2SO_4	5	9.76×10^6	75	7,930	2.3	3
15	" tartrate	$Na_2C_4H_4O_6.2H_2O$	6.5	3.56×10^6	49	5,630	2.77	2
16	" potassium tartrate	$KNaC_4H_4O_6.4H_2O$	6.2	2.24×10^6	31	6,480	3.19	2
17	" sulfite	Na_2SO_3	8.5	5.56×10^6	13	9,060	3.2	1
18	" oxalate	$Na_2C_2O_4$	5.5	4.44×10^6	34	15,950	4.59	3

under the heading "Degree." The chemicals are listed in the order of "degree" values. Values lower than 1 represent lower production of mutations, and values higher than 1 higher production, as compared with the effect of NaCl. All tests were repeated once, and the two sets of results are in close agreement, particularly as regards the order of the chemicals on the list. Table 13 gives the data from one set of tests.

Allowing for the considerable degree of experimental error, it can be assumed that the chemicals listed from number 5 to number 11, inclusive, have effects similar to that of NaCl, whereas those above 5 on the list inhibit the mutagenic action of $MnCl_2$ and those below 11 stimulate it. Evidently, the different effects of these salts cannot be ascribed to differences between organic and inorganic chemicals, since 2 of the 7 organic salts tested are at the top of the list, 2 are in the middle, and 3 near the bottom. It is also evident that differences in pH cannot account for the observed effects. There does appear, however, to be a good correlation between the degree of effect and the number of Na or K atoms contained in the molecule. In salts with two Na atoms, or one Na and one K atom, the degrees of effect approach those produced by either 0.3 M NaCl or 0.3 M KCl. From this it appears that the stimulating effects of the solutions listed from 12 to 20 in Table 13 are due to the presence of either two atoms of Na or one atom of Na and one of K per molecule—that is, they are correlated with concentration of Na or of Na and K ions in the solution.

Buffers

Some of the results reported in Table 13 indicate that, within the range that is not too injurious to bacteria, the pH of solutions used in washing is not responsible for the observed effects on mutagenic action of $MnCl_2$. This was confirmed in additional experiments, two of which are reported in Tables 14 and 15.

In the experiment shown in Table 14, the bacteria were washed in 0.15 M or 0.5 M solutions of either acetate or phosphate buffer of various pH. The 0.5 M acetate buffer of pH 3.5 was prepared by adding 0.65 ml of 0.5 M sodium acetate to 9.35 ml of 0.5 M acetic acid; for pH 4.5 the proportions were 4.22 ml to 5.78 ml; and for pH 5.5 they were 8.85 ml to 1.15 ml. The 0.5 M phosphate buffer of pH 5.5 was prepared by adding 0.4 ml of 0.5 M Na_2HPO_4 to 9.6 ml of 0.5 M KH_2PO_4; for pH 6.5 the proportions were 3.2 ml to 6.8 ml; and for pH 7.5 they were 8.5 ml to 1.5 ml. The 0.15 M buffers were made by diluting the 0.5 M buffers in distilled water.

TABLE 14. EFFECTS OF WASHING IN ACETATE OR PHOSPHATE BUFFERS OF DIFFERENT pH ON THE NUMBER OF MUTANTS PER 10^8 BACTERIA SURVIVING TREATMENT WITH 0.04% $MnCl_2$

Buffer	pH	0.15 M			0.5 M		
		Survivors		Mutants per 10^8	Survivors		Mutants per 10^8
		Total	%		Total	%	
Acetate	4.5	6.45×10^7	70	90	1.72×10^7	37	122*
	5.5	5.65×10^7	61	3,142	3.00×10^7	65	394
Phosphate	5.5	1.82×10^7	79	748	3.92×10^6	42	11,100
	6.5	1.95×10^7	85	1,760	3.86×10^6	42	11,300
	7.5	1.27×10^7	55	6,550	3.56×10^6	38	5,340*

*Indicates that clumping of bacteria was observed after washing. This tends to give higher values for mutants.

The results of this experiment show that the pH of the chemical used in washing had an effect but was not primarily responsible for the differences in mutagenic activity, because washing in acetate and phosphate buffers of the same pH produced quite different results. It is interesting to note that at pH 5.5 the 0.15 M concentration of acetate buffer gave a high value whereas the same concentration of phosphate buffer gave a low value, but that with the 0.5 M concentrations the relation between the two values was reversed. It appears probable that we are dealing here with an intricate set of conditions that affect permeability of bacterial cells and their uptake of $MnCl_2$.

Table 15 presents data indicating that the mutagenic action of $MnCl_2$ was entirely suppressed when the agent was dissolved in 0.15 M sodium phosphate buffer of pH 5.6. The frequency of mutants obtained with this treatment was not significantly greater than the spontaneous mutation frequency.

TABLE 15. NUMBER OF MUTANTS PER 10^8 BACTERIA SURVIVING TREATMENT WITH $MnCl_2$ PLUS SODIUM PHOSPHATE BUFFER (pH 5.6), AS COMPARED WITH EFFECT OF BUFFER ALONE. BACTERIA WERE NOT WASHED BEFORE TREATMENT.

Treatment	Survivors		Mutants
	Total	%	
Sodium phosphate 0.15 M	7.15×10^7	100	6
Sodium phosphate 0.15 M + $MnCl_2$ 0.04%	5.3×10^7	75	9
Sodium phosphate 0.03 M + $MnCl_2$ 0.04%	7.2×10^7	100	11

$MnCl_2$ Concentration, Duration of Treatment, and Temperature

In several experiments in which the concentration of $MnCl_2$ was varied, it was observed that the mutagenic effect was proportional to the concentration up to a certain point but was not affected by further increase in concentration. Figure 2 presents data of an experiment in which unwashed bacteria were treated with various concentrations of $MnCl_2$. The break in the curve occurs at a concentration of about 0.005 per cent, which is equivalent to 2.5×10^4 M.

The results of two experiments shown in Figure 3 indicate that the mutagenic effect of $MnCl_2$ was related to the duration of the treatment. The bac-

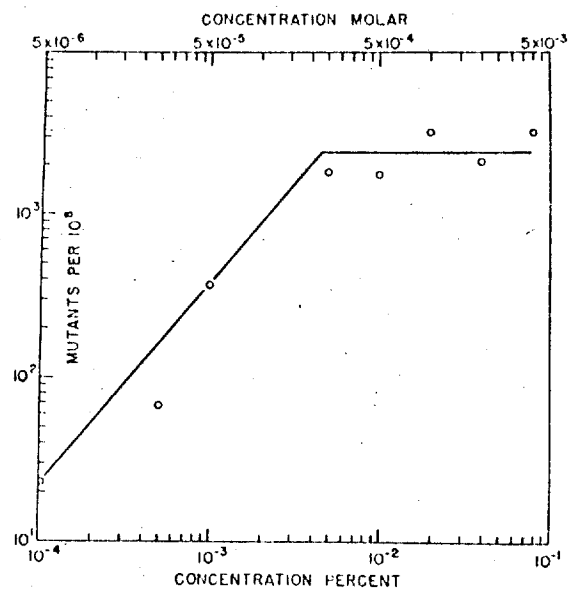

FIG. 2. Relation between frequency of mutations and concentration of $MnCl_2$.

teria were not washed in either experiment. In one of them we used a concentration of 5×10^{-5} M, which does not give the maximal effect, as can be seen from Figure 2. In the other we used a concentration of 2×10^{-3} M, which does give the maximal effect. Both experiments showed that the mutagenic effectiveness of $MnCl_2$ increased with duration of treatment up to 30 minutes, and then was not altered with longer treatment.

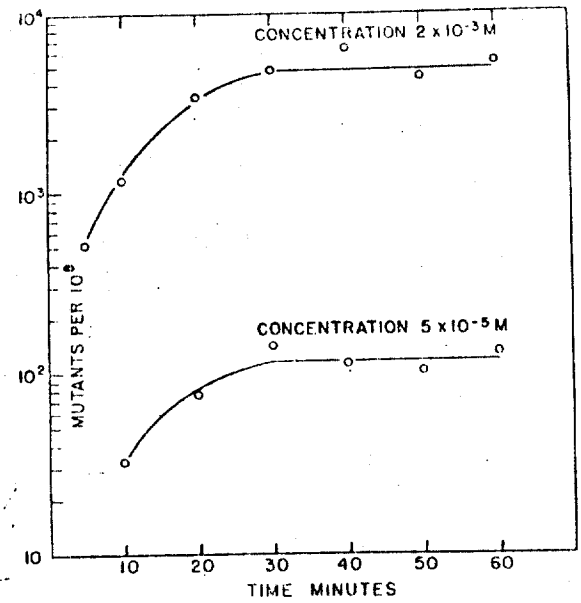

FIG. 3. Relation between frequency of mutations and length of treatment in $MnCl_2$. Two concentrations of $MnCl_2$ were tested.

Figure 4 represents the results of an experiment in which bacteria were washed in 0.3 M NaCl and then treated with 0.04 per cent $MnCl_2$ at various temperatures. A strong correlation between temperature and mutagenic effect is obvious.

Washing after Treatment

The question of whether the full effect of $MnCl_2$ is accomplished during treatment was studied by experiments in which the bacteria were washed in various solutions after treatment. Results of such an experiment are shown in Table 16. They indicate that the treatment-induced reaction that was responsible for the genetic changes continued after the treatment had ended—that is, after the samples of bacteria in the solution of $MnCl_2$ had been diluted 1 to 10 and plated on broth-agar plates. Washing in water decreased the frequency of induced mutants, and this de-

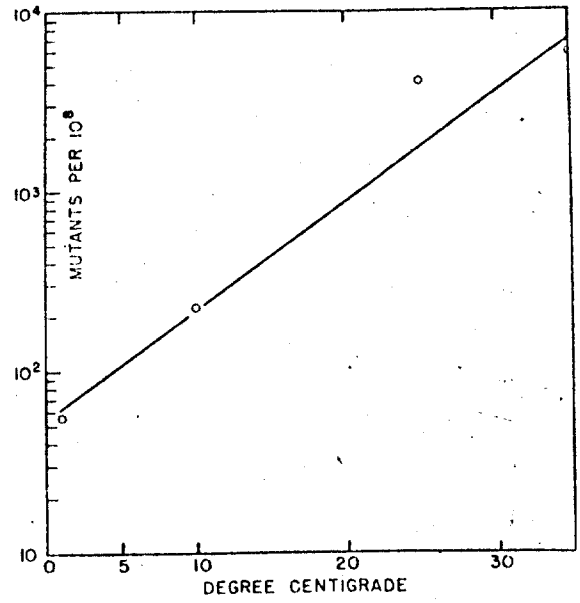

FIG. 4. Relation between frequency of mutations induced by $MnCl_2$ and temperature during the treatment.

crease was slight when washing was continued for one hour but considerably greater when it was continued for four hours. This suggests that internal conditions created in bacteria by treatment with $MnCl_2$ persist for a considerable period of time, and during that time can be modified by washing. Washing in water for four hours is more effective than one hour's washing, whereas one hour's washing in either NaCl or $MgCl_2$ is sufficient to stop further induction of mutants. It appears that $MgCl_2$ is more efficient than NaCl in reducing the mutagenic effect of $MnCl_2$.

TABLE 16. EFFECTS OF WASHING AFTER TREATMENT ON MUTAGENIC ACTION OF $MnCl_2$. BACTERIA WERE NOT WASHED BEFORE TREATMENT. WASHING WAS DONE AT ROOM TEMPERATURE EXCEPT WHERE OTHERWISE INDICATED.

Washing	Time (hours)	Survivors		Mutants per 10^8
		Total	%	
None	..	8.55×10^6	45	5,000
Water	1	2.85×10^7	37	4,256
Water	4	3.48×10^7	45	2,420
NaCl 0.15 M	1	4.25×10^7	56	2,130
NaCl 0.15 M	4	3.68×10^7	48	2,060
$MgCl_2$ 0.15 M	1	3.73×10^7	49	1,730
$MgCl_2$ 0.15 M	4	2.5×10^7	33	1,890

Discussion

The data presented are indicative of an unusual situation, in which the effectiveness of a substance as a mutagen is greatly influenced by previous treatment of the bacteria to which it is applied. It has been shown that the mutagenic effect of $MnCl_2$ was high when bacteria were washed before treatment in high concentrations of certain chemicals, low when they were washed in low concentrations of the same chemicals or in some other chemicals, and entirely suppressed when they were washed in still other chemicals. It has been shown, too, that other conditions relating to the physiology of the bacteria—namely, whether they were treated in the growing stage or the resting stage, or were grown with or without aeration—also had a determining effect on the mutagenic results of treatment with $MnCl_2$.

These strong influences of washing and of physiological condition on the genetic reaction of bacteria to chemical treatment suggest that we may be dealing with differences in permeability of the bacteria and in their uptake of this chemical, which are induced by various methods of washing or brought about by different conditions of growth.

Studies by Roberts and Aldous (1951), carried on with our Sd-4 strain of *coli* and under the same conditions as some of our experiments, indicate that situations which favor the uptake of $MnCl_2$ also favor high mutability, and suggest that the genetically active component of $MnCl_2$ is bound to specific sites within bacterial cells. These workers concluded that manganese is adsorbed on proteins or nucleic acids of the cell in a loosely bound complex. The quantity adsorbed is determined mainly by the concentration of manganese and other competing ions, and by the number of available adsorption sites, but is not greatly influenced by temperature. As a second step, some temperature-dependent chemical change occurs, which results in a mutation.

How the genetic effect is brought about we are not yet in a position to speculate. Since it is known that manganese plays either a direct or an indirect role in several enzymatic reactions, and also that these reactions may be modified by the presence of Ca, Co, Be, U, Mg, and Zn, it may well be that the induction of mutations by $MnCl_2$ is initiated by some reaction involving a certain enzyme or group of enzymes. However, it is just as possible that the mutagenic process is related to some other reaction, affecting the metabolic processes of the cell, which in turn govern the stability of the genetic system. By continuing the genetic and biochemical study of factors influencing the mutagenic action of $MnCl_2$, it may be possible to answer some of the many questions that still remain.

The data available at present suggest that $MnCl_2$ does not induce gene changes directly but acts in some indirect way. This is indicated by three lines of evidence. First, the effect of $MnCl_2$ is not specific. The studies reported so far were made with mutants affecting streptomycin dependence, but we have done tests with seven other types of mutation and found $MnCl_2$ to be mutagenic in these cases as well. They included mutations from sensitivity to resistance to T1 phage; mutations from inability to ability to ferment lactose; and back-mutations from five nutritional deficiencies involving leucine, methionine, methionine-threonine, tryptophane, and cystine. The work with nutritional deficiencies was done in cooperation with Miss Elise Cahn. Results of these studies show that $MnCl_2$ induced changes in all the genetic "loci" tested, indicating that its action is of a general kind, which increases mutability in many or all loci of the genetic system.

A second line of evidence that the mutagenic action of this chemical is indirect is provided by the finding that similar patterns of genetic effect are produced by treatment with $MnCl_2$, ultraviolet rays, and X-rays. Earlier work (Demerec, 1946) has shown, with ultraviolet- or X-ray-induced mutants involving resistance to phage T1, that the total number of mutations is not expressed until the number of bacteria has increased by a factor of 1000–4000, that is, until after the treated bacteria have passed through between ten and twelve divisions. Some mutants appear with each division following irradiation, but the bulk of them shows up late. About four divisions are required for expression of 25 per cent of the induced mutations.

The same general situation applies to radiation-induced mutations of other loci that have been studied—involving changes in streptomycin resistance, lactose fermentation, and nutritional deficiencies for leucine, methionine, and methionine-threonine—although each locus has its characteristic pattern of behavior. In the case of lactose fermentation, all the induced mutations are expressed early, after one or two divisions; whereas mutations to streptomycin resistance require three to four divisions for complete expression and mutations from dependence require about six to seven divisions. In leucine-deficiency experiments, about 50 per cent of mutations are

expressed in 1½ to 2¾ divisions and total expression requires six to eight divisions; in methionine-deficiency, about 50 per cent are expressed in eight to ten divisions and all in 10 to 14; and in methionine-threonine, about 50 per cent are expressed in 4¾ to 5¼ divisions and all in 7 to 11 divisions.

More recent studies carried out in our laboratory have demonstrated a similar delay in appearance of mutants induced by treatment with $MnCl_2$, a convincing indication that the pattern of expression of induced mutations is characteristic of the mutating locus and does not depend on the treatment by which the mutations are induced.

TABLE 17. FREQUENCIES OF MUTANTS INDUCED BY TREATMENTS WITH 0.04% $MnCl_2$ AND ULTRAVIOLET RAYS (800 ergs/mm²). RESULTS OF TWO EXPERIMENTS. PROCEDURE IS EXPLAINED IN TEXT.

Treatment	Survivors %	Mutants per 10^8	Survivors %	Mutants per 10^8
$MnCl_2$	39.8	17,379	52.4	10,100
$MnCl_2$ + U.V.	0.09	14,400	0.45	4,360
U.V. + $MnCl_2$	0.4	17,600	0.13	11,100
U.V.	16.8	7,380	13.3	5,200

The third and most suggestive line of evidence against direct mutagenic action of $MnCl_2$ is the observation that the effects of treatment with both ultraviolet rays and $MnCl_2$ are not additive. The results shown in Table 17 were obtained in a representative experiment in which Sd-4 bacteria were treated with either one or both of these mutagens. When treatment was with $MnCl_2$ only, or with $MnCl_2$ followed by ultraviolet, the bacteria were washed in 0.3 M solution of NaCl, resuspended in 0.04 per cent $MnCl_2$, kept for one hour, and either plated immediately or treated with ultraviolet (800 ergs/mm²) and then plated. For the treatments with ultraviolet alone or followed by $MnCl_2$, the washed pellet was resuspended in 0.3 M solution of NaCl and irradiated with 800 ergs/mm². After irradiation the suspension was divided into two parts, which were centrifuged. One pellet was resuspended in broth and plated on broth-agar plates without streptomycin. The other was resuspended in .04 per cent $MnCl_2$ and kept at 37° C for one hour before plating. Plates were incubated for six days, after which the nondependent mutants were scored. It was evident that the effects of treatment with the two mutagens were not cumulative; in fact, in one experiment the mutation frequency produced by $MnCl_2$ + U.V. treatment was lower than those resulting from either $MnCl_2$ or ultraviolet alone.

At present we are inclined to interpret the genetic effect of $MnCl_2$ as due to changes induced outside the gene system, in either the nucleus or the cytoplasm of the bacterial cell. It is assumed that these changes affect the metabolic activity of cells in such a way as to make the gene systems less stable than they normally are, and so increase the rate of mutation.

We have evidence to show that the relation described in this paper between physiological state of the treated cells and mutagenic effectiveness of the chemical used in treatment is not a general one. We found that the mutagenicity of beta-propio lactone, another potent mutagen, was not affected by washing the bacteria in various concentrations of NaCl, $CaCl_2$, dextrose, or $MgCl_2$ before treatment. Neither did pretreatment of bacteria increase the mutagenic effect of the following chemicals, which are known to be slight mutagens: cadmious chloride, cerous sulfate, cobaltous chloride, manganous lactate, and chromic chloride. So far, pretreatment has been found effective only in the case of the highly mutagenic manganous and ferrous compounds, although it appears that similar pretreatment may produce different results with these two groups of compounds.

SUMMARY

The mutational changes observed in most of the experiments reported here were from streptomycin dependence (strain B/r/Sd-4) to nondependence in *E. coli*. A method utilizing these changes has proved very suitable for quantitative study of induced mutability.

It has been found that manganous chloride ($MnCl_2$) is a very potent mutagen, inducing considerable numbers of mutants under conditions of treatment that produce very little or no killing.

The mutagenic effect of $MnCl_2$ is influenced by washing the bacteria before treatment. Washing in water, or in hypotonic solutions of NaCl, KCl, $CaCl_2$, sucrose, or dextrose decreases the frequency of induced mutants, whereas washing in hypertonic solutions of these compounds increases it. Temperature during washing makes no difference to the effect. Exposure to water after washing in NaCl lessens the stimulating action of the NaCl.

Washing in similar concentrations of various other salts before treatment with $MnCl_2$ results in lower mutation frequencies than washing in NaCl or KCl, in approximately the following de-

scending order: $CaCl_2$, $BeFl_2$, UO_2Cl_2, $CdCl_2$, $CrCl_3$, $CoCl_2$, $MgCl_2$, and $ZnCl_2$.

Tests involving 18 organic and inorganic salts of sodium indicated that the effect of washing on mutagenic action of $MnCl_2$ depends on the concentration of Na ions in the washing solution rather than on its pH.

Effects of washing in acetate or phosphate buffers were tested. The presence of sodium phosphate buffer in the $MnCl_2$ treatment solution entirely suppresses mutagenic action.

The presence of NaCl in the $MnCl_2$ solution greatly inhibits its mutagenic action, whereas the presence of dextrose either does not affect this action or, under certain conditions, even increases it.

The degree of mutagenic effect of $MnCl_2$ is not so great in growing bacteria as in resting bacteria, nor in bacteria grown in unaerated cultures as in those grown under aeration. It is also much lower in bacteria grown in M9 synthetic medium than in those grown in broth.

Degree of mutagenic effect is proportional to concentration of $MnCl_2$, up to a concentration of about 0.005 per cent, after which further increases are ineffective. It is also proportional to length of treatment, up to about 30 minutes.

Within the range between 1° and 37° C, mutagenic action is greatly increased by higher temperature during treatment.

The combined effects of treatment with $MnCl_2$ and with ultraviolet radiation are not additive.

Tests involving seven types of mutation in addition to streptomycin-resistance showed $MnCl_2$ to be mutagenic in every case, indicating that its mutagenic effect is general.

Tests with several other chemical mutagens showed that their effectiveness is not modified or increased by washing the bacteria before treatment.

Results obtained by us and by Roberts and Aldous (1951) indicate that the mutagenic action of $MnCl_2$ depends on the cell's uptake of this compound, and suggest that the genetically active component of $MnCl_2$ is bound to specific sites within the bacterial cell.

It is suggested that $MnCl_2$ affects the metabolic activity of bacterial cells in such a way as to make the gene systems more unstable than they otherwise are.

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DISCUSSION

LEDERBERG: The most striking feature of the mutagenicity of $MnCl_2$ is the apparent absence of a concomitant bactericidal effect, in contrast to the bactericide of other mutagens. This might, however, be more apparent than real. Not only is the effect of Mn likely to be excited during subsequent growth of the treated cells, but the cells may be heterogeneous with respect to susceptibility to or uptake of the manganous ion. If 60 per cent to 80 per cent of the cells were immune to Mn, the survival of these cells would mask any correlated lethal and mutagenic effects in the residue.

A mechanism which deserves consideration is that bound Mn may catalytically activate free radical formation from H_2O_2 or other products of cell metabolism.

DEMEREC: It is entirely possible that a fraction of bacteria of the Sd-4 strain that we use is susceptible to injury and killing by $MnCl_2$, and that the mutants we observed in the experiments were induced in that fraction of the cells. It would not have to be large; the presence of one per cent or even fewer susceptible cells would be enough to account for our results, and if a high rate of killing should occur among them our experimental methods would not be sufficiently sensitive to detect it. However, I do not see any a priori reason why a mutagen should be toxic to a cell exposed to it. In a living cell there should be many metabolic processes going on that might be sufficiently upset by certain agents to initiate genetic changes without upsetting vital functions and causing death.

PAIGEN: It has been suggested in previous discussion that the manganous ion acts as a mutagen by precipitating the DNA of the bacterial cell, and so setting up an abnormal state which

could give rise to mutation. Since such precipitation would almost surely have drastic effects on the ability of the cell to reproduce, it would seem that this mechanism would be denied by the relative nontoxicity of the manganous ion under the conditions used.

It is possible that the explanation of the mutagenic effect of the manganous ion lies in another direction. It is very suggestive that acetate and phosphate buffers of the same pH should have drastically different effects on the rate of mutation—especially so when this fact is considered in the light of the inhibitory effects of polyvalent cations, particularly Zn and Mg, on the mutation process. The function of such cations as cofactors for many of the enzymes concerned with phosphate metabolism, and the increasing evidence of the role of high-energy phosphate compounds in the synthesis of macromolecules, suggest that the mutagenic activity of the manganous ion is the result of its effect on the phosphate metabolism of the cell, and perhaps more specifically on the availability of high-energy phosphates for use in synthetic reactions.

J. Dental Res. 41(1): 3-11, 1962

The Effect of Manganese Chloride on Amelogenesis

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A search of the literature revealed that enamel defects have been produced by a variety of methods. Many of the methods, however, are not attractive for routine laboratory study, and, furthermore, the incidence of the defects produced is frequently low. On the other hand, a procedure reported by Wessinger and Weinmann[1] is simple and effective. Enamel defects were induced 100 per cent of the time in the incisors of rats by the subcutaneous injection of manganese chloride within a specified dose range.

The guinea pig and hamster, as well as the rat, are well suited for the study of the production and development of dental defects because their incisors also are continuously forming and erupting. In these animals, histogenic and organogenic alterations induced by experimental procedures will eventually emerge into the oral cavity, permitting an examination of the clinical aspect of such faults. In the present investigation, this feature was exploited to determine whether manganese chloride injections affected the incisors of rats, guinea pigs, and hamsters in a similar manner.

EXPERIMENTAL METHODS

Rats.—Twenty rats were each injected subcutaneously with 1 cc. of an aqueous solution of manganese chloride. The dosage received by each animal was 400 mg. of manganese chloride per kilogram of body weight. The incisors were examined weekly or biweekly until the completion of the experiment. Fourteen of the rats were sacrificed within the first month following the injections, and their jaws were prepared for histologic study. The six remaining rats were allowed to survive until the defects became evident in the oral cavity. The skulls of these animals were then preserved.

Guinea pigs.—The first guinea-pig group consisted of fourteen experimental animals, two sodium chloride–injected controls, and two uninjected controls. Each experimental guinea pig was injected subcutaneously with 1 cc. of an aqueous solution of manganese chloride. The dosages ranged from 150 to 490 mg. manganese chloride per kilogram of body weight. Two control animals were each injected with 1 cc. of a sodium chloride solution, the chloride concentration of which was equivalent to 300 mg. of manganese chloride per kilogram of body weight. The two remaining control animals were not injected. The incisors were examined weekly for defects. When defects became clearly visible on the erupting incisors, the animals were sacrificed and their skulls preserved.

This investigation was supported by U.S.P.H.S. research grant D-970 from the National Institute of Dental Research, National Institutes of Health.

Presented at the 38th annual meeting of the International Association for Dental Research, Chicago, Illinois, March 17, 1960.

Received for publication June 13, 1960.

Later, an additional eight guinea pigs were injected with dosages of sodium chloride containing chloride equivalents of from 400 to 700 mg. of manganese chloride per kilogram of body weight. These animals, together with two untreated controls, were observed and treated in a manner similar to the preceding guinea-pig group.

Hamsters.—Twenty hamsters each received a subcutaneous injection of manganese chloride. The dosages administered ranged from 150 to 550 mg. manganese chloride per kilogram of body weight. The control group consisted of two sodium chloride–injected hamsters and three untreated animals. These animals were examined every 2–5 days for about 2 months for defects on the incisors.

RESULTS

Rats.—Amelogenic disturbances and enamel defects were evident either histologically or grossly in the incisors of each of the twenty rats injected with manganese chloride. These results are in accord with the findings of Wessinger and Weinmann.[1] The defects observed grossly appeared first on the lower incisors at the gum line on the thirty-fifth day postinjection. Bilateral defects were visible in both the upper and lower incisors by the fiftieth day postinjection. The emergence of the faults on the lower incisors into the oral cavity always preceded that of the upper incisors. Only one hypoplastic defect occurred on each incisor. Each was positioned centrally on the labial surface of the tooth. The lesions varied in their diameters, thus attesting to the diversity of the response of the individual rats to the same dosage based on body weight. Figures 1 and 2 show the histologic and gross aspects of the defects.

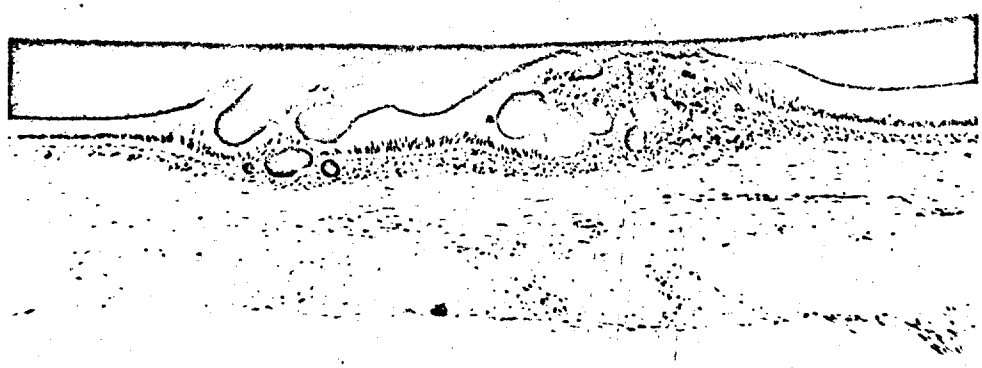

FIG. 1.—Disrupted amelogenesis in the incisor of a rat sacrificed 7 days following the injection of 400 mg $MnCl_2 \cdot 4H_2O$/kg body weight. (H & E stain; Orig. Mag. ×140.)

Guinea pigs.—Six of the fourteen experimental guinea pigs died within 5 days following the administration of manganese chloride. The remaining eight animals were sacrificed at the termination of the study. On the fifty-sixth day postinjection, the first defects became evident in the incisors at the gum line. By the seventy-third day postinjection, all the emerging incisors exhibited dental faults. Each of the incisors was marred by two shallow grooves that traversed the enamel perpendicular to the long axis of the tooth. In addition, all except four of the incisors displayed hypoplastic enamel defects. The hypoplastic lesions invariably developed in relation to the grooves.

The dual grooving of each incisor appeared to be the mildest structural alteration to occur. The distance from the deepest point of one groove to the deepest point of the other was characteristically about 1.5 mm. Of the two grooves, the one nearer to the incisal edge (i.e., distal) tended to be the less definite. In some instances, a furrowing of the dentin occurred, which, together with the proximal enamel groove, formed a constricted ring around the tooth (Fig. 3). Such dentinal blemishes were most marked

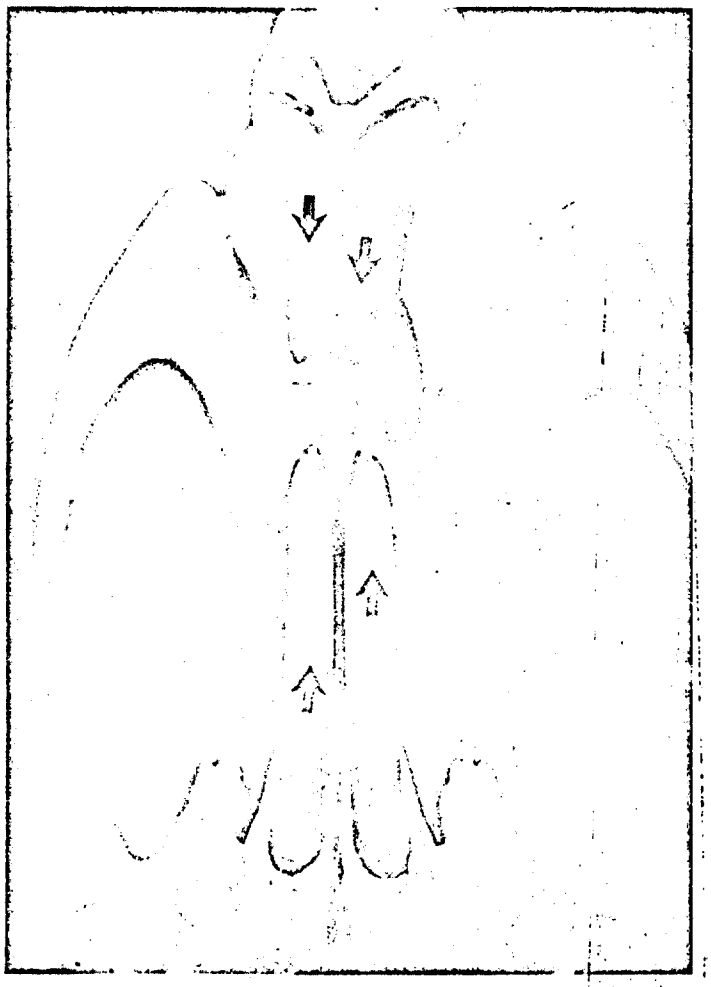

FIG. 2.—Bilateral enamel hypoplasia in the incisors of a rat sacrificed 46 days after the injection of 400 mg $MnCl_2 \cdot 4H_2O$/kg body weight.

in the upper incisors. Sagittal sections through these defects revealed a normal thickness of enamel and dentin. The grooves, therefore, were considered to be a result of disturbed morphogenesis.

The hypoplastic faults that developed in the incisors of the guinea pigs were usually bilaterally symmetrical and were consistently of greater magnitude in the lower incisors than in the upper incisors. The extent of the defects varied and did not correlate with the dose level of manganese chloride administered. The area most prone to be affected by hypoplastic pitting appeared to be the lateral aspect of the distal groove. In each instance of enamel hypoplasia, this part of the tooth was damaged. The next most common sites for the appearance of hypoplasia were the mesial surface of the distal groove and the lateral surface of the proximal groove. More extensive lesions were marked by hypoplastic pitting of the enamel throughout the distal groove and

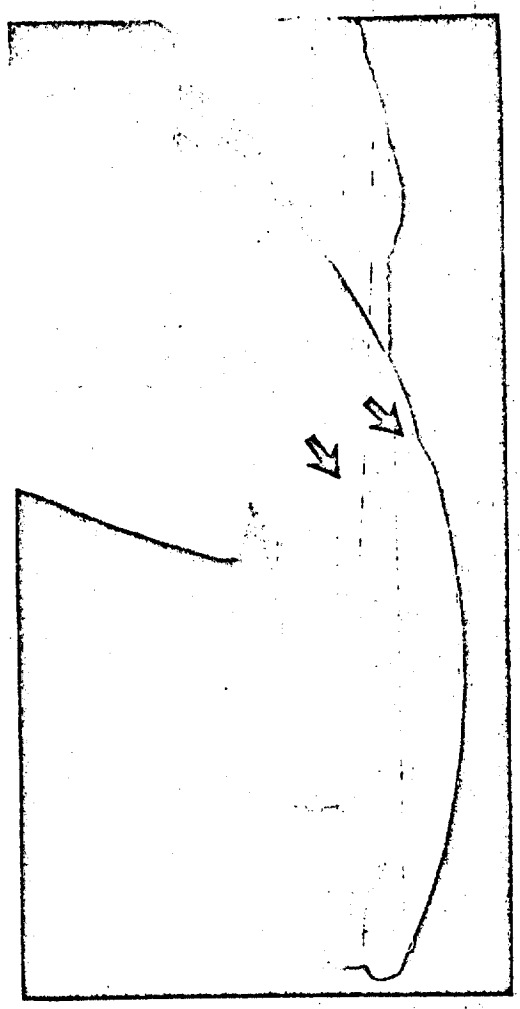

FIG. 3.—Groove defect of the enamel and dentin in the upper incisor of a guinea pig sacrificed 79 days following the administration of 200 mg $MnCl_2 \cdot 4H_2O$/kg body weight. No enamel hypoplasia is evident.

laterally between the proximal and distal grooves. The most severe damage to an incisor included, in addition, hypoplastic defacement of the enamel surface in the proximal groove and the total area between the two grooves (Figs. 4 and 5).

The control animals continued to produce normal incisors with one exception. Eighty-four days postinjection, the lower incisors of a sodium chloride–injected control animal exhibited hypoplastic defects unaccompanied by grooves (Fig. 6). The history of this animal, unique from that of the other guinea pigs, showed that, after the initiation of the experiment, a firm, indurated nodule developed posterior to the ramus of the mandible and inferior to the right ear. Twenty-six days postinjection, a biopsy procedure was performed. A histologic examination revealed a chronic granulomatous inflammatory lesion involving a portion of the parotid gland and possibly a lymph node. Tests for acid-fast bacilli and fungi proved to be negative. However, numerous streptococci were shown to be present by the Brown and Brenn staining procedure.

In order to rule out the sodium chloride injection as being responsible for the enamel defects, a group of eight guinea pigs was injected with dosages of sodium chloride containing chloride equivalents of from 400 to 700 mg. of manganese chloride per kilogram of body weight. These animals, together with two untreated controls, were ob-

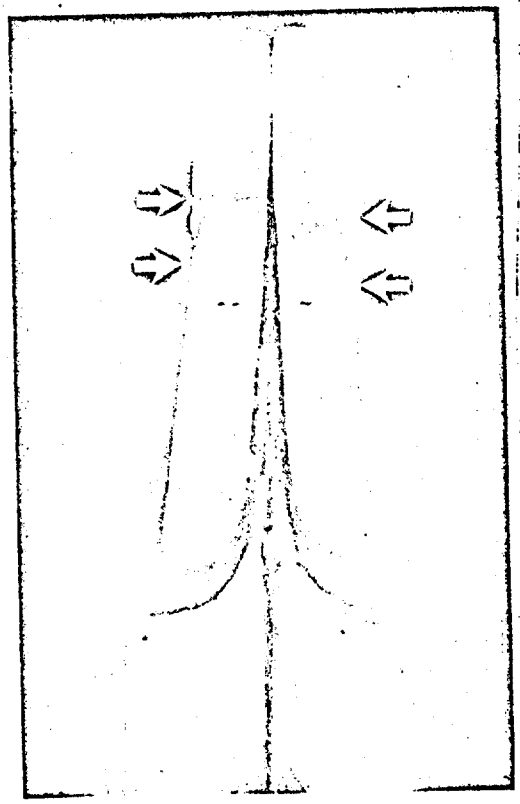

FIG. 4.—Arrows indicate proximal and distal grooves on the lower incisors of a guinea pig sacrificed 86 days following the injection of 150 mg $MnCl_2 \cdot 4H_2O$/kg body weight. Hypoplastic pitting is seen in the distal grooves and on the lateral aspect of the teeth.

served for approximately 3 months. No defects developed on the incisors. A study is now in progress to determine whether streptococcal infections will effect dental lesions in the guinea pig.

Hamsters.—No hypoplastic enamel lesions developed in either experimental or control animals. The only aberrations noted in the incisors were sporadic episodes of depigmentation. This occurred in the control as well as in the experimental hamsters and was not related to the experimental procedures.

DISCUSSION

Of the six guinea pigs that received dosages of 400 mg. manganese chloride per kilogram of body weight or higher, only one survived. These results indicate the lethal dose for the guinea pigs to be close to 400 mg. per kilogram. The lethal dose for rats was shown to be approximately twice this amount by Wessinger and Weinmann.[1] In respect to rabbits, Findlay[2] found 5.5 mg. per kilogram of body weight to be the lethal level. The lethal dosage for hamsters was not established by our study, but the evidence indicates it to be higher than 550 mg. per kilogram of body weight. It is inter-

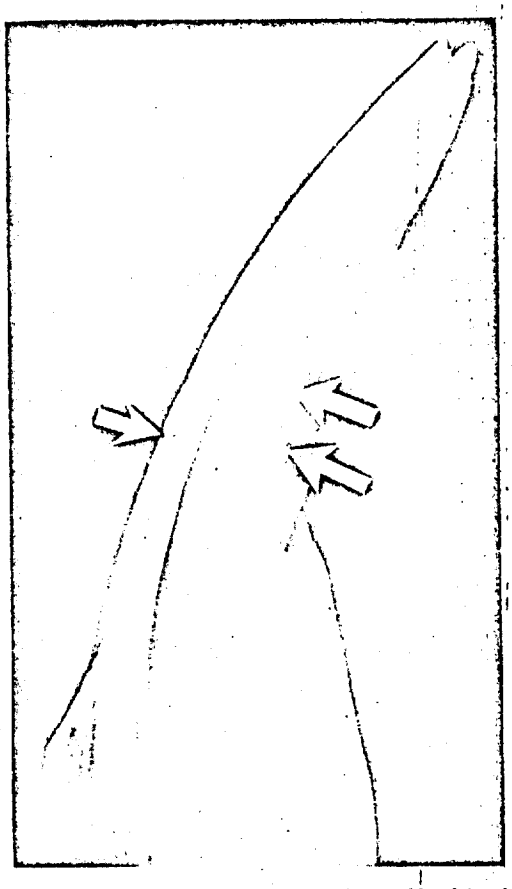

FIG. 5.—Enamel hypoplasia in the incisors of a guinea pig sacrificed 79 days after the injection of 200 mg $MnCl_2 \cdot 4H_2O$/kg body weight.

esting that the manganese tolerance should be so varied among these animals when the concentration of manganese in individual tissues is so constant that it is almost characteristic of the individual organ regardless of its species origin.[3] It may be that the difference in tolerance to manganese chloride is due to a variation in excretion rate and intracellular turnover.

The absence of a relationship between the degree of enamel damage and the dosage of manganese might be attributed to variations in ameloblast metabolism at the time of insult. Töndruy[4] has pointed out that ameloblasts are most liable to injury when in their highest metabolic phase of cytomorphosis. However, it would also seem possible to envision a regional fluctuation in metabolic activity occurring at the same relative level of ameloblastic development.

Whether the amelogenic faults that were produced were direct results of excess manganese or were caused by indirect effects is not known. Support for direct interference by manganese may be drawn from the works of Maynard,[5] Cotzias and Greenough,[6] and Borg.[7] They found that excess manganese containing radioactive forms moved into all mitochondrial pools following intravenous administration. Furthermore, the turnover of the mitochondrial manganese was very high in comparison

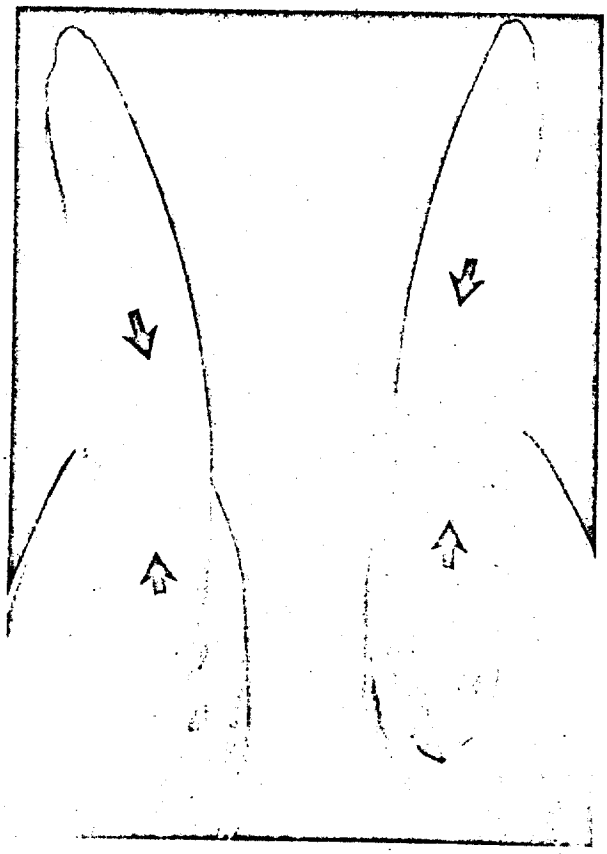

FIG. 6.—Enamel hypoplasia in the lower incisors of the NaCl-injected control guinea pig, sacrificed 58 days following the removal of an abscess.

with that of the nuclear manganese, which indicates the existence of a highly organized intracellular pathway for manganese. Cotzias[3] advanced the idea that perhaps a rapid turnover of a homeostatically controlled substance such as manganese might in itself be an adequate cause of degeneration. This could account for ameloblast damage, as well as for the parenchymal damage found in the liver and kidneys by Findlay, Ravera, and co-workers.[8]

Greenberg and Campbell[9] have found that, 3 days after the administration of manganese, the skin and bone, respectively, accumulate the highest manganese concentrations in the body. Their report, however, did not include observations concerning teeth. Fore and Morton,[10] studying ox bone, discovered that most of the manganese was associated with the inorganic matrix rather than with the organic portion. Here, then, is a twofold indication that manganese may be directly implicated in the production of amelogenic defects, first, because the enamel organ is a derivative of the skin and, second, because the inorganic matrix of enamel has many features in common with the inorganic matrix of bone.

Indirect sources of damage to amelogenesis following manganese chloride injections may also be associated with a toxic effect on the liver and/or kidneys, and it has been reported that massive oral intake of manganese compounds interferes with calcium and phosphorus absorption and results in rickets.[11, 12]

Regardless of whether enamel defects are produced by the direct or indirect effects of manganese chloride in the rat and guinea pig, the amelogenic process in the hamster appears to continue to function with impunity. Furthermore, the distribution of the hypoplastic enamel defects in the incisors of the guinea pig differs from that of the rat. In addition to the difference among these animals in their tolerance to manganese chloride, it is evident that amelogenesis in the rat, guinea pig, and hamster is not similarly affected by the same dosage of manganese chloride based on body weight. Further investigation of some of the factors discussed might well assist in a better understanding of the amelogenic process.

SUMMARY

The effect of manganese chloride on the amelogenesis of the incisors was studied in groups of rats, guinea pigs, and hamsters. The experimental animals received subcutaneous injections of manganese chloride, and the control animals were injected with sodium chloride solutions or remained untreated. The erupting incisors were observed periodically for the appearance in the oral cavity of any defects. Only in respect to the rats were sections prepared for a histologic examination.

The histologic and clinical studies revealed that defects developed in 100 per cent of the manganese chloride–treated rats. The hypoplastic enamel lesions observed clinically made their appearance in the oral cavity between the thirty-fifth and fiftieth days postinjection. No morphogenic alterations occurred.

Hypoplastic enamel defects became evident clinically in the guinea-pig incisors between the fifty-sixth and seventy-third days postinjection. These faults developed in 100 per cent of the surviving animals. Only four of the upper incisors remained free from hypoplastic damage. However, all the incisors exhibited a dual grooving. These grooves manifested a disturbance of morphogenesis. In each instance, the hypoplastic pitting developed in relation to the grooves.

Amelogenesis in the hamsters was unaffected by the administration of manganese chloride.

The assistance of Carlyle Costello is gratefully acknowledged.

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Abnormal Glucose Tolerance in Manganese-deficient Guinea Pigs [1,2]

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ABSTRACT The effects of low dietary manganese during pre- and postnatal development on glucose utilization of young adult guinea pigs is reported. Glucose tolerance tests were performed on deficient and control guinea pigs, using both oral and intravenous glucose administration. Deficient guinea pigs showed decreased utilization of glucose and in consequence had a diabetic-like glucose curve in response to glucose loading. Control animals and deficient guinea pigs, given dietary manganese equivalent in amount to that supplied control animals for 2 months, showed normal responses to glucose administration.

In our studies of the effects of low dietary manganese on the prenatal and postnatal development of the guinea pig (1–3) it was noted that the pancreatic gland in neonatal animals was often absent or disproportionately reduced in size. Animals having the most extreme pancreatic abnormalities at birth died within the first few postnatal hours or days. Others, less handicapped, survived to adult life. A description of the morphological changes in the pancreatic tissues of manganese-deficient animals has been presented in another report by the authors (4).[2]

These observations have been followed by a study of glucose tolerance of surviving young adult animals, which were the offspring of females fed a diet of less than 3 ppm manganese throughout pregnancy. Before these animals were tested for glucose utilization, they were maintained postnatally on the same low manganese regimen. Certain of the deficient animals were subsequently fed the control diet for a 2-month period and retested to determine the reversibility of the defective glucose tolerance noted previously for manganese-deficient animals. This present paper reports the physiological response of the manganese-deficient, deficient-supplemented, and control guinea pigs to glucose loads administered both orally and intravenously.

EXPERIMENTAL

The animals used in this study were the offspring of female guinea pigs fed diets containing either less than 3 ppm manganese or 125 ppm manganese in the form of manganese sulfate. The composition of the ration and the management of the colony have been presented previously by Tsai and Everson (2).

Oral glucose tolerance tests were performed on 15 pairs of young adult animals. Each pair consisted of a deficient and a control animal of the same sex and age. Initial blood samples were taken after a 20-hour fast. Venous blood was obtained by clipping the nail bed of a toe, as described by Vallejo-Freire (5). A glucose load equivalent to 200 mg/100 g of body weight was given as a 20% aqueous solution. This was fed by dropper and was consumed in less than 2 minutes. Animals were anesthetized using sodium pentobarbital and subsequent sampling was performed at 30 minutes, and 1, 2, 3, and 4 hours. Glucose was determined by Nelson's modification of the Somogyi method (6).

Intravenous glucose tolerance tests were made on eight matched pairs of young adult animals. Using a modified Popovic technique (7, 8), a sterile no. 10 polyethylene cannula [3] was placed in the descending aorta via the carotid artery. The exterior-

Received for publication June 6, 1967
[1] Supported in part by Public Health Service Research Grant no. HD-00429-10 from the National Institutes of Health.
[2] Presented in part at the 51st annual meeting of the Federation of American Societies for Experimental Biology, Chicago, 1967.
[3] Intramedic polyethylene tubing no. 10. Clay-Adams, Inc., New York.

ized cannula filled with sterile heparinized saline was heat-sealed and coiled on the back of the animal's neck. Complete post-surgical recovery, including return to normal body weight usually occurred within 5 to 10 days. The animals were then fasted for 18 hours and initial blood samples were removed via the cannula, using a 27-gauge needle and a disposable syringe. Unanesthesized animals were given a test load of glucose, equivalent to 100 mg/100 g of body weight. This was administered as 40% glucose in sterile physiological saline and was infused for 3 minutes. Samples were withdrawn at 15, 30, and 45 minutes and 1, 2, 3, and 4 hours. Animals were unrestrained and showed no adverse reactions to the manipulations performed during testing. Glucose was determined by both the Somogyi and glucose oxidase methods.[4] Following evaluation of the glucose tolerance, approximately one half of the deficient animals were killed to obtain tissue samples. The remaining animals (hereafter referred to as supplemented animals), were fed the control diet for 2 months. Intravenous glucose tolerance tests were then repeated on these animals using the procedures outlined above.

Peripheral utilization of glucose was calculated by the method of Amatuzio et al. (9), and Student's *t* test was applied to determine the significance of differences observed (10).

RESULTS

Oral glucose tolerance tests showed a distinctly different type of response between the manganese-deficient and control guinea pigs. Representative curves are illustrated in figure 1. The fasting glucose values of deficient animals averaged 40 mg/100 ml higher than those of control animals (table 1). Blood glucose remained elevated for the entire test period in all manganese-deficient guinea pigs and in several cases the 4-hour level exceeded the fasting value by more than 50 mg /100 ml blood. The control animals showed a more normal response to oral administration of glucose with the peak

[4] Glucostat, Worthington Biochemical Corporation, Freehold, New Jersey.

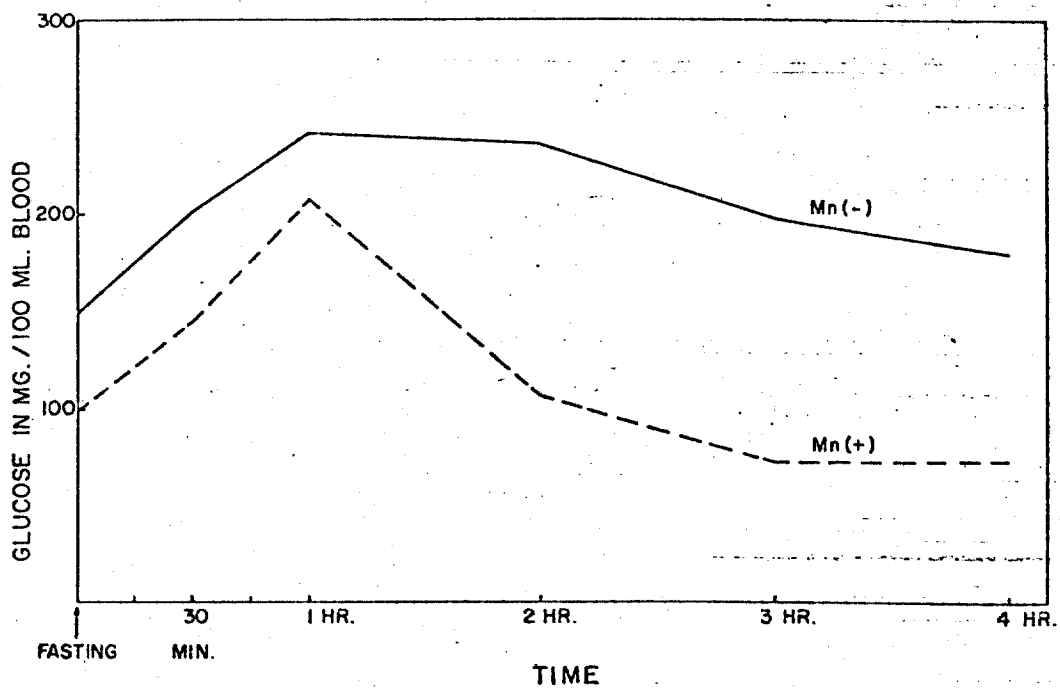

Fig. 1 Glucose tolerance curves of guinea pigs. (Oral administration of glucose, sodium pentobarbital anesthesia, blood sampling by nail-bed clipping.)

TABLE 1

Fasting blood sugar levels in manganese-deficient and control guinea pigs

Regimen of animals	Method of blood sampling			
	Nail-bed clipping		Cannulation	
	Mean	Range	Mean	Range
	mg/100 ml blood		*mg/100 ml blood*	
With manganese	130	95–165	95	85–105
Without manganese	165	130–200	125	110–140

elevation occurring at 30 minutes or 1 hour after glucose intake. Two or three hours following glucose administration, blood glucose values were approximately equal to fasting levels for control animals.

Ambiguous responses to oral glucose tolerance tests were shown by supplemented animals. Incomplete reversal of abnormal patterns appeared in all but a few of the animals tested and no clear-cut pattern of response could be determined. Curves suggestive of improvement when compared with initial test results were observed for several animals.

While there was an unmistakable difference in glucose utilization between deficient and control guinea pigs tested by oral feeding of glucose and nail-bed clipping of anesthetized animals, aberrant values resulting from trauma were obtained at varying times throughout the testing period when the above procedures were used. It was also recognized that the use of an anesthetic introduced possible variables in alimentary absorption and peripheral utilization (11). Sampling of blood for glucose concentrations using nail-bed clipping without anesthesia proved unsatisfactory in the guinea pig. The recognition of these procedural defects led to the repetition of the experiments using animals having indwelling cannulas. Mean fasting values and ranges found for nail-bed clipped and cannulated deficient and control guinea pigs are presented in table 1.

The results of the intravenous glucose tolerance tests are presented in figure 2. Data given in table 1 showed lower mean fasting blood glucose values for deficient and control animals when glucose was administered and blood sampling carried out by cannulation. Less variation in fasting values was noted with intravenous testing. The effect of diet was similar in the 2 methods of testing, in that the manganese-deficient animals had higher glucose values at the end of an 18- or 20-hour fast than controls.

Glucose tolerance curves illustrated in figure 2 confirmed the evidence obtained first by oral testing, that there is a marked difference between the two ration groups in ability to utilize glucose. Manganese-deficient animals maintained elevated glucose concentrations throughout the 4-hour test period. The effect of 2 months of adequate dietary intake of manganese on the ability of these previously deficient animals to handle glucose is demonstrated in figure 2. The broken line in this figure represents the normal glucose tolerance curve of the supplemented cannulated animals given intravascular glucose. Supplementation appeared to correct previously identified abnormalities of glucose utilization in these animals. To determine whether additional glucose loading would show any functional inadequacies, a duplicate load of glucose was administered to several supplemented cannulated animals at the end of the first test period. A typical response curve following this procedure is shown in figure 3. The curves obtained from both control and supplemented animals subjected to repeated doses of glucose appeared to be normal.

The rate of disappearance of glucose from the blood has been expressed in terms of percentage decrease per minute (9, 12, 13). Glucose decreased in the blood of normal subjects at a rate of 1 to 3% per minute. Peripheral underutilization, as in diabetes, is indicated by values of less than 1 % decrease per minute (14–16). Data from the 3 groups of cannulated guinea pigs under investigation show that the deficient animals lost glucose from

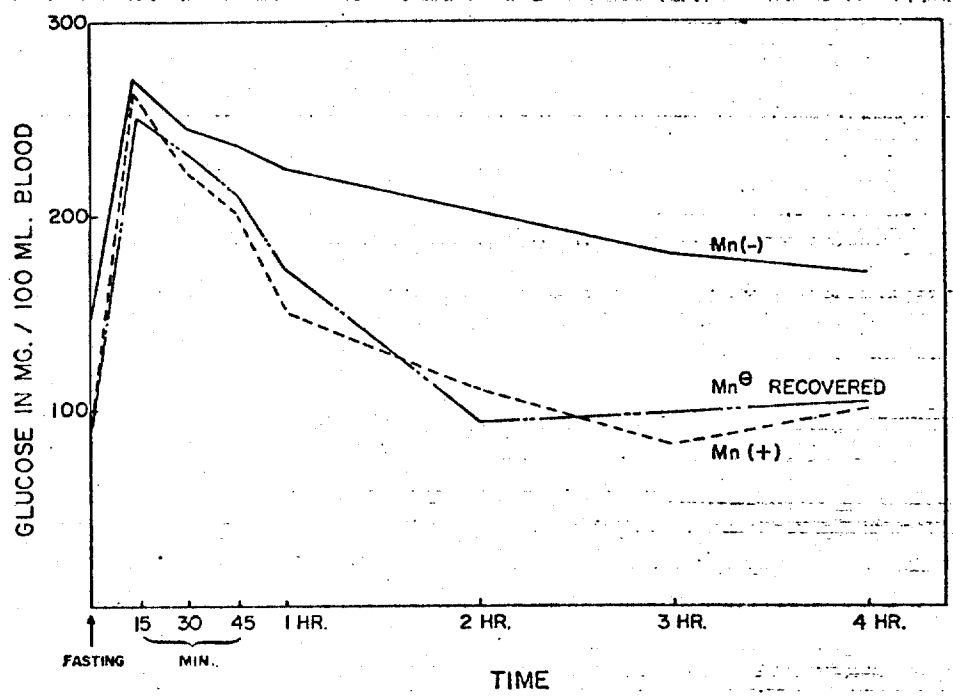

Fig. 2 Glucose tolerance curves of cannulated guinea pigs.

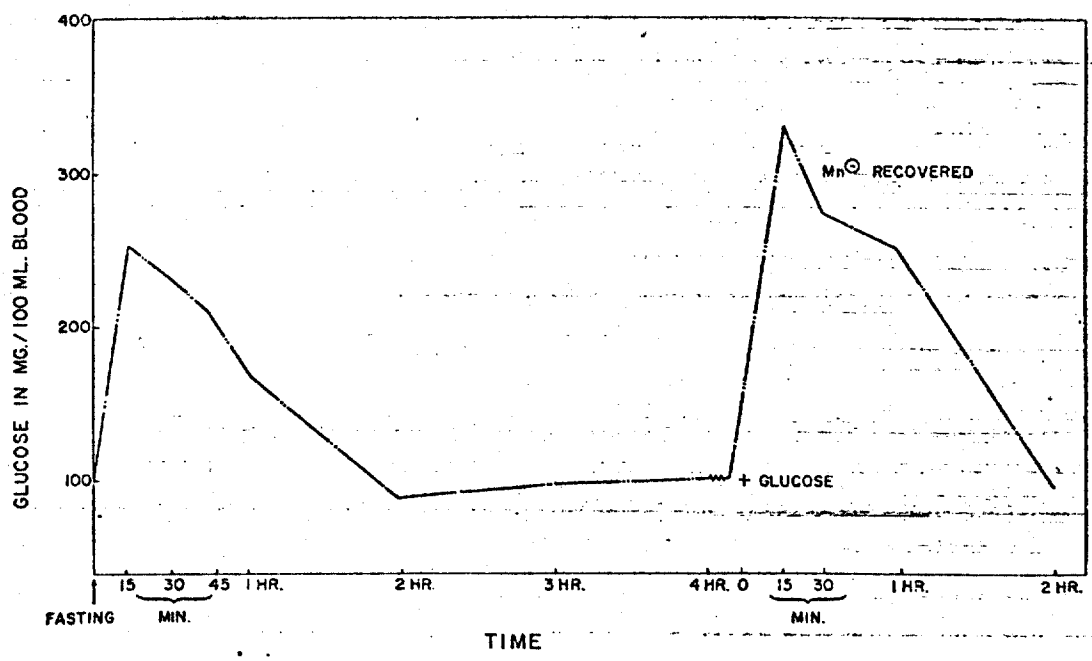

Fig. 3 Glucose tolerance curves of supplemented guinea pigs following repeated administration of glucose via cannula placed in the carotid artery.

the blood at less than 0.5% per minute, whereas the rate of loss for control and supplemented animals exceeds 1.7% per minute. These differences were found to be significant at the 0.1% level.

DISCUSSION

Manganese has been reported by other investigators to influence glucose utilization. Its administration to diabetic subjects has been found to have a hypoglycemic effect (17, 18). "Toxic" levels of dietary manganese have been observed to result in hypoglycemina in normal rabbits (19) and dogs (20). Pancreatectomy and diabetes have been correlated with decreased manganese levels in blood and tissues (21–24). The precise nature of the involvement of manganese is not clear and whether the metal is related to insulin synthesis, need, or secretion is not known.

Our previous studies of manganese deficiency in the guinea pigs have shown that congenitally deficient animals have reduced amounts of mucopolysaccharides in cartilage tissue. It is of interest in this respect that decreased concentrations of stainable mucopolysaccharides have been reported in the skin of young rats born to diabetic mothers (25). Schiller and Dorfman (26) have suggested that insulin may regulate utilization of glucose in the synthesis of mucopolysaccharides. Whether the reduced glucose utilization found in the manganese-deficient guinea pig is directly or indirectly related to the connective tissue defect established in manganese-deficient animals, is still under investigation.

It appears from these studies that manganese supplementation completely reversed the reduced glucose utilization reported.

ACKNOWLEDGMENT

The authors acknowledge the participation of Yiota Bakouli during the early phases of the study of oral glucose tolerance testing in the guinea pig.

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THE EXPERIMENTAL PRODUCTION OF BILIARY CIRRHOSIS BY SALTS OF MANGANESE.

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Received for publication February 27th, 1924.

CHRONIC manganese poisoning among industrial workers has now been recorded on the Continent, in America, and more recently in this country by Charles (1922). The clinical symptoms, which are not unlike those associated with progressive lenticular degeneration, have been fully described, though little is yet known in regard to the pathological changes occurring in this form of poisoning. So far Casamajor (1916) has described the only case in man where a pathological examination has been made after death. In addition to lesions in the medulla biliary cirrhosis was present.

Attempts to produce chronic manganese poisoning in animals have not been very successful. Reiman and Minot (1920) fed dogs on rhodenite (manganese metasilicate) for more than a year without producing any pathological changes in the tissues, while Drinker, Shaw and Drinker (1923) found that cats showed fibrosis of the liver after receiving a single massive dose of manganese, though no pathological details are given.

Numerous experiments, however, have been carried out on the fate of single doses of manganese injected into animals. Thus Lund, Shaw and

Drinker (1923) showed that manganese dioxide and manganese metasilicate have little permanence as foreign substances in the body; they are arrested in the liver to the practical exclusion of other organs, and are thence excreted in the bile, largely in the form of a soluble manganese carbonate. When large doses are administered parenterally a very small amount of manganese may appear in the urine.

In view of the absence of precise knowledge in regard to the toxic effects of manganese it seemed to be of interest to investigate the histological changes produced in animals by salts of this substance. For this purpose the soluble manganous chloride was employed.

I. EXPERIMENTS ON RABBITS.

A. *The Effect of the Parenteral Administration of Large Doses.*

Subcutaneous injections of a solution of manganous chloride in water were given to ten rabbits with the usual aseptic precautions. The actual dose of manganous chloride varied from 10 to 60 mg. In every case death took place in from twenty-two to forty-eight hours after the injection. Manganous chloride is thus extremely toxic to rabbits, as is shown by Table I:

TABLE I.

	Weight of rabbit in gm.	Doses in mg.	Period of survival in hours.
1	1800	10	48
2	1450	15	40
3	1400	20	48
4	1550	25	36
5	1500	35	40
6	1250	40	42
7	1750	45	28
8	1450	50	22
9	1400	55	24
10	1850	60	26

A few hours after the injection the animals became quiet and listless. They refused food, gradually became weaker, and eventually passed into a semi-comatose state. The urine after death was loaded with albumin, together with red blood-cells, casts, and a trace of bile. On post-mortem examination the appearances were closely similar in all cases. The liver was always slightly enlarged, friable, and of a pale yellow colour, often with reddish mottlings. The spleen varied considerably in size, but as a rule was enlarged and of a deep red colour. The kidneys were pale, the lungs and intestines congested. Microscopically the liver showed degeneration of the parenchyma, amounting in some areas to definite necrosis. Sections stained with Sudan III showed the presence of fatty changes, especially at the periphery of the lobules. A few of the Kupfer cells were swollen and prominent, but there was no undue proliferation of the endothelial cells. In some sections small haemorrhagic foci could be noted scattered throughout the liver substance. In the kidneys the main changes were found in the glomeruli.

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Many of the tufts were congested, and in several there was blood in the capsular space. The effect on the tubular cells was unequal. In some cases they were apparently normal, while in others there was fatty change. In the spleen there was congestion of the sinuses; the other organs showed no constant histological changes.

B. *The Effect of Repeated Subcutaneous Injections.*

Preliminary experiments had shown that after the subcutaneous injection of 5 mg., rabbits, although obviously unwell for a few hours, finally recovered completely.

Two series of young rabbits were therefore inoculated subcutaneously with solutions of manganous chloride. The first series received 3 mg., the second series 5 mg. every second day. The main pathological effects, apart from the occasional occurrence of local necrosis at the site of injection, were a new formation of connective tissue in the liver and proliferation of the epithelium lining the bile-ducts.

1. *Experiments in which 3 mg. were given every second day.*—Ten rabbits were used in this experiment. The first rabbit was killed ten days after the beginning of the experiment, the others at intervals up to the sixtieth day of the experiment. For some hours after each injection the animals were quiet and refused food. As a rule they increased only very slightly in body-weight during the course of the experiment—in many cases in fact they showed an actual decrease.

All the animals were free from coccidiosis, and in only two cases were there tape-worm cysts in the mesentery. At post-mortem examination the animals were found to be rather poorly nourished. There was occasionally an icteric tint in the subcutaneous tissues, but no evidence of ascites. The liver was slightly enlarged, smooth and rather friable, deep red in colour or with red and yellow mottlings. There was no thickening of the capsule and no pathological adhesion to surrounding structures. The spleen was normal in size, but of a deep purple-red colour. The kidneys were pale but of average size. The lungs, heart, pancreas, suprarenals and intestine showed no naked-eye changes. On microscopical examination the liver showed some fatty degeneration restricted chiefly to the periphery of the lobules. The extent of the fatty change appeared to vary with the period which had elapsed since the time of the last injection and the death of the animal, being more extensive when the animal was killed a few hours after an injection. The vessels in the portal tracts were congested. The capsule was not thickened. In the portal tracts, however, there were masses of small round-cells, together with the formation of new fibrous tissue. Numerous new bile-capillaries were present and each of these twigs was surrounded by freshly-formed fibrous tissue. Many of the larger bile-ducts were dilated, while the epithelial cells lining the ducts showed numerous mitotic figures. In some animals the bile-duct epithelium was thrown into folds, the appearance being similar to that described by Vaughan Harley and Barratt (1896) as having been produced in cats by the experimental obstruction of the hepatic ducts.

In two rabbits the bile-duct epithelium had taken on a papillomatous

appearance. Although very careful search was made in these two animals for evidence of coccidial infection, none could be found. The amount of round-cell infiltration and fresh fibrous-tissue formation was much greater in the livers of those animals which had received five or more injections. The splenic sinuses were engorged with red cells, but there was no increase of fibrous tissue. The kidneys exhibited only some slight fatty change in the tubular cells.

2. *Experiments in which 5 mg. were injected subcutaneously every second day.*—The rabbits were very unwell for about twenty-four hours after the injections. Of the rabbits used in this experiment one was found to be suffering from coccidiosis and was therefore discarded. Particulars of the rabbits are shown in Table II:

TABLE II.

	Weight in gm. at beginning of experiment.	Weight in gm. at end of experiment.	Date of death in days.
31	1520	1380	17
32	1440	1400	17
33	1100	1020	21
34	1220	1080	22
35	1450	1300	23
36	1540	1200	25
37	1400	1350	Suffering from coccidiosis.
38	1420	1400	31
39	1420	1280	35
40	1500	1200	35
41	1630	1640	21
42	1700	1480	26
43	1750	1730	38
44	1560	1430	58
45	1420	1400	2
46	1950	1980	45
47	1870	1920	Still alive.
48	1230	1200	42
49	1380	1420	35
50	1180	970	50
51	1310	1260	50
52	1150	1050	39

In the case of rabbits Nos. 31–40 the experiments were carried out in the Research Laboratory of the Royal College of Physicians of Edinburgh, the later experiments in London. Control rabbits, examined both in Scotland and in London, failed to show pathological changes in the internal organs similar to those exhibited by the experimental animals. With two exceptions all the rabbits underwent a progressive diminution in weight as the result of the injections. The urine contained albumin, casts and sometimes a trace of bile. Rabbits Nos. 44 and 50 showed rigidity of the hind limbs a few days before death.

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On post-mortem examination there was no evidence of ascites though jaundice was sometimes present. The liver was enlarged, soft and friable. It was either deep red or reddish-yellow in colour. The gall-bladder was not unduly distended. The capsule was not thickened, nor was there any abnormal adhesion to surrounding structures.

The spleen did not show any definite enlargement; it was purplish-red in colour. The kidneys were normal in size but pale. The other organs presented no naked-eye lesions.

Microscopically the changes were very similar in every case with the exception of rabbit No. 45, which only showed diffuse fatty change in the liver. In all the others the lesions in the liver varied in degree rather than in kind. The liver-cells, except those immediately round the central vein, stained poorly. At the periphery of the lobules, in some animals, many of the hepatic cells were shrunken and atrophied while the intralobular capillaries were greatly congested. The Kupfer cells were not enlarged. In the portal spaces there was an excess of small round-cells together with freshly formed fibrous tissue, strands of which divided up the liver substance into areas which for the most part corresponded with pre-formed lobules. The bile capillaries were proliferated, each bile-duct being surrounded by newly formed connective tissue.

In the spleen the sinuses were packed with red blood-corpuscles, while many of the endothelial cells contained much blood-pigment. There was no obvious formation of fresh fibrous tissue. In the kidneys the glomeruli were congested and in many cases there was hæmorrhage into Bowman's capsule. The intertubular vessels were deeply congested. The tubular cells showed fatty changes somewhat irregularly distributed throughout the section. The lumina of many of the tubules were filled with casts, but only a very slight formation of fresh fibrous tissue could be detected in a few animals.

Rabbit No. 50 is of interest, for during the course of the experiment it became pregnant and gave birth to five apparently normal young. Two of these were killed at birth and two when fifteen days old. The livers of all were quite healthy. The mother had cirrhosis.

The main change produced in rabbits by the subcutaneous injection of manganous chloride is thus seen to be a form of liver cirrhosis of the biliary type.

Certain objections have, however, been urged against the use of rabbits for work on cirrhosis. Thus Ophüls (1908) has stated that owing to the frequency of coccidiosis in the livers of rabbits, all cases of cirrhosis in these animals must be looked upon with suspicion. This objection has been combated by Gye and Purdy (1922), who, in their work on the effects of colloidal silica on the rabbit, point out that the lesions produced by coccidiosis are circumscribed, and that even when the liver is infected there is little difficulty in fixing their limit. In only one animal in the present series was there any evidence of coccidiosis.

A more serious objection has, however, been made by Wells (1920), who finds that the repeated injection in rabbits of almost any foreign protein material, such as Witte's peptone, foreign blood or emulsions of organs may be followed by the occurrence of cirrhotic changes in the liver. "Peptone" injections in dogs and guinea-pigs failed to cause a similar cirrhosis.

Rabbits would thus seem to be especially liable to develop cirrhotic changes in the liver under comparatively slight provocation. Before drawing any definite conclusion as to the effects of manganous chloride on the liver, it therefore seemed desirable to investigate the effects of this salt on other laboratory animals. Experiments were accordingly carried out on guinea-pigs and rats.

II. EXPERIMENTS ON GUINEA-PIGS.

Preliminary experiments had shown that in proportion to their body-weight guinea-pigs are relatively less susceptible than rabbits to the toxic action of manganous chloride.

Six guinea-pigs were given subcutaneous injections of 3 mg. of manganous chloride dissolved in distilled water every other day. Particulars of the animals are given in Table III:

TABLE III.

	Weight of animal in gm. at beginning of experiment.	Weight of animal in gm. at end of experiment.	Period of survival in days.
1	285	220	12
2	380	350	29
3	300	310	32
4	290	225	34
5	270	270	39
6	260	270	44

Guinea-pig No. 1 died from an intercurrent broncho-pneumonia. The liver showed slight fatty change, but no increase in fibrous tissue.

All the other animals showed some degree of fibrosis of the liver, the change being most pronounced in guinea-pig No. 5. In this animal the liver was reduced in size and showed red and yellow mottlings. The other organs were macroscopically normal. Microscopically in the liver there were seen to be patches where the parenchyma cells were replaced by masses of loose connective tissue of a somewhat less cellular character than that seen in the rabbit. The fibrosis was well seen in the region of the portal tracts and was associated with proliferation of the smaller bile-ducts. The liver parenchyma had undergone fatty degeneration. There was no enlargement or proliferation of the Kupfer cells. The microscopical appearances of the liver are shown in Fig. 4, from which it will be seen that the type of lesion produced is similar to that met with in the rabbit. The changes met with in livers of the other guinea-pigs varied only in degree from that illustrated in Fig. 4. Control guinea-pigs kept under similar conditions and receiving the same food showed no fibrosis of the liver. A slight degree of proliferation of the bile-duct epithelium was seen in two of the animals examined, but it was never so definite as in the rabbit.

III. EXPERIMENTS ON RATS.

A few experiments were also carried out on rats. Ten rats were given 3 mg. of manganous chloride by subcutaneous injection every other day.

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Unfortunately subcutaneous injections of manganous chloride in the rat are very liable to produce local necrosis. In the first four weeks of the experiments four of the animals died from septic infection. Neither macro- nor microscopically were there any characteristic lesions in these rats. In the remaining six animals there was present in all more or less fibrosis of the liver. In rats 9 and 10 there was noticed a certain rigidity of the hind legs and head, though choreiform movements were not seen at any time. Particulars of the animals are given below:

Rat 2 died on the 38th day of the experiment. Weight 92 gm.—a decrease of 28 gm. The liver was enlarged and yellow in colour, with some reddish mottling. There was jaundice, but no ascites. The spleen was greatly enlarged, weighing 1·19 gm. Microscopically the liver showed a very slight increase in fibrous tissue, which was almost entirely restricted to the region of the portal tracts. There was very little round-celled infiltration. The other organs were normal, with the exception of the spleen, which showed congestion.

Rat 3 died on the 42nd day of the experiment. Weight 63 gm.—a loss of 31 gm. The liver was yellowish-white in colour, the spleen slightly enlarged. Microscopically the changes in the liver, illustrated in Fig. 5, were closely similar to those in rat 2.

Rat 4 died on the 49th day of the experiment. Weight 72 gm.—a loss of 25 gm. There was jaundice but no ascites. The liver was enlarged and yellow in colour. The other organs appeared normal except for broncho-pneumonic patches in the right lung.

Microscopically there was evidence of a well-marked perilobular fibrosis, which divided the liver up into monolobular areas. The connective tissue was loose in structure and was associated with a considerable degree of round-celled infiltration. There was also a slight degree of proliferation of the bile-capillaries, as is seen in Fig. 6.

Rat 7 died on the 53rd day of the experiment. Weight 82 gm.—a loss of 31 gm. Both the naked-eye and microscopic appearances were very similar to those in rat 4.

Rat 9, killed when moribund on the 84th day of the experiment. Weight 58 gm.—a decrease of 37 gm. The animal was emaciated. There was no œdema or ascites, but jaundice was present. The liver was normal in size, firm, and of a lemon-yellow colour. The outer surface was slightly roughened. There were no pathological adhesions. The spleen, which was a little enlarged, was of a deep red colour. The right lung showed patches of broncho-pneumonia. The other organs appeared normal. Microscopically the liver showed an intense fibrosis, not confined to the portal regions, but extending into the lobules and dividing up the liver-cells into small irregular areas. The fibrous tissue contained a number of small round-cells. In some parts of the liver the fibrous tissue was so dense that practically no liver-cells remained. In other parts a few liver-cells were noticeable, many of them of a hypertrophic character with giant nuclei. All the surviving liver-cells showed fatty degeneration. The other organs did not show any abnormal changes.

Rat 10 killed on the 86th day of the experiment, moribund. Weight 86 gm.—a loss of 24 gm. The liver was normal in size. It was of a yellow

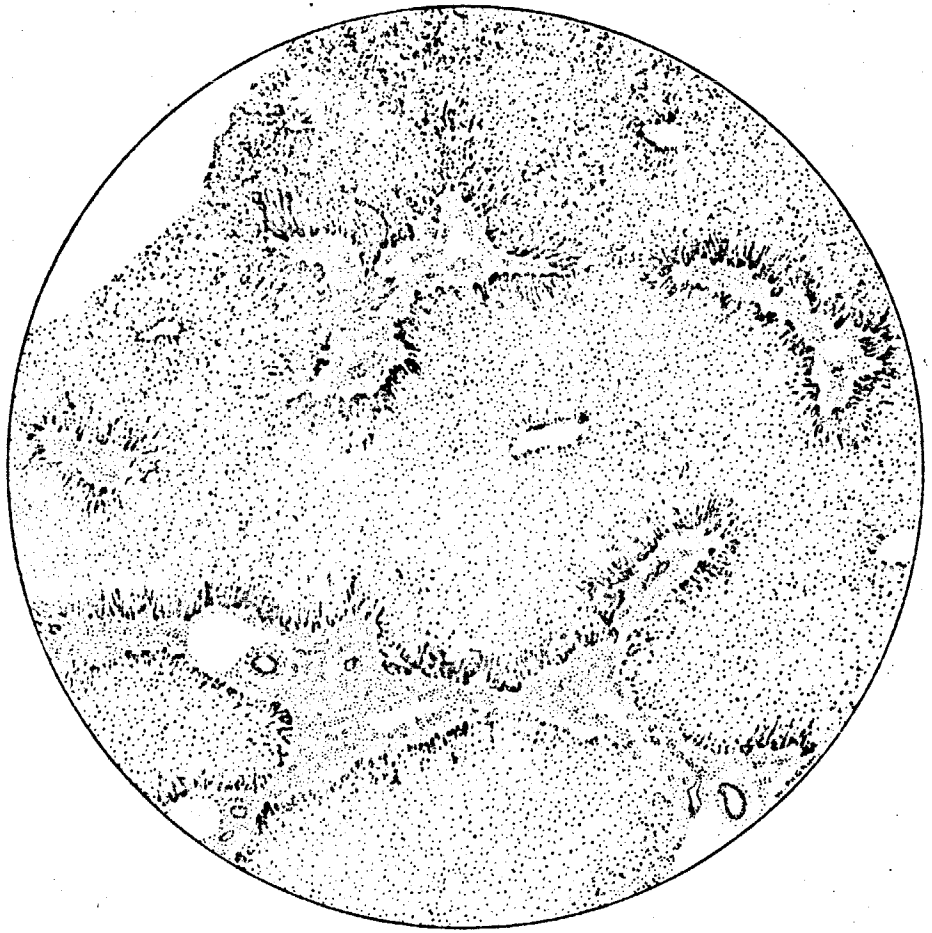

FIG. 1. Scale 56 : 1.

Stained iron-hæmatoxylin and eosin.

Liver of rabbit 15, which received subcutaneous injections of 5 mg. of manganous chloride for 5 weeks. The fibrosis is closely related to the portal spaces and is accompanied by round-celled infiltration and a formation of fresh bile-capillaries.

Findlay.

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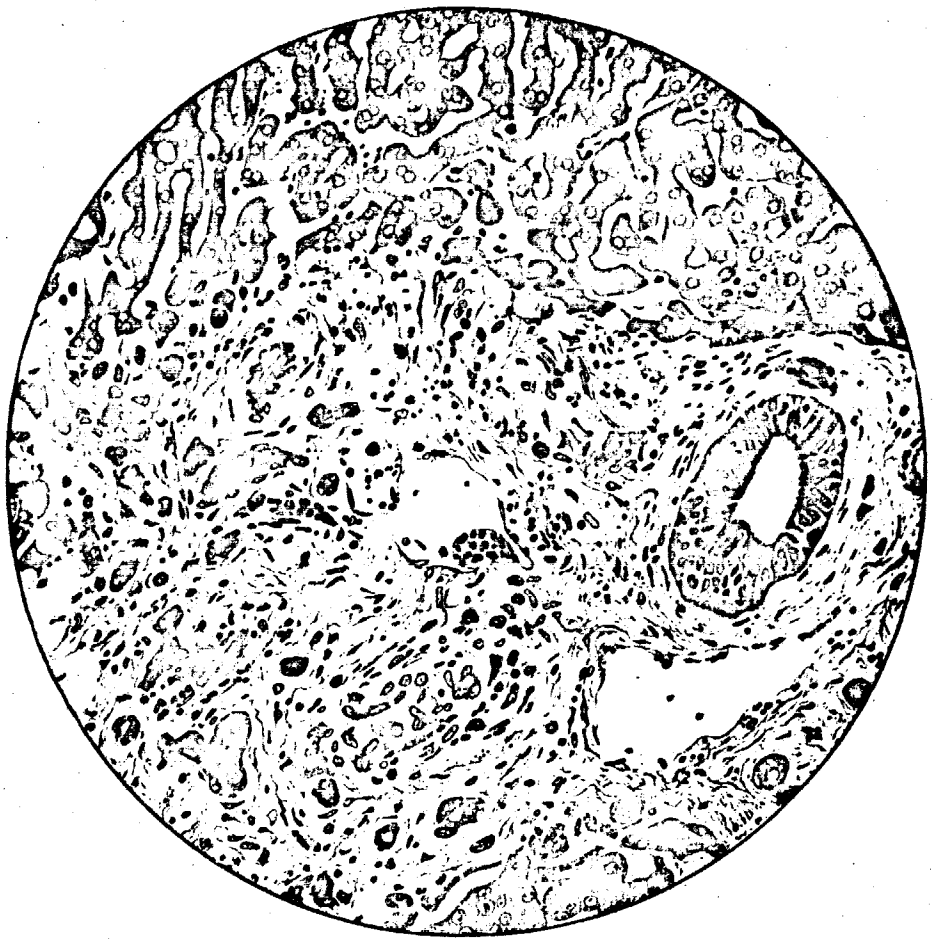

FIG. 2. Scale 260 : 1.

Stained iron-hæmatoxylin and eosin.

Liver of rabbit 51, which received subcutaneous injections of 5 mg. of manganous chloride for 9 weeks. The epithelium of the bile-duct forms papillary processes.

Findlay.

FIG. 3. Scale 300 : 1.

Stained iron-hæmatoxylin and van Gieson.

Liver of rabbit 46, which received subcutaneous injections of 5 mg. of manganous chloride for 6 weeks. Note the formation of new bile-capillaries and the proliferation of the epithelium of the larger bile-duct.

Findlay.

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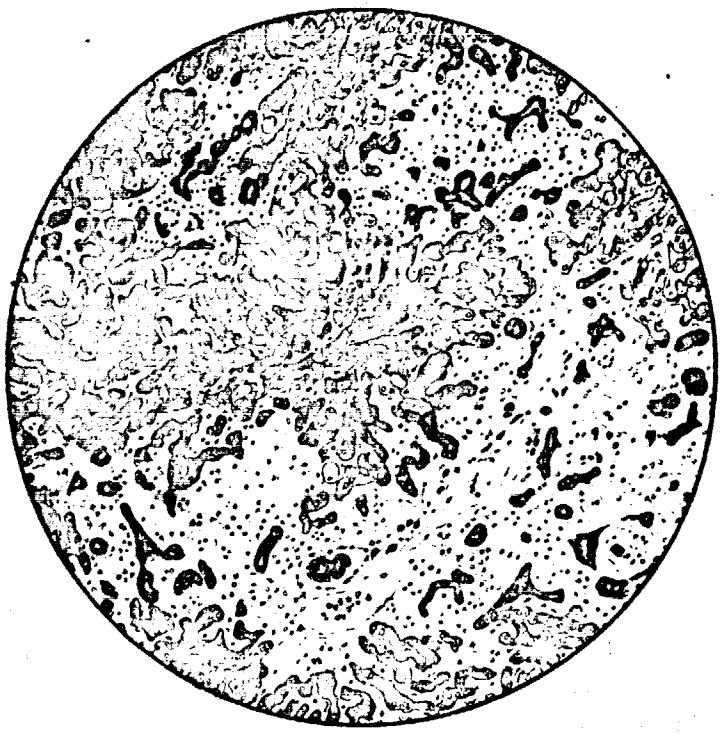

Fig. 4. Scale 110 : 1.

Stained iron-hæmatoxylin and van Gieson.

Liver of guinea-pig 5, which received subcutaneous injections of 3 mg. of manganous chloride for $3\frac{1}{2}$ weeks. The freshly formed fibrous tissue is most marked in the portal spaces; it should be compared with that shown in Fig. 6.

Findlay.

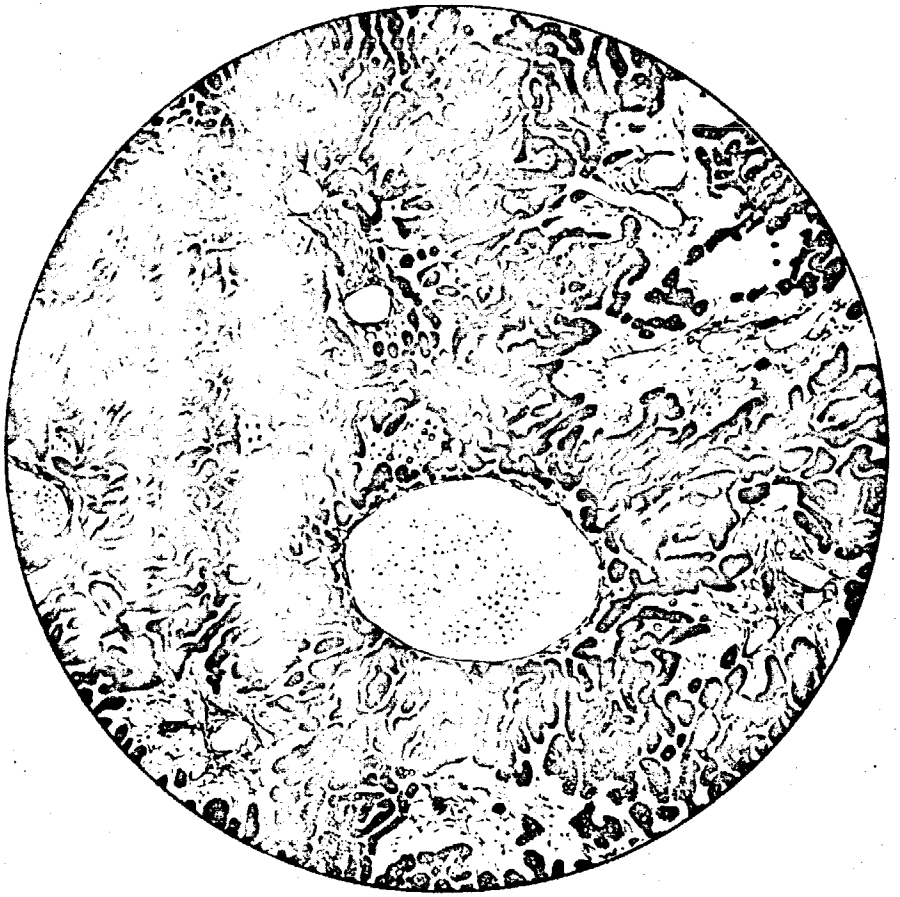

Fig. 5. Scale 130 : 1.

Stained iron-hæmatoxylin and van Gieson.

Liver of rat 3, which had received subcutaneous injections of 3 mg. of manganous chloride for $4\frac{1}{2}$ weeks. Note the early formation of fresh fibrous tissue in relation to the portal spaces.

Findlay.

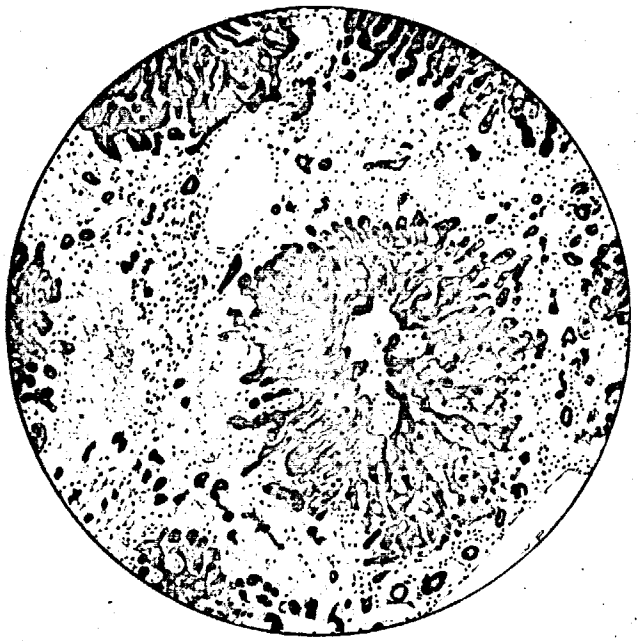

FIG. 6. Scale 40 : 1.

Stained iron-hæmatoxylin and van Gieson.

Liver of rat 4, which had received subcutaneous injections of 3 mg. of manganous chloride for 7 weeks. The freshly formed fibrous tissue forms a complete ring round the periphery of the lobules.

Findlay.

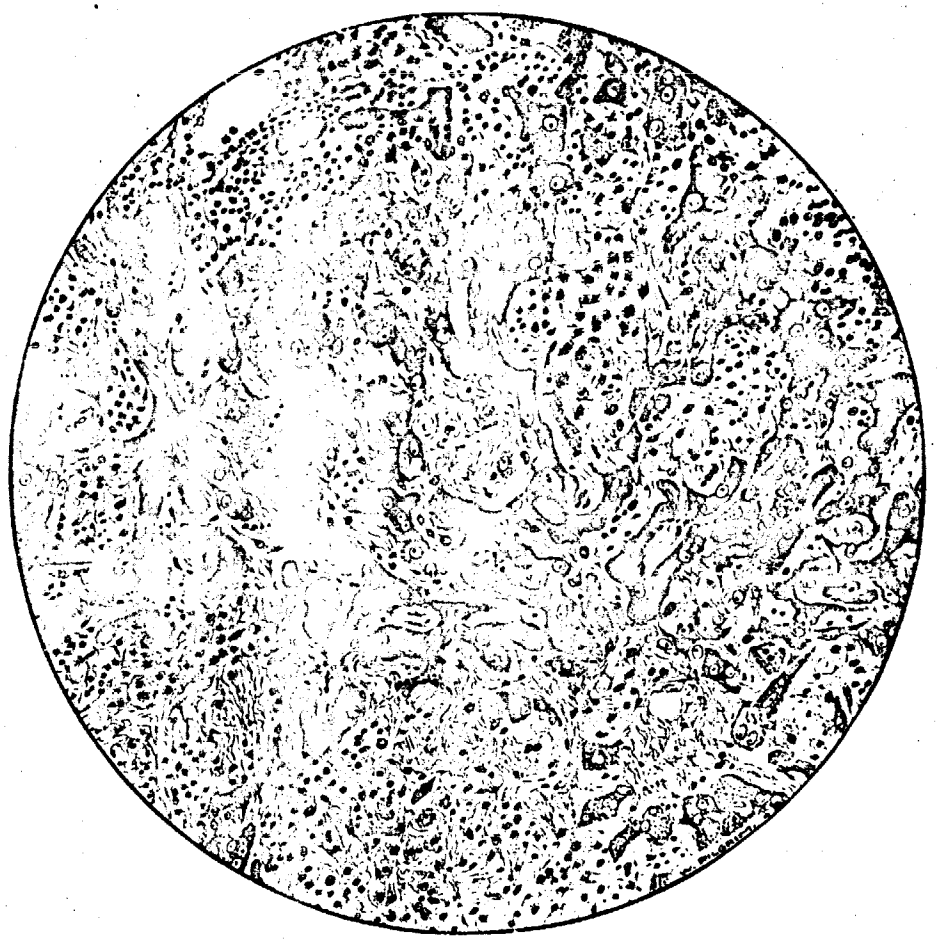

FIG. 7. Scale 40 : 1.

Stained iron hæmatoxylin and van Gieson.

Liver of rat 9, which had received subcutaneous injections of 3 mg. of manganous chloride for 12 weeks. The freshly formed fibrous tissue has invaded the lobule. Note the hypertrophic liver-cells.

Findlay.

colour with reddish mottling. The other organs appeared macroscopically normal. Microscopically, in the liver there was a moderate degree of cirrhosis confined to the periphery of the lobules, similar to that described in rat 4.

An attempt was also made to produce a similar cirrhotic condition in rats by feeding them on manganous chloride.

Twelve rats were given from 0·3–0·4 gm. of manganous chloride per day in addition to their ordinary diet. The animals remained in good condition, and in many cases continued to increase in weight during the course of the experiment. Death usually took place after from six to ten weeks, and in every case a certain degree of cirrhosis of the liver was found. The histological appearances were entirely similar to those described in the rats which had been injected with manganous chloride. In three of the rats fed on manganous chloride there was rigidity of the limbs for some days before death, while in all jaundice was noted at the post-mortem.

The livers of a number of control rats kept under similar conditions were entirely free from fibrosis.

CONCLUSIONS.

When injected subcutaneously in large doses manganous chloride is extremely toxic to the parenchyma of the liver and kidney. In small repeated doses its toxic action is chiefly exerted on the liver. It has been possible to produce by repeated subcutaneous injections of manganous chloride an experimental form of liver cirrhosis in rabbits, rats and guinea-pigs, while a similar condition has also been brought about in rats by the oral administration of the same salt. In its earliest stages the fibrous overgrowth in the liver is confined to the periphery of the lobules in relation to the portal spaces, the type of cirrhosis being very definitely monolobular. In its later stages the freshly formed fibrous tissue invades the whole of the lobules. Jaundice is often present. A possible clue to this action of manganous chloride may be found in the fact that manganese salts are very largely excreted from the body by means of the bile.

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Treatment of "rheumatic" endocarditis with manganese chloride by the Walbum method

by

Viktor Gorlitzer.

Among septic diseases endocarditis rightly occupies a special place. Endocarditis - and here we will only report on the group known as "rheumatic" endocaridits - is a pathologic state the development of which can only be regarded as being due to the introduction into the heart of inflammatory agents from inflamed tissues through the blood, via the large circulation and the coronary circulation. Even though this disease is caused by inflammatory agents, the reaction of the blood is very different from that observed in almost all other septic diseases. The blood picture, which almost always reveals distinct proliferation of leukocytes, leftward displacement and toxic granulation of neutrophile leukocytes in spetic process, shows no leukocytosis or only slight leukocytosis in endocarditis. Among neutrophiles, which relatively often are not increased either, there are not many immature forms. A particularly striking feature is the absence or slight degree of toxic granulation; eosinophile leukocytes can frequently be detected, unlike in other toxic diseases. Thus a drop of blood taken from the finger tip shows the blood picture of an apparently healthy person, almost a picture of "profound tranquility of health", yet on the inside membrane of the heart a very serious inflammation is taking place. However, according to Naegeli (1) this inflammation, in contrast to the large

absorption surface in pneumonia, abscesses, empyema, suppuration of serous membranes, has very small absorption surfaces, which bring about the discharge of inflammatory products into the surrounding area of the blood. Gloor (2), who has studied the qualitative changes of leukocytes in septic diseases with particular thoroughness, found that for instance in endocarditis lenta, despite the severe septic disease, the pathologic, toxic alterations of leukocytes were very slight and he attributed the absence of toxic granulation to the peculiarity of the inflammatory foci. These are small in endocarditis and they are further distinguished by the fact that they reveal only slight round cell infiltration and they do not suppurate. "The leukocytes have no opportunity to go into action as combatting or defensive cells, and this is why they are little altered". Thus it would seem that the inflamed internal membrane of the heart only produces small quantities of inflammatory products and discharges only small quantities into the circulation. These inflammatory products act as endogenously formed stimulants for the defensive systems against infection known at present. The stimulus which advances from the inflamed endocardium to the sites of formation of defensive bodies (leukocytes, reticuloendothelial system), thus appears only slight, with an insufficient alerting effect, and the antibodies mobilized by this stimulus are often inadequate. The slight reaction of the leukocyte system appears to support this view. Therefore a treatment which would add an exogenous stimulus to an apparently insufficient endogenous stimulus would assume particular importance. This stimulant therapy, however, from the onset runs into an obstacle which is very difficult to overcome.

This is the problem of dosage. The effectiveness of stimulant therapy is determined by an optimal dose, a more or less narrow zone of optical concentration. The optimal dosage (concentration) of the stimulant should reach the optimal mobilization of the defensive systems. This optimal concentration is a function of the severity of the infection on the one hand, and the resistance of the infected subject on the other (Walbum, 3). The varying intensity of the infection is caused by the difference in the quality, the quantity and the virulence of the pathologic agents that have penetrated. These factors have their own characteristics in each individual case of illness and they cannot be influenced or mitigated in advance, before the pathogenic agent breaks into the organism - unless by prophylaxis or immunization. Therefore we are confronted with the task of intensifying the resistance of the affected organism as effectively as possible. The forces of resistance are subject to variations, both physiologic and pathologic. By determination of the opsonic index at various times of the day the present author (6) has investigated the daily variations in the opsonic force. Both in patients with various diseases and in patients suffering from septic diseases the opsonic index in the majority of cases was relatively high in the morning after refreshing sleep, and relatively low after larger meals, that is after luncheon and in the evening. Based on the relative elevation of the opsonic force in the morning, by analogy we concluded a relative increase in defensive bodies in the morning. In order to mobilize as many defensive bodies as possible and in order to achieve a particularly favorable situation for the affected organism by a significant preeminence of the forces of

resistance towards the pathologic agents, recently we have always administered the stimulant to the patient in the morning. It appears that better results can be achieved in this manner. This measure is recommended regardless of the nature of the stimulant used. So far as the selection of the stimulant to be used is concerned, we first of all used the agents most commonly employed in this country, that is colloid silver solutions and dye solutions. Good results were repeatedly obtained with these preparations. However, some apparently resistant cases led us to seek other agents. In metal salt therapy according to Walbum's method we found what we believe to be a valuable aid and used it particularly in the form of a 0.02 mol solution of manganese chloride. The good solubility of this crystalloid, its evenly diluted solutions distinguish this preparation by an always uniform concentration and exact dosages resulting therefrom. The extremely broad experimental foundations of metal salt therapy laid down by Walbum led us to attach considerable hope to the testing of these agents, a hope which was not disappointed.

As preparation in case of insomnia we administered Pantopen 0.02 rectally or subcutaneously on the evening before the metal injection and on the following morning between 7 and 8 we administered up to 1.5 cc, but as a rule 1.0 cc of a 0.02 mol solution of manganese chloride intravenously. The patient, who took no food during the night, remained fasting until the injection and during the forenoon only received tea with a small amount of cognac to overcome the feeling of hunger. Luncheon, which consisted of easily digestible foods, was served as late as possible

(in the first hours of the afternoon). In order to keep the patient as quiet as possible, the injection was usually given on a day without visitors, or else visitors to the patient were asekd to stay only briefly with patients on the day of the injection. Success was striking on repeated occasions.

Case 1. 20 year old female patient, K.W., became ill on Dec. 30, 1930 with tonsillitis and fever. Fever persisted after that date. Since the patient suffered pains in the lumbar regions and attacks of severe palpitations in the middle of January 1931, she was admitted on January 16th to the Ist Internal Medicine Service of the Vienna City Hospital.

Current status: Powerful bone and muscle structure, pallor, moist, coated tongue, throat mucosa reddened, tonsils cherry-size, no visible pus. Heart: heart beat over 5th intercostal space, median clavicular line, left sternal border, lower IIIrd left rib, systolic murmur over the apex, otherwise heart sounds clear. Right lumbar region slightly sensitive to percussion. Urine: albumin negative, normal in other respects as well. 10,200 leukocytes, 3% stabs, 72% polymorph neutrophile leukocytes, 18% lymphocytes, 3% monocytes, 2% eosinophiles, 2% basophile leukocytes.

1.0 cc of a 0.02 mol solution of manganese chloride was administered on January 19th. From January 20th to January 29th the temperature curve showed a continuous drop. On this and the following day there was a brief elevation of temperature up to 38.2° with the onset of menstruation, but this subsided spontaneously after 2 days down to subfebrile temperatures. On February 14th, 1931 the patient was released free of symptoms.

Reference was made at the beginning to the special nature of the blood picture in "rheumatic" endocarditis compared to other septic diseases. Now, on the basis of recent new findings, "rheumatic" endocarditis can even more than before be give a special place among bacteremias. Attempts have been made for years to cultivate the pathogenic agent of this disease from the blood of patients with "rheumatic" endocarditis on bouillon, and blood and serum agar media. While in all other septic diseases a varying percentage of positive cultures were obtained (streptococci, staphylococci, coli bacilli), so far it has not been possible to obtain convincing bacterial cultures from "rheumatic " endocarditis.

The works of Reitter (4) referring to the close relationship between rheumatic fever and tuberculosis suggest the extreme probability of important etiologic links between acute "rheumatic" polyarthritis and tuberculosis resulting from the discovery by Reitter of tubercle bacilli in punctates from acutely inflamed joints and from the successful culturing of tubercle bacilli from the blood of patients with acute polyarthritis (Reitter and Löwenstein, 5). Bearing these new results in mind, in "rheumatic" endocarditis, which develops as a sequel to "rheumatic" polyarthritis and then frequently dominates the clinical picture, a search should also be made for tubercle bacilli in the blood. Recently Löwenstein has in fact repeatedly found tubercle bacilli in the blood in cases of "rheumatic" endocarditis.

A particularly severe case of "rheumatic" endocarditis belonging in this category was admitted on April 23, 1931 to the Ist Internal Medicine Service.

Case 2, Sch. M., 20 year old female patient, has suffered from inflammation of the joints since her 6th year. From January 12 to February 28, 1929 the patient was already hospitalized for heart symptoms due to a heart defect. No fever during that period with the exception of one day. After release from the hospital the patient had frequent sore throats. In the middle of April 1931 the patient felt very tired, had a slight sore throat, followed by back pains and a general feeling of oppression, particularly in the chest area.

Present status (April 23, 1931): subicterus of sclera, cyanosis of the lips, pallor,poor nutritional state, tongue moist, coated, throat mucosa red, tonsils of medium size. Lungs: loud percussion sound, vesicular respiration. Slight bilateral basal bronchitic sounds. Heart: heart beat in the 6th intercostal space, almost in left anterior axilla, upper/IIIrd left rib, right sternal border, tympanic first sound over apex, systolic murmur, rolling diastolic murmur, dull sound over the base, arrhythmic pulse rate of up to 112. Left hand joint red, swollen, painful, other joints free. Liver: 3 fingers' breadth under the costal arc. Spleen normal. Kidneys: not sensitive to percussion, no edema. Urine: albumin weakly positive; sparse leukocytes, epithelia in sediemnt, no erythrocytes, no cylinders. Increased urobilinogen. 8800 leukocytes, 2% stabs, 73% polymorph neutrophile leukocytes, 18% lymphocytes, 6% monocytes, 1% eosinophile leukocytes. April 24: proximal interphalangeal joint of the right big toe red, swollen, painful. April 26: slight swelling of

left knee. Until early April 27 continuous fever of up to 39°C. On April 27 at 7:30 1 cc of a 0.02 mol solution of manganese chloride was administered intravenously. Fever and all symptoms disappeared since April 27. Blood culture for tubercle bacilli according to Löwenstein on April 24 gave a positive result 15 days after beginning of cuture. Since then 9 blood cultures for tubercle bacilli on May 7, May 13, May 20, May 26, June 3, June 10, June 26, July 8, July 27, in other words up to July 17, made at intervals of 1-2 weeks, remained negative. The patient remained free of fever until May 5. On this day she developed pains in the left elbow joint; temperature rose again (although only to 37.9°). On May 8 at 7:20 the patient again received 1 cc of the 0.02 mol solution of manganese chloride. Fever once again disappeared until June 9. Because of the reappearance of fever up to 38.1°, on June 8 at 7:15 the patient once again received 1 cc of the 0.02 ml manganese chloride solution intravenously. This was followed by disappearance of fever, weight increase of 5 kg and permanent feeling of well-being until release from hospital on July 29.

The success achieved in this patient appears all the more remarkable since the patient was admitted in an extremely critical condition.

On the basis of the bacteriologic findings we believe that in this case we were able to unmask the otherwise so invisible enemy and render it harmless. This assumption is further supported by the fact that the patient presented herself at the outpatient clinic at the end of September 1931. She reported that after her

release from hospital she had never had any fever, had felt continuously well and had gained further weight. Considered from this angle, manganese chloride treatment assumes far greater importance. The favorable results obtained with manganese chloride in such "rheumatic" endocarditis, considered retrospectively, could be due to a suppression of the tubercular infection, thus confirming the elective curative effect of manganese chloride against tubercular infections in animal experiments (Walbum).

Tubercle bacilli were detected by Löwenstein in the circulating blood in 2 other patients with "rheumatic" endocarditis (case 3, T.D., 24 years old and case 4, J.A., 16 years old). Both patients received 2 manganese chloride injections and reacted well; both had left the hospital free of fever and recovered, before the results of the blood cultures were known.

One patient (case 5, B.A., 28 years old, recurrent endocarditis, stenosis of left ostii venosi, mitral valve insufficiency, stenosis of left ostii arteriosi, insufficiency of semilunar aortic valves) had persistent fever and was in a perilous state. Despite the administration of colloid silver rectally, 2.0 g quinine, up to 1.2 g amidopyrine, the fever persisted. Next manganese chloride was administered. After the second manganese chloride injection there was a distinct drop in temperature, finally the fever disappeared and the patient improved. The patient, who was followed as an outpatient for 6 months, has since remained continuously free of symptoms provided she avoids physical strain.

Manganese chloride administered intravenously in the morning produced good results in 11 other cases of endocarditis. The drug was taken 9 times in one case, 4 times in another case, twice in 6 cases and once in 3 cases.

The fact that we administered manganese chloride even in severest cases explains that 3 cases must be reported in which the patient died of cardiac insufficiency or some other complication, even though long after the last manganese chloride injection.

Case 17. The 55 year old male patient P.J. had had joint pains following a cold while in military service in 1916. He took cures at thermal baths. At the beginning of Janurary 1930 several joints were again swollen. Admitted to hospital on January 20, 1930. Present status: pallor, subicterus of sclera and the common integument. Cyanosis of the lips, powerful bone and muscle structure. Tongue moist, with thick white coating, throat mucosa dark red. Blood pressure 110 mm Hg. Heart: heartbeat in 6th intracostal space, 1 finger's breadth outside the median clavicular line, in the middle of the sternum, lower IIIrd left rib, first sound not clear over the apex. Later cardiac findings: tympanic first sound over the apex, low diastolic rolling murmur, arrhythmia. Upon admission redness, swelling and painfulness of both hand joints, the right knee and ankle joint. Fever over 38°. A single injection of manganese chloride (1 cc of a 0.02 mol solution) was administered on January 21st at 7:30. Absence of fever from this day onward. On March 17th sudden development of right hemiplegia. An embolism of the artery of the left sylvian fossa was assumed.

Death on March 20. The autopsy, performed by Prof. Erdheim, M.D., revealed mitral buttonhole and mitral insufficiency with extreme calcification of the entire valvular ring and insignificant recent verrucose endocarditis. Small thrombus in left heart tube. Extreme hypertrophy of right heart ventricle, severe brown induration, congestion of the liver, very slight congestion of the liver, spleen. Acute enteritis of the colon. No edema. Completely new encephalomalacia following embolism in the left artery of the sylvian fossa.

Case 18. 31 year old female patient P.A. had suffered from rheumatic fever in 1916, 1921 and 1927. A heart defect was already detected in 1916. At the beginning of February 1931 she had a sore throat and fever (40°), 8 days later inflammation of all joints of both hands and the right knee. Admitted to hospital on February 14, 1931. Present status: redness, swelling, pain in joints of both hands, the metacarpophalangeal joints on both sides, later the left elbow and knee joint were affected. Tonsils plum-size, no visible pus. Heart: heart beat in 5th intercostal space, 1 finger's breadth beyond the median clavicular line, lower IIIrd rib, middle of the sternum; raw, scratching systolic murmur over the base, particularly over the upper part of the corpus sterni, decreasing in intensity towards the apex. Lungs: vesicular respiration, no accessory sounds, loud percussion sound. Reduced chlorides in urine, increased leukocytes in sediment. Culture: coli bacilli. February 17: heart: blowing systolic murmur over upper part of sternum (timbre of the murmur changed). Feb. 20: Lungs: right bottom rear, penetrable dullness over area 3 fingers' breadth wide, distinct small-vesicular and crepitating

sounds; left bottom rear, bronchial respiration, slight crepitation. February 27: dullness more pronounced at right bottom rear over area 4 fingers' breadth wide, weaker respiration over this area. Pleura: puncture yielded ca. 3 cc slightly cloudy serous exudate. Rivalta ++, culture sterile. Diagnosis: rheumatic fever, endocarditis, bilateral infiltration of lower pulmonary lobes, right exutative pleurisy. Aspirin ineffective. Manganese chloride administered in doses of 1 cc of a 0.02 mol solution on February 23 and 27 led to brief drop in temperature. Pulmonary symptoms gradually increased and despite the administration of cardiac-active agents, cardiac insufficiency developed. On March 2, bronchitic sounds over entire lung. On March 14 collapse, pulmonary edema, death. Because of the sudden collapse the possibility of an embolism was considered in a branch of the pulmonary artery. The autopsy, performed by Dr. Feyrter, revealed the following: concretio cordis of entire pericardium. Recurrent verrucose endocarditis of the aortic and mitral valves, valvular insufficiency, stenosis of the mitral ostium. The verrucose deposits could be stripped off, although with difficulty. Excentric hypertrophy of the heart in every part. Bilateral cloudy serous effusion in free pleural cavity. Purulent bronchitis, edema of the lungs. Fresh foci of lobular pneumonia in all lobes of the lungs, partly confluent in left upper lobe. Compression atelectasia in basal portion of both lower lobes. Chronic cystitis.

Female patient, W.M., 25 years old, had had rheumatic fever at the age of 13. A cardiac defect was discovered four years ago. On April 2, 1931 the patient had a sore throat and fever up to

39.5. Admitted to hospital on April 30, 1931. This patient also suffered from endocarditis. On May 10, penetrable dullness over the left, bottom rear portion of the lungs, and at the same time a small-vesicular crepitating sound. The patient received 4 injections of 0.02 mol manganese chloride solution in a dose of 0.8 to 1.5 cc. The last injection was given on May 15. The patient's fever disappeared, but the pulmonary complications persisted and despite the administration of cardiac-active drugs it was not possible to overcome the gradually developing cardiac insufficiency. The patient died on May 29, The autopsy, performed by Dr. Löw-Beer, revealed the following: recurrent endocarditis of the mitral valve with mitral buttonhole and valvular insufficiency. Small verrucae in a state of development on the aortic valves. Dilatation of the left atrium and severe excentric hypertrophy of the right ventricle. Extreme congestion of the lungs, multiple small earlier infarctions in the left lower lobe with old, softened thrombi in the left peroneal vein. Unfortunately the mediastinal and peribronchial lymphnodes were not examined.

In the 3 cases described above, the endocarditis was in the course of development. Since aside from aspirin or Atophanyl and cardiac-active agents only manganese chloride was administered and this agent was the only stimulant given against endocarditis, it is possible that the development of the endocarditis was caused by the effect of the manganese chloride. Case 17, in particular, appears to support this view, in which after a single injection of manganese chloride 8 weeks before death a prompt and continuous loss of fever was achived. This patient, who could be regarded as convalescent, died suddenly and was autopsied (3 days after

the appearance of the paralysis) as a result of the embolism in the left artery of the sylvian fossa. Aside from insignificant recent verrucose deposits there was an abundance of old scar tissue on the mitral valve.

The dosage of the stimulant therapy remains an unsolved problem, since at present we are still not in a position to know the desirable optimal dose in individual cases. Therefore, since it is impossible to carry out experiments in series on individuals of roughly the same constitution, characteristics and an infection of the same intensity, and one must on the contrary expect to find considerable diversity of these factors, we must limit oursevles to collecting experience with dosages of an agent which seems to us effective. Possibly this contribution to the results of empirical observation will make it possible to give a dose of a particularly effective agent very close to the optimal concentration.

The treatment of "rheumatic" endocarditis, even the severest forms, with manganese chloride, seems to us very promising, the more so since this preparation worked in a case in which treatment with colloid silver had been ineffective.

Summarizing the results, we observed a good effect of intravenously administered manganese chloride injections according to Walbum in 17 cases of "rheumatic" endocarditis.

Figures

1.K. Waltraude, case 1, temperature curve.

2-4. Marie Sch., Case 2, temperature curves 1, 2 and 3.

Lösung = solution.

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See p. 111.

Wien. Klin. Wchnschr. 45: 107-11, 1932

Aus der I. internen Abteilung des Krankenhauses der Stadt Wien. (Vorstand: Prof. C. Reitter)

Ein Beitrag zur Behandlung der Endocarditis „rheumatica" mit Manganchlorid ad modum Walbum

Von Dr. Viktor Gorlitzer, Assistent

Unter den septischen Erkrankungen gebührt der Endokarditis mit Recht eine Ausnahmsstellung. Die Endokarditis — hier soll nur von jener Gruppe berichtet werden, welche „rheumatische" Endokarditis genannt wird — bietet

einen Krankheitszustand, dessen Entstehung nicht anders als durch Verschleppung von Entzündungserregern aus entzündeten Geweben auf dem Wege der Blutbahn, auf dem Wege des großen Kreislaufsystems und Koronarkreislaufsystems in das Herzinnere verursacht, gedacht werden kann. Obwohl also diese Erkrankung durch Entzündungserreger entstanden ist, verhält sich die Blutreaktion dieser Krankheit sehr verschieden von fast allen anderen septischen Erkrankungen. Das Blutbild, welches bei septischen Prozessen fast immer eine deutliche Vermehrung der weißen Blutkörperchen, Linksverschiebung und toxische Granulation der neutrophilen Leukozyten aufweist, zeigt bei der Endokarditis keine oder meist nur eine mäßige Leukozytose; unter den Neutrophilen, welche auch relativ oft nicht vermehrt sind, finden sich nicht reichlich Jugendformen; besonders auffallend ist das Fehlen oder die geringe Ausbildung der toxischen Granulation; eosinophile Leukozyten sind zum Unterschied von anderen septischen Erkrankungen häufig nachweisbar. So zeigt der der Fingerbeere entnommene Blutstropfen das Blutbild eines scheinbar gesunden Menschen, fast ein Bild „tiefen gesundheitlichen Friedens", und doch brennt auf der inneren Haut des Herzens eine sehr folgenschwere Entzündung. Diese Entzündung hat aber nach Naegeli[1] im Gegensatz zu den großen Resorptionsflächen bei Pneumonie, Abszeß, Empyem, Eiterungen von serösen Häuten nur sehr kleine Resorptionsflächen, welche die Abgabe der Entzündungsprodukte an das umgebende Gewebe, bzw. an das Blut vermitteln. Gloor,[2] welcher die qualitativen Veränderungen der Leukozyten bei septischen Erkrankungen besonders eingehend studierte, fand z. B. bei Endocarditis lenta trotz der schweren septischen Erkrankung die pathologische, toxische Leukozytenveränderung sehr gering und führte das Fehlen der toxischen Granulation auf die Besonderheit der Entzündungsherde zurück. Diese sind bei der Endokarditis klein und sind außerdem dadurch ausgezeichnet, daß sie nur geringe Rundzelleninfiltrationen aufweisen und nicht vereitern. „Die Leukozyten haben gar keine Gelegenheit, als Kampf- und Abwehrzellen in Aktion zu treten, darum sind sie auch nur wenig verändert." Es dürften demnach von der entzündeten Herzinnenhaut nur geringe Mengen von Entzündungsprodukten gebildet und in die Blutbahn abgegeben werden. Diese Entzündungsprodukte wirken als endogen gebildete Reizkörper für die bisher bekannten Infektionsabwehrsysteme. Der Reiz, welcher von dem entzündeten Endokard zu den Abwehrkörperbildungsstätten (leukozytäre, retikuloendotheliale Systeme) dringt, scheint daher nur gering, nur ungenügend alarmierend zu wirken, die durch diesen Reiz mobilisierten Antikörper werden oft unzureichend sein. Die geringe Reaktion des Leukozytensystems scheint diese Auffassung zu stützen. Deshalb könnte einer Behandlung, welche zu dem anscheinend zu geringen endogenen Reiz einen exogenen Reiz hinzufügt, eine besondere Bedeutung zukommen. Diese Reizkörpertherapie stößt aber vom Anfang an auf ein sehr schwierig zu überwindendes Hindernis. Es ist dies die Frage der Reizdosierung. Die Wirksamkeit der Reizkörpertherapie ist durch eine optimale Dosis, eine mehr oder weniger schmale Zone optimaler Konzentration bedingt. Die optimale Dosierung (Konzentration) des Reizkörpers soll die optimale Mobilisierung der Abwehrkörper erreichen. Dieses Optimum der Konzentration ist abhängig von der Schwere der Infektion einerseits, von dem Widerstandsvermögen des Infizierten anderseits (Walbum).[3] Die verschiedene Heftigkeit der Infektion wird bedingt durch die jeweilige Verschiedenheit der Qualität, der Quantität, der Virulenz der eingedrungenen Krankheitserreger. Diese Faktoren sind für jeden einzelnen Krankheitsfall für sich charakteristisch, sie sind nicht im voraus — wenn nicht durch Prophylaxe, Immunisierung — vor dem Krankheitserregereinbruch in den Organismus zu beeinflussen, zu mildern. Es ergibt sich daher die Aufgabe, das Widerstandsvermögen des befallenen Organismus möglichst wirksam zu steigern. Die Widerstandskräfte sind Schwankungen — sowohl physiologischen als auch pathologischen — unterworfen. Durch Bestimmung des opsonischen Index zu verschiedenen Tageszeiten habe ich[6] die Tagesschwankungen der opsonischen Kraft untersucht. Sowohl bei Pat. mit verschiedenen Krankheiten als auch bei an septischen Krankheiten Leidenden war der opsonische Index in der Mehrzahl der Fälle am Morgen nach erfrischendem Schlafe relativ erhöht, nach größeren Mahlzeiten, also nach dem Mittagessen und am Abend relativ erniedrigt. Auf Grund der relativen Erhöhung der opsonischen Kraft am Morgen habe ich per analogiam auf eine relative Vermehrung der Abwehrkörper am Morgen geschlossen. Um so viel als möglich Abwehrkörper zu mobilisieren, um durch ein bedeutendes Ueberwiegen der Widerstandskräfte gegenüber den Krankheitserregern für den befallenen Organismus eine besonders günstige Situation zu erreichen, habe ich in letzter Zeit den Reizkörper stets am Morgen dem Kranken einverleibt. Es scheint, daß dadurch bessere Resultate erzielt werden können. Diese Maßnahme ist — was immer für ein Reizkörper auch gewählt werden mag — empfehlenswert. Was nun die Wahl des zu verwendenden Reizkörpers anbetrifft, so haben wir zunächst die in unserem Lande hauptsächlichst gebrauchten Mittel, das sind kolloidale Silberlösungen, Farbstofflösungen, benützt. Und wir haben mit diesen Präparaten wiederholt gute Resultate erhalten. Manche anscheinend refraktäre Fälle veranlaßten uns aber nach anderen Mitteln zu suchen. Wir fanden in der Metallsalztherapie ad modum Walbum eine, wie wir glauben, besonders wirksame Helferin und verwendeten im besonderen 0·02 mol. Lösung von Manganchlorid. Das gute Lösungsverhältnis dieses Kristalloids, die gleichmäßig verdünnten Lösungen von Manganchlorid zeichnen dieses Präparat durch seine stets gleichmäßige Konzentration und die dadurch bedingte genaue Dosierbarkeit aus. Die von Walbum geschaffene, außerordentlich breite experimentelle Grundlage der Metallsalztherapie ließ uns mit besonderer Hoffnung an die Erprobung dieses Mittels herangehen; eine Hoffnung, welche nicht enttäuscht wurde.

Als Vorbereitung gaben wir bei Schlaflosigkeit am Abend vor der Metallsalzinjektion meist Pantopon 0·02 rektal oder subkutan und verabreichten am nächsten Morgen zwischen 7 und 8 Uhr 0·8 bis 1·5 ccm, meist 1·0 ccm einer 0·02 mol. Lösung von Manganchlorid intravenös. Der Pat., welcher während der Nacht keine Nahrung genommen hatte, blieb bis zur Injektion nüchtern und bekam auch am Vormittag nur bei Bedarf zur Verjagung des Hungergefühls Tee mit etwas Kognak. Das Mittagessen, welches aus leicht verdaulichen Speisen bestand, wurde möglichst spät (in den ersten Nachmittagstunden) gegeben. Um dem Pat. möglichste Ruhe zu verschaffen, wurde die Injektion meist an einem „besuchslosen" Tag verabreicht oder die Besucher des Kranken aufgefordert, am Injektionstag nur kurze Zeit bei dem Pat. zu verweilen. Die Erfolge waren wiederholt sehr auffallend.

Fall 1. Die Pat. K. W., 20 Jahre alt, erkrankte am 30. Dezember 1930 an Angina tonsillaris, Fieber. Sie blieb seither fiebernd. Da Mitte Jänner 1931 Schmerzen in beiden Lendengegenden

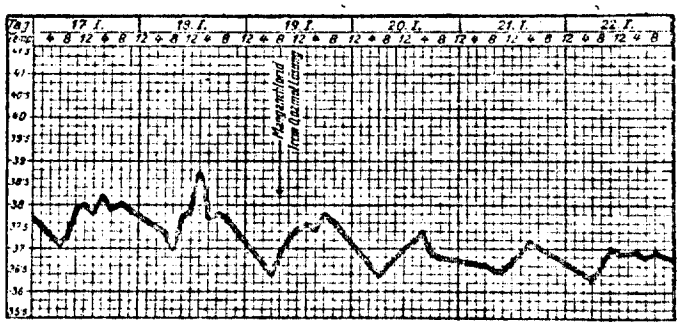

Abb. 1. K. Waltraude, Fall 1, Temperaturkurve

und anfallsweise starkes Herzklopfen auftraten, wurde die Pat. am 16. Jänner auf die I. interne Abteilung des Krankenhauses der Stadt Wien eingeliefert.

Status praesens: Kräftiger Knochen-Muskelbau, Blässe, Zunge feucht, belegt, Rachenschleimhaut gerötet, Tonsillen kirschengroß, Eiterpfröpfe nicht sichtbar. Cor: Ictus im 5. I. C. R., in der Mclv.-L., l. St. R., u/III. linken Rippe, über der Spitze ein systolisches Geräusch, sonst reine Töne. Rechte Lendengegend leicht klopfempfindlich. Harn: Albumen negat., auch sonst o. B. 10.200 Leukozyten, 3% stabkernige, 72% segmentkernige neutrophile Leukozyten, 18% Lymphozyten, 3% Monozyten, 2% eosinophile, 2% basophile Leukozyten.

Manganchlorid, 1·0 ccm einer 0·02 mol. Lösung wurde am 19. Jänner verabreicht. Die Temperaturkurve zeigt ab 20. Jänner eine bis zum 29. Jänner dauernde Entfieberung. An diesem und am nächstfolgenden Tage ist mit Eintritt der Menstruation noch eine kurzdauernde Temperatursteigerung bis 38·2° festzustellen, welche nach 2 Tagen bis auf subfebrile Temperaturen spontan zurückgeht. Am 14. Februar 1931 wird Pat. ohne Beschwerden entlassen.

Wenn wir eingangs auf eine Besonderheit des Blutbildes bei Endocarditis „rheumatica" gegenüber den anderen septischen Erkrankungen hingewiesen haben, dann können wir auf Grund von neuen, in letzter Zeit erhobenen Befunden um so mehr eine Sonderstellung der Endocarditis „rheumatica" unter den Bakteriämien fordern. Jahrelang wurde versucht, mit Hilfe von Bouillon-Anreicherung, mit Hilfe von Blut-, Serum-Agar-Nährboden aus dem Blute von Endocarditis „rheumatica"-Kranken die für diese Krankheit pathogenen Erreger zu züchten. Wenn bei allen anderen septischen Erkrankungen ein mehr oder minder hoher Prozentsatz von positiven Kulturen (Streptokokken, Staphylokokken, Bacterium coli) erreicht wurde, konnte bei Endocarditis „rheumatica" keine bisher überzeugende Bakterienkultur erzielt werden.

Hatten die Arbeiten von Reitter[4] auf enge Beziehungen des akuten Gelenksrheumatismus zur Tuberkulose aufmerksam gemacht, dann wurden wesentliche und ätiologische Zusammenhänge der Polyarthritis „rheumatica" acuta mit der Tuberkulose durch das Auffinden von Tbc.-Bazillen im Punktat von akut entzündeten Gelenken durch Reitter und schließlich durch das Gelingen von Kulturen von Tuberkelbazillen aus dem Blute von Polyarthritis acuta-Kranken (Reitter und Löwenstein)[5] außerordentlich wahrscheinlich. Diese neuen Forschungsergebnisse beachtend, mußte bei der im Gefolge der Polyarthritis „rheumatica" acuta auftretenden und dann oft das Krankheitsbild beherrschenden Endocarditis „rheumatica" auch bei dieser Erkrankung nach Tuberkelbazillen im Blute gefahndet werden. Tatsächlich ist in letzter Zeit wiederholt in Fällen von Endocarditis „rheumatica" von Löwenstein Tbc.-Bazillämie festgestellt worden.

Ein hieher gehörender, besonders schwerer Fall von Endocarditis „rheumatica" wurde am 23. April 1931 auf die erste interne Abteilung eingeliefert.

Fall 2. Pat. Sch. M., 20 Jahre alt, litt seit ihrem 6. Lebensjahr wiederholt an Gelenksentzündungen. Vom 12. Jänner bis 28. Februar 1929 stand Pat. wegen Herzbeschwerden auf Grund eines Herzfehlers bereits auf der hiesigen Abteilung in Behandlung. Damals mit Ausnahme eines einzigen Tages kein Fieber. Nach der Spitalsentlassung hatte die Pat. häufig Halsschmerzen. Mitte April 1931 fühlte sich die Pat. sehr müde, hatte etwas Halsschmerzen, bald traten Rückenschmerzen, ein allgemeines Unbehagen besonders auf der Brust auf.

Status praesens (23. April 1931): Subikterus der Skleren, Zyanose der Lippen, Blässe, reduz. Ernährungszustand, Zunge feucht, belegt, Rachenschleimhaut gerötet, Tonsillen mittelgroß. Pulmo: Lauter Klopfschall, vesikul. Atmen, basal bdsts. geringgradige bronchitische Geräusche. Cor: Ictus im 6. I. C. R. fast in der vord. Axill. L., o/III. link. Rippe, rechter Sternalrand, über der Spitze paukender 1. Ton, systol. Geräusch, rollendes diastol. Geräusch, dumpfe Töne über der Basis, Puls arhyth. bis 112. Linkes Handgelenk gerötet, geschwollen, schmerzhaft, übrige Gelenke frei. Hepar.: 3 Qnf. unter dem Rippenbogen. Lien: O, Renes: nicht klopfempfindlich, keine Oedeme. Harn: Albumen schwach positiv, im Sediment spärlich Leukozyten, Epithelien, keine Erythrozyten, keine Zylinder, Urobilinogen vermehrt. 8800 Leukozyten, 2% stabkernige, 73% segmentkernige neutrophile Leukozyten, 18% Lymphozyten, 6% Monozyten, 1% eosinoph. Leukozyten. 21. April: Das proximale Interphalangealgelenk des 3. linken Fingers und das Metatarsophalangealgelenk der rechten Großzehe gerötet, geschwollen, schmerzhaft. 26. April: Geringe Schwellung des linken Knies. Bis 27. April früh kontinuierliches Fieber bis 39° C. Am 27. April um 7½ Uhr wird 1 ccm einer 0·02 mol. Lösung von Manganchlorid intravenös verabreicht. Das Fieber und alle Beschwerden sind seit 27. April geschwunden. Die Blutkultur nach Löwenstein auf Tbc. am 21. April abgenommen, ergibt 15 Tage nach dem Anlegen der Kultur ein positives Resultat (rasches Wachstum). Blutkultur auf Tbc. am 29. April abgenommen, wurde 21 Tage nach dem Anlegen der Kultur positiv. Seither blieben alle 9 Blutkulturen auf Tbc., abgenommen am 7. Mai, 13. Mai, 20. Mai, 26. Mai, 3. Juni, 10. Juni, 26. Juni, 8. Juli, 17. Juli, also bis zum 17. Juli, in einem Zwischenraum von ein bis zwei Wochen

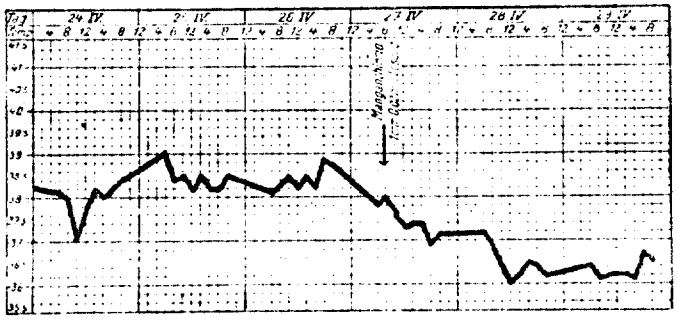

Abb. 2. Sch. Marie, Fall 2, Temperaturkurve 1

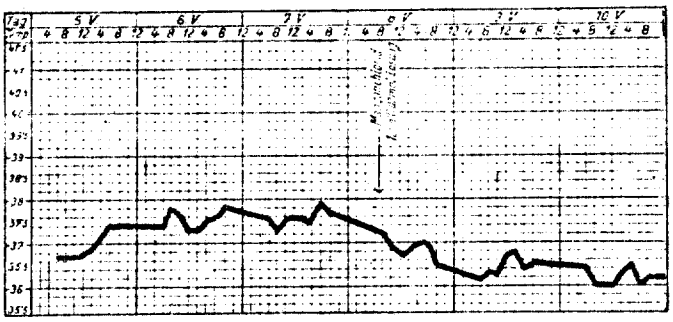

Abb. 3. Sch. Marie, Fall 2, Temperaturkurve 2

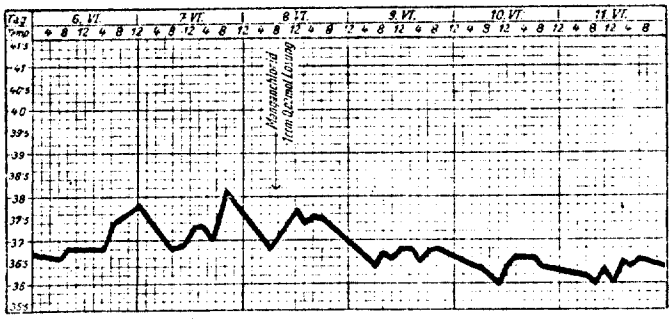

Abb. 4. Sch. Marie, Fall 2, Temperaturkurve 3

angelegt, negativ. Die Patientin bleibt bis 5. Mai entfiebert. An diesem Tage Auftreten von Schmerzen im linken Ellbogengelenk; neuerlicher Temperaturanstieg (allerdings nur bis 37·9°). Am 8. Mai um 7 Uhr 20 Min. wieder 1 ccm der 0·02 mol. Lösung von Manganchlorid. Wieder Entfieberung bis zum 6. Juni dauernd. Wegen wiederauftretendem Fieber bis 38·1° wird am 8. Juni um 7 Uhr 15 Min. nochmals 1 ccm der 0·02 mol. Manganchloridlösung intravenös injiziert. Darauffolgende Entfieberung, Gewichtszunahme von 5 kg und dauerndes Wohlbefinden bis zu der am 29. Juli erfolgenden Spitalsentlassung.

Der bei dieser Pat. erzielte Erfolg erscheint um so bemerkenswerter, als die Pat. in einem außerordentlich hinfälligen Zustand eingeliefert worden war.

Auf Grund der bakteriologischen Befunde glauben wir in diesem Falle den sonst so unsichtbaren Feind demaskiert und pathogen unwirksam gemacht zu haben. Diese Ver-

mutung wird noch dadurch gestützt, daß sich die Pat. Ende September 1931 in der Ambulanz der Abteilung wieder vorgestellt hat. Sie gibt an, nach der Spitalsentlassung nie mehr Fieber gehabt, sich stets wohlgefühlt und weiter an Gewicht zugenommen zu haben. Von diesem Gesichtspunkt aus betrachtet, gewinnt die Manganchlorid-Behandlung eine weit größere Bedeutung. Die mit Manganchlorid erzielten günstigen Resultate bei einer derartigen Endocarditis „rheumatica" könnten — retrospektiv beurteilt — auf einer Zurückdrängung der Tbc.-Infektion beruhen, die elektive Heilwirkung von Manganchlorid gegen Tbc.-Infektion im Tierexperiment (Walbum) bestätigend.

Außerdem wurden in weiteren 2 Fällen von Endocarditis „rheumatica" (Fall 3: T. D., 24 Jahre alt, und Fall 4: J. A., 16 Jahre alt) Tuberkelbazillen im strömenden Blute von Löwenstein festgestellt. Beide Pat. haben je 2 Manganchloridinjektionen erhalten und haben darauf gut reagiert; beide Pat. haben bereits, noch ehe uns die Bakkulturergebnisse bekanntgeworden waren, das Spital entfiebert und erholt verlassen.

Eine Pat. (Fall 5: R. A., 28 Jahre alt, Endocarditis recurrens, Stenosis ostii venosi sinistri, Insufficientia valvulae mitralis, Stenosis ostii arteriosi sinistri, Insufficientia valvularum semilunarium aortae) hatte kontinuierliches Fieber und zeigte einen gefährlichen Krankheitszustand. Trotz Verabreichung von kolloidalem Silber rektal, Chinin 2·0 g, Amidopyrin bis 1·2 g keine Entfieberung. Es wurde nun Manganchlorid gegeben. Nach der zweiten Manganchloridinjektion deutliche Temperatursenkung, schließlich Entfieberung und Besserung. Pat., nach einem halben Jahr in der Ambulanz der Abteilung untersucht, ist seither dauernd fieberfrei und bei Vermeidung von körperlicher Anstrengung beschwerdefrei.

Manganchlorid, am Morgen intravenös injiziert, ergab in weiteren elf Fällen von Endokarditis gute Erfolge. Und zwar wurde das Mittel in einem Falle neunmal, in einem Falle viermal, in sechs Fällen zweimal, in drei Fällen einmal einverleibt.

Da wir auch in den schwersten Fällen Manganchlorid verabreicht haben, erklärt es sich, daß wir auch über drei Fälle nun berichten werden, welche — allerdings lange Zeit nach der letzten Manganchlorid-Injektion — infolge von Herzinsuffizienz oder infolge einer anderen Komplikation gestorben sind.

Fall 17: Der 55jährige Pat. P. J. hatte im Jahre 1916 nach einer Verkühlung während des Militärdienstes Gelenksschmerzen; hernach Kuren in Heilbädern. Anfangs Jänner 1930 neuerliches Auftreten von mehrfachen Gelenksschwellungen. Spitalsaufnahme am 20. Jänner 1930. Status praesens: Blässe, Subikterus der Skleren und des allgemeinen Integuments, Zyanose der Lippen, kräftiger Knochen-Muskelbau. Zunge feucht, dick weiß belegt, Rachenschleimhaut düsterrot; Tonus: 110 mm Hg. Cor: Ictus im 6. I. C. R. 1 Qui. außerhalb der Mdelv.-L., Mitte des Sternum, u/III. link. Rippe, über der Spitze der 1. Ton unrein. Späterer Cor-Befund: paukender 1. Ton über der Spitze, leises diastol. Rollen, Arrhythmie. Bei der Einlieferung: Rötung, Schwellung, Schmerzhaftigkeit beider Handgelenke, des rechten Knie- und Sprunggelenkes. Fieber über 38°. Es wird eine einzige Injektion von Manganchlorid 1 ccm einer 0·02 mol. Lösung am 21. Jänner um 7½ Uhr verabreicht. Vom selben Tag an Entfieberung, nur eine einmalige Temperatursteigerung bis 37·5° am 26. Jänner, seither dauernd entfiebert. Am 17. März plötzliches Auftreten einer rechtsseitigen Hemiplegie. Es wird eine Embolia arteriae fossae Sylvii sinistri angenommen. Am 20. März: Exitus letalis. Die von Herrn Professor Dr. Erdheim durchgeführte Obduktion ergab Knopflochstenose der Mitralis und Insuffizienz der Mitralis mit enormer Verkalkung des ganzen Klappenrandes und unbedeutende rezente verrukose Endokarditis. Kleiner Thrombus im linken Herzohr. Enorme Hypertrophie des rechten Herzventrikels. Beträchtliche Herzfehlerdehnungen. Stauung der Leber, sehr geringe der Milz. Akute Enteritis des Dickdarmes. Kein Oedem. Ganz frische Enzephalomalazie bei Embolie in der linken Arteria fossae Sylvii.

Fall 18. Die Pat. P. A., 35 Jahre alt, litt in den Jahren 1916, 1921 und 1927 an Gelenksrheumatismus, schon im Jahre 1916 wurde ein Herzfehler festgestellt. Anfangs Februar 1931 Angina, Fieber 40°, 8 Tage später Entzündung sämtlicher Gelenke beider Hände und des rechten Kniegelenkes. Spitalsaufnahme am 14. Februar 1931. Status praesens: Rötung, Schwellung, Schmerzhaftigkeit beider Handgelenke, der Metakarpophalangealgelenke II. V. beiderseits, später wurden noch das linke Ellbogen- und Kniegelenk befallen. Tonsillen pflaumengroß, Eiterpfröpfe nicht sichtbar. Cor: Ictus im 5. I. C. R. 1 Qui. außerhalb der Mdelv.-L., u/III. link. Rippe, Mitte des Sternum, über der Basis, besonders über dem oberen Teil des Corpus sterni ein rauhes, kratzendes, systol. Geräusch, gegen die Spitze an Intensität abnehmend. Pulmo: vesikul. Atmen, keine Nebengeräusche, lauter Klopfschall, im Harn Chloride vermindert, im Sediment Leukozyten vermehrt; kulturell Bacterium coli. Am 17. Februar: Cor: Ueber dem oberen Teil des Sternums ein blasendes, systol. Geräusch (Timbre des Geräusches geändert). Am 20. Februar Pulmo: rechts, hinten, unten 3 Qui. breite, durchschlagbare Dämpfung, deutliches kleinblasiges und Knisterrasseln; links, hinten, unten bronchiales Atmen, spärliches Knistern. Am 27. Februar: Dämpfung rechts, hinten, unten intensiver, 4 Qui. breit, daselbst abgeschwächtes Atmen; Pleura-Probepunktion ergibt etwa 3 ccm leicht getrübtes, seröses Exsudat. Rivalta +++, kulturell: steril. Diagnose: Polyarthritis acuta, Endocarditis, Infiltratio lobi infer. pulmon. utriusque, Pleuritis exsudat. dextra. Aspirin ohne Effekt. Manganchlorid je 1 ccm einer 0·02 mol. Lösung, am 23. und am 27. Februar verabreicht, zeigt am 28. Februar eine kurzdauernde Temperatursenkung. Die pulmonalen Beschwerden nehmen allmählich zu und trotz reichlich verwendeter Cardiaca stellt sich alsbald eine Cor-Insuffizienz ein. Am 2. März über der ganzen Lunge bronchitische Geräusche. Am 14. März: Kollaps, Lungenödem, Exitus letalis. Wegen des plötzlich aufgetretenen Kollapses wurde noch an eine Embolie in einen Pulmonalisast gedacht. Die von Herrn Assistenten Dr. Feyrter vorgenommene Obduktion ergab: Concretio cordis cum pericardio totalis. Rekurrierende Endocarditis verrucosa der Aorten- und Mitralklappen, Insuffizienz der Klappen, Stenose des Mitralostiums. Die warzigen Auflagerungen abstreifbar, wenn auch nicht leicht. Exzentrische Hypertrophie des Herzens in allen seinen Teilen. Trüb-seröser Erguß im freien Brustfellraum beidseits. Eitrige Bronchitis, Oedem der Lungen. Frische lobuläre pneumonische Herde in allen Lungenlappen, im linken Oberlappen z. T. konfluierend. Kompressionsatelektase in den basalen Anteilen beider Unterlappen. Chronische Cystitis.

Fall 19. Die Pat. W. M., 25 Jahre alt, hatte im 13. Lebensjahr Gelenksrheumatismus. Vor 4 Jahren wurde ein Herzfehler festgestellt. Am 21. April 1931 Angina, Fieber bis 39·5°. Spitalsaufnahme am 30. April 1931. Auch diese Pat. litt an Endokarditis. Am 10. Mai trat über der Lunge links, hinten, unten eine durchschlagbare Dämpfung auf, daselbst kleinblasiges und Knisterrasseln. Die Pat. bekam 4 Injektionen von 0·02 mol. Manganchloridlösung in einer Dosis von 0·8 bis 1·5 ccm. Die letzte Injektion erfolgte am 15. Mai. Die Pat. wurde zwar fieberfrei, die pulmonale Komplikation blieb aber bestehen und trotz verabreichter Cardiaca konnte der allmählich einsetzenden Herzinsuffizienz nicht mit Erfolg entgegengewirkt werden. Die Pat. starb am 29. Mai. Die Obduktion, welche Herr Assistent Dr. Löw-Beer durchführte, ergab: Rekurrierende Endokarditis der Mitralis mit Knopflochstenose des Ostiums und Insuffizienz der Klappe. In Organisation befindliche Wärzchen auf den Aortenklappen. Dilatation des linken Vorhofes und beträchtliche exzentrische Hypertrophie des rechten Ventrikels. Enorme Stauung der Lungen, multiple kleine ältere Infarkte im linken Unterlappen bei älteren erweichten Thromben in den Wadenvenen (links). Konfluierende Lobulärpneumonie im linken Unterlappen. — Die mediastinalen und peribronchialen Lymphdrüsen wurden leider nicht untersucht.

In den eben beschriebenen drei Fällen war die Endokarditis in Organisation befindlich. Da außer Aspirin oder Atophanyl und Cardiaca nur Manganchlorid gegeben wurde, und dieses Mittel als einziger Reizkörper gegen die Endokarditis verabreicht worden war, könnte die Organisation der Endokarditis durch die Manganchloridwirkung bedingt worden sein. Besonders der Fall 17, bei welchem nach einer einzigen Manganchloridinjektion acht Wochen ante exitum prompte und dauernde Entfieberung erzielt worden war, scheint diese Annahme zu stützen. Dieser Kranke, welcher schon als Rekonvaleszent gelten konnte, kam infolge der Embolie in die linke Arteria fossae Sylvii plötzlich (drei Tage nach dem Auftreten der Lähmung) zur Autopsie. Neben unbedeutenden rezenten verrukösen Auflagerungen fand sich reichlich, allerdings auch älteres, Narbengewebe an der Mitralklappe.

Die Dosierung bei der Reizkörpertherapie bleibt nach wie vor eine ungelöste Frage, da wir bisher keineswegs in der Lage sind, für den Einzelfall die jeweilige, anstrebenswerte, optimale Dosis zu erkennen. Wir müssen uns daher darauf beschränken — da uns Reihenversuche mit Individuen von annähernd gleicher Konstitution, Disposition und gleich starker Infektion nicht möglich sind

und wir vielmehr mit großen Verschiedenheiten dieser Faktoren rechnen müssen —, Erfahrungen mit der Dosierung eines uns wirksam erscheinenden Mittels zu sammeln. Vielleicht wird durch dieses Beitragen der erreichten Resultate verschiedener Beobachter der Empirie es ermöglicht, dereinst eine dem Konzentrationsoptimum sehr naheliegende Dosis eines besonders wirksamen Mittels anzugeben.

Die Behandlung der Endocarditis „rheumatica", auch der schwersten Formen, scheint uns mit Manganchlorid recht aussichtsreich. Dies um so mehr, als dieses Präparat in einem Fall noch wirkte, in welchem die Therapie mit kolloidalem Silber ohne Erfolg geblieben war.

Die Arbeitsergebnisse zusammenfassend, fanden wir in 17 Fällen von Endocarditis „rheumatica" mit Hilfe von intravenösen Manganchloridinjektionen ad modum Walbum eine gute Beeinflussung der Erkrankung.

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Proc. Soc. Exp. Biol. 86: 223-227

Copper Metabolism. XII. Influence of Manganese on Metabolism of Copper.* (21056)

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The clinical and pathological similarity between the manifestations of chronic manganese poisoning in man and the changes which occur in hepatolenticular degeneration has been noted several times(1-3). Hepatolenticular degeneration is now known to be characterized by aminoaciduria, hypocupremia, hypercupriuria and an increase in the amount of copper in the tissues(4-9). Studies on the metabolism of copper in man or in animals with manganese toxicity have not been reported, however.

It was, therefore, considered to be of interest to determine whether or not the administration of large amounts of manganese could be shown to influence the metabolism of copper in animals. In this report observations are presented on the level of copper in the plasma, urine and tissues of rats given large amounts of manganese. The influence of manganese on the red blood cells, the urinary excretion of amino acids and the content of iron in the plasma and tissues were also studied. A preliminary report of this work has been published(10).

Methods. Eighty-three male weanling rats of the Sprague-Dawley strain were divided into 4 groups (Table I), housed in individual

* This investigation was supported by a research grant from the National Institutes of Health, U. S. Public Health Service.

TABLE I. Observations on Copper and Iron Content of Various Tissues and on Urinary Excretion of Amino Acids. Ten animals in each group, sacrificed at 86 days.

	A Control	B High Mn	C High Cu	D High Mn + Cu
Body wt, g	371 ± 9.1	171 ± 7.2	333 ± 0.9	125 ± 5.0
Plasma copper, μg %	107 ± 3.6	142 ± 3.9	126 ± 3.6	194 ± 11.7
Urine " * μg/day	1.8	1.0	3.1	1.8
Liver " μg/g	5 ± 0.2	5 ± 0.2	39 ± 14.1	670 ± 66.4
μg/rat	69 ± 3.1	29 ± 1.3	457 ± 161.4	3070 ± 276.6
Kidney " μg/g	8 ± 0.6	5 ± 0.2	23 ± 3.9	55 ± 23.4
μg/rat	19 ± 1.7	7 ± 0.2	51 ± 7.0	71 ± 29.1
Brain " μg/g	3.6± 0.21	4.4± 0.15	3.9± 0.10	5.0± 0.21
μg/rat	6.6± 0.25	7.3± 0.22	6.8± 0.11	8.0± 0.24
Plasma iron,* μg %	301	112	265	128
Liver " μg/g	160 ± 11.0	40 ± 2.2	130 ± 5.0	40 ± 2.0
μg/rat	2120 ± 198.0	200 ± 3.2	1620 ± 67.7	200 ± 11.1
Kidney " μg/g	78 ± 2.8	32 ± 2.2	75 ± 2.8	41 ± 4.1
μg/rat	194 ± 11.5	47 ± 3.6	161 ± 6.4	54 ± 4.8
Urine amino N,* mg/day	1.4	0.8	1.2	0.6

* Value obtained from pooled specimens.
Figures refer to mean ± stand. error.

cages, and fed the following basal diet *ad libitum*: purified casein, 20%; sucrose, 64%; lard, 11%; and salt mix(11), 5%. Vitamins were added to the basal diet as follows (mg/kg of diet): thiamin hydrochloride, 12.5; riboflavin, 6; nicotinic acid, 60; pyridoxine hydrochloride, 10; calcium pantothenate, 25; inositol, 5; para-aminobenzoic acid, 5; pteroylglutamic acid, 15; vit. E, 7.6; and vit. K, 7.6. Vit. A in an amount of 23000 units and vit. D in an amount of 4250 units per kilogram of diet were also added.

The general design of the experiments and the schedule of the intraperitoneal injections are recorded in Fig. 1 together with the growth curves. All animals in groups A, B, and C survived the duration of the experiment. Three animals in group D died between the 68th and 83rd days. The remaining animals in this group were thin, inactive, and failed to gain weight. Therefore, on the 86th day 10 animals in each group were anaesthetized with ether, phlebotomized by cardiac puncture and the organs removed for chemical analysis. The remaining animals continued to survive and on the 120th day of the experiment they were sacrificed by ether anaesthesia. Total body copper and iron analyses were performed on these rats. *Hemoglobin* was determined by the oxyhemoglobin method in the Evelyn photoelectric colorimeter. The other hematologic determinations were performed as outlined by Wintrobe(12). The method employed for the measurement of plasma copper has been described by Gubler, *et al.*(13); for plasma iron by Hamilton *et al.*(14); for tissue iron by Gubler *et al.*(15); and for total body copper and iron by Chase *et al.*(16). A modification(17,18) of the method of Folin was used for the determination of urinary alpha amino-nitrogen. Twenty-four-hour urine samples were collected from ten rats on the 84th day of the experiment. The samples were pooled and used for the determination of both alpha amino-nitrogen and copper(19).

Results. The administration of manganese alone was associated with a significant increase ($P < 0.01$) in the concentration of copper in the plasma and brain and a decrease in the urinary excretion of copper (Table I). The concentration of copper in the liver was not significantly influenced, and the concentration of copper in the kidneys was decreased. The administration of large amounts of copper was associated with a significant increase ($P < 0.01$) in the concentration of copper in the plasma, urine, liver, and kidneys but not in the brain. When large amounts of both copper and manganese were given (group D)

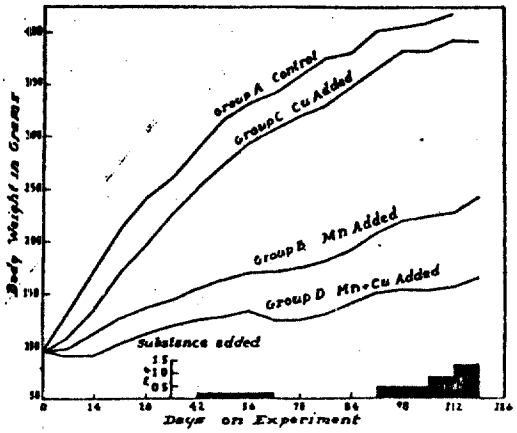

FIG. 1. Influence of manganese, copper, and copper in addition to manganese on growth of rats. All animals received the basal diet and the following supplements: Group A, none; group B, 4% manganese chloride; group C, 0.1% copper sulfate; group D, 4% manganese chloride and 0.1% copper sulfate. In addition, the animals in group B were given manganese chloride ($Mn\ Cl_2 \cdot 4\ H_2O$) intraperitoneally in amounts indicated; those in group C were given "Cupralene" in this way and those in group D both manganese chloride and "Cupralene." These compounds were injected 3 times weekly. Half the animals in each group were sacrificed at 86 days, the remainder at 120 days. As a result those sacrificed at 86 days received a total of 4.5 mg $Mn\ Cl_2$ intraperitoneally while the remainder received a total of 25.5 mg. The total amounts of "Cupralene" received by the animals sacrificed at these different times were also 4.5 and 25.5 mg, respectively.

the concentration of copper in the plasma, liver, kidneys, and brain was significantly increased ($P < 0.01$) as compared with the values in the animals given the same amount of supplementary copper (group C). A decreased urinary excretion of copper was associated with this retention of copper in the tissues.

The concentration of iron in the plasma, liver and kidneys was significantly lower ($P < 0.01$) in the animals given manganese, either alone (group B) or with copper (group D). Aminoaciduria was not observed in any of the 3 experimental groups.

As shown in Table II, the administration of manganese alone (group B) had little influence on total body copper; the administration of large amounts of copper (group C) caused an increase in total body copper. When large quantities of both copper and manganese (group D) were given simultaneously, the total body copper was approximately twice that found in the animals receiving the same amount of copper without added manganese. Since these animals were considerably smaller than the animals in group C, the amount of copper expressed in terms of $\mu g/100$ g of body weight was increased about 5-fold.

The administration of manganese alone (group B) was associated with a significant decrease ($P < 0.01$) in the total amount of iron in the body and a significant decrease ($P < 0.01$) in the amount of iron per 100 g of body weight. When supplementary copper was given in addition to manganese, the decrease in iron (mg/100 g of body weight) was partially overcome, but the total body iron was less than in the animals in group B. The administration of copper alone (group C compared with group A) had no significant influence on total body iron.

The hematologic observations are summarized in Table III). The animals receiving large amounts of manganese (group B) developed a mild anemia which was microcytic and slightly hypochromic and was accompanied by a slight increase in reticulocytes. The animals given large quantities of copper (group C) failed to develop anemia or morphologic alterations in the erythrocytes. When large amounts of copper were given in addition to manganese (group D) the hematologic changes observed were similar to those which occurred when large amounts of manganese alone were given.

Discussion. From the data presented it is evident that in the presence of a large amount of dietary copper, the administration of large quantities of manganese is associated with a marked increase in the retention of copper in the body, particularly in the liver and spleen but also in the plasma and brain.

The administration of large quantities of manganese, either with or without the administration of excessive amounts of copper was associated with the development of microcytic, hypochromic anemia. The concentration of iron in the body of the rats fed large amounts of manganese was significantly reduced, particularly in the liver. Therefore, it is quite possible that the anemia was the result of a mild deficiency of iron. However,

TABLE II. Total Body Copper and Iron. Ten animals in each group, sacrificed at 120 days.

	A Control	B High Mn	C High Cu	D High Mn + Cu
Body wt, g	419 ± 10.9	234 ± 12.1	392 ± 7.8	166 ± 8.7
" copper, μg/100 g	126 ± 3.5	193 ± 8.9	470 ± 31.3	2319 ±378.3
μg/rat	572 ± 16.4	445 ± 18.5	1833 ±115.0	3741 ±537.0
" iron, mg/100 g	4.72± 0.177	3.62± 0.247	4.81± 0.108	4.32± 0.193
mg/rat	19.6 ± 0.71	8.6 ± 0.85	18.8 ± 0.38	7.1 ± 0.11

Values represent mean ± stand. error.

the degree of reduction in the plasma iron level and in the total body iron would not seem to be sufficient to account for the development of anemia on this basis. It should be noted that the anemia associated with an excessive intake of manganese is also morphologically similar to the anemia which appears in swine deficient in copper(20). It is possible that manganese forms a complex with copper thus making the copper physiologically unavailable or that the manganese in some manner blocks the action of copper-containing enzymes which are essential for normal erythropoiesis. Such an interaction has been postulated by Marston(21) to account for the observation that moderate amounts of molybdenum, administered to rats given rations which supply sufficient copper to provide for maximal growth and erythropoiesis, will increase materially the copper concentration in the tissues and precipitate the symptoms of copper deficiency.

In regard to the possible role of manganese and copper in the pathogenesis of hepatolenticular degeneration, it is worthy of note that, although in our experimental animals the tissue levels of copper were increased to a degree comparable to that observed in patients with hepatolenticular degeneration(8), a syndrome simulating this disease was not produced. Neurologic abnormalities, corneal pigmentation, aminoaciduria, and histologic alterations† in the brain were not observed. Furthermore, the plasma copper level was increased rather than decreased, and the administration of manganese was associated with a decrease rather than an increase in the excretion of copper in the urine. In the animals receiving excessive manganese (group B), copper (group C), and copper and manganese together (group D) acute widespread necrosis of the liver with varying degrees of regeneration of liver parenchyma was observed.† However, increase in fibrous tissue, such as is found in the livers of patients with hepatolenticular degeneration was not present. It cannot, of course, be concluded from these relatively short term studies in rats that copper and manganese either do or do not play a role in the pathogenesis of the lesions observed in hepatolenticular degeneration.

† E. J. Eichwald, unpublished observations.

Summary. 1. Administration of large amounts of manganese to rats was associated with an increase in the concentration of copper in plasma and brain, a decrease in the urinary excretion of copper, a decrease in the concentration of copper in the kidneys, no alteration in the concentration of copper in the liver, and microcytic, hypochromic anemia. The total amount of copper in the body was not increased. 2. Administration of large amounts of copper was accompanied by an increase in total body copper and an increase in the concentration of copper in the plasma, urine, liver, and kidneys but not in the brain. Anemia did not occur. 3. Simultaneous administration of large amounts of both manganese and copper resulted in a marked increase in total body copper, an increase in the concentration of copper in the plasma, liver, kidneys and brain, and microcytic, hypochromic anemia. The total amount of copper in the body of these animals was twice as great as in rats given the same amount of supplemental copper alone. The concentration of copper (μg/100 g of body weight) was increased 5-fold. 4. It is suggested that manganese may form a complex with copper which makes the latter unavailable or that in some manner it blocks the action of copper-containing enzymes.

TABLE III. Hematologic Studies. Ten animals in each group.

	A Control	B High Mn	C High Cu	D High Mn + Cu
Red cell count $\times 10^6/mm^3$	8.91 ± .323	9.40 ± .192	8.66 ± .238	10.06 ± .334
Hemoglobin, g %	17.5 ± .19	11.7 ± .64	17.6 ± .32	12.8 ± .75
Vol packed red cells, ml/100 ml	51 ± .8	37 ± 1.3	49 ± .7	40 ± 1.9
Mean corpuscular vol, $c\mu$	57 ± 1.7	40 ± 1.8	58 ± 1.3	40 ± 2.7
Mean corpuscular hemoglobin, $\mu\mu g$	20 ± .6	12 ± .5	21 ± .3	13 ± .9
Mean corpuscular hemoglobin conc., %	35 ± .4	32 ± 1.0	36 ± .2	32 ± 1.2
Reticulocytes, %	2.6 ± .23	5.7 ± .57	2.7 ± .20	6.8 ± 1.19

Values represent mean ± stand. error.

The authors are grateful to Dr. A. Gibson, Merck and Co., Rahway, N. J., for the supply of B vit.; to Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y., for pteroylglutamic acid; to Dr. E. L. Sevringhaus, Hoffmann La Roche, Inc., Nutley, N. J., for fat soluble vitamins; and to Dr. J. M. Carlisle, Merck and Co., for the "Cupralene".

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Received April 21, 1954. P.S.E.B.M., 1954, v86.

German Translation

40. Acute and Chronic Heavy Metal Poisoning

Communication I: Manganese Poisoning

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(Received for publication on Aug. 10, 1925).

From: Archiv fur Experimentelle Pathologie und Pharmakologie, Vol. 10, No. 5/6, pp. 265-280, 1925/26.

The purpose of the present investigations (see note) is to check whether the action of heavy metal compounds is related to the way in which the metal is bound in the molecule of the compound (Note: This study is the first one in a series concerned with the effect of heavy metals). The following Mn compounds were studied: $MnCl_2$, Mn citrate and a complex Mn salt of a pyrocatechol derivative (Heyden compound No. 730, supplied to us by the Heyden Chemical Plant and which will be chemically defined elsewhere); thus, the Mn compounds studies include an inorganic salt, an aliphatic compound, and a complex aromatic compound. The $MnCl_2$ solution was prepared by us as an exact 0.5% solution from individual components. According to our analysis, Mn citrate had a divalent Mn content of 12.45%; the complex aromatic Mn Compound had an 8.2% Mn content. The solutions used had an 0.5% Mn content (for Mn citrate) and a 1% Mn content (for the aromatic Mn compound).

The three Mn compounds did not exhibit any differences in their

acute effects, both in regard to dosage and poisoning pattern. Mice, guinea pigs and rabbits died within a period of at most 12 hours, when given a subcutaneous injection of 50 mg/kg body weight. The animals always displayed symptoms of paralysis starting at the rear extremities, which were moved with difficulty and partially dragged; paralysis then increased, spread to the forward extremities and the animals died with asphyxiating cramps. However, differences in the effect of the three Mn compounds could be observed in chronic poisoning tests, in which the animals received an injection of 10 mg Mn/kg at definite intervals. In this case, under otherwise similar conditions, Mn citrate (the aliphatic complex salt) was by far the most toxic, followed far behind by $MnCl_2$, which again was slightly more toxic than the complex aromatic Mn compound. Particularly striking symptoms could not be observed in the chronic poisoning tests; the intervals were selected in such a way that the injections were stopped when the animals displayed signs of reluctance to eat.

In order to get an idea about the action mechanism of the Mn compounds, we had to find out whether any relationship existed between the distribution of the toxic compound in the organism, the toxic effect and any pathological-anatomic changes in individual organs. For this purpose, we had to work out at first a chemical method which would allow us to carry out large-scale series investigations. We proceeded in such a way that we developed the common method used for qualitative Mn determination into a quantitative method: namely, the Mn present in ash was oxidized to permanganate, and the latter determined colorimetrically.

The organs, as long as they were not too large, were dissolved

in concentrated nitric acid and/or incinerated (reduced to ash) until the solution was more or less clear; this was done in an oblique-standing Jena glass incineration flask (of 100-150 cc volume) provided with a long and wide neck. The acid was added through a number of dropping funnels mounted at such a distance behind the flask that nothing from the iron ring fasteners exposed to the acid fumes could enter into the flask; the funnels were equipped with a correspondingly long spout. At first we used, for incineration purposes, mixtures of HNO_3 with concentrated H_2SO_4, which later proved to be unnecessary. Special caution must be exercised during incineration of feces, urine, blood, pelt and fat samples in view of the violent reaction of nitric acid. Large-size organs, such as stomach and intestines with their contents, were covered with concentrated HNO_3 in a dish, so that after standing for sometime, a completely homogenous and consistent paste was formed, aliquots of which were weighed and processed further.

With feces samples, usually an amount of 10 g was dissolved in concentrated nitric acid, and one must be careful that the reaction is not too violent, since otherwise the mass begins to foam and spills over easily. After prolonged heating with concentrated HNO_3, a clear colored solution is also obtained in this case.

The total amount of urine samples obtained was processed by adding 50-100 cc concentrated HNO_3 to the urine with great caution, since very strong foaming takes place occasionally; for this reason, small portions of the urine were added to the acid. The acid-urine mixture was then placed into the dropping funnel and allowed to flow gradually into the flasks containing about 5 cc concentrated H_2SO_4. Heating

was also stopped here when the total amount of urine had been concentrated to a volume of 10-15 cc; evaporation must be constantly watched since violent foaming takes place without any visible external reason, which can be reduced by adding small amounts of water. Strong pounding during evaporation can be avoided by using a few boiling chips.

Pelt reacted very violently with nitric acid, and the conversion to nitrogen oxides took place suddenly, sometimes with the appearance of fire; for this reason, we have always treated pelt with acid in large dishes. Fat tissue had to be carbonized first with sulfuric acid, since explosions may easily take place during the violent nitration process.

The nitric acid solutions obtained as described above, mostly with a volume of 100-150 cc, were converted in large porcelain crucibles (high form), from which no traces of Mn could be extracted with acids, and concentrated as far as possible on a water bath. The residue was evaporated to dryness over an open flame and calcined in order to completely destroy any organic matter left; when sulfuric acid had been adden to the substances tested, this acid of course had to be evaporated cautiously. In case of very compact material, such as intestinal contents, a large amount of residue remained during carbonization, the processing of which was difficult, and therefore only small portions of these samples were used in the analysis.

The calcined residue was warmed slightly with concentrated HCl in order to dissolve again any MnO_2 which may have been formed; after adding a little water, the hydrochloric acid solution was again concentrated to dryness on the water bath. The residue was mixed

with 2 cc concentrated H_2SO_4 to remove chlorine ion and heated until the appearance of white sulfuric acid vapor. At this stage, all of the manganese present is in the form of $MnSO_4$. Chlorine ions must be moved, since they exert a disturbing effect during the subsequent oxidation with lead peroxide.

The sulfuric acid solution was then transferred to an Erlenmeyer flask, diluted to 50 cc, mixed with 5 cc concentrated HNO_3 and heated to the boiling point. Then, after removing the flame, about 1 g lead peroxide was added and the mixture was kept boiling for several minutes, thereby allowing the oxidation of $MnSO_4$ to permanganate. The lead peroxide used must be free of Mn.

It was found in the first experiments that the calcination with H_2SO_4 and HNO_3 (at most 5 cc H_2SO_4 and very large amounts of HNO_3 were used) was incomplete. Larger amounts of H_2SO_4 would have had to be removed prior to oxidation of Mn to permanganate, since the latter is decomposed with oxygen formation, by strong sulfuric acid solutions. Treatment with HNO_3 was continued until the solution was water-clear and no coloring (carbonization) occurred during concentration until large amounts of H_2SO_4 vapors had evolved. When this solution was greatly (at least 10-fold) diluted, and any nitrosyl-sulfuric acid formed and nitrogen oxides still present removed by boiling, and a permanganate solution added, decolorization was very often observed immediately or after a short time. A permanganate solution containing the same amount of H_2SO_4 retained its color. For this reason, we could not use this simple process and had to switch to the more complex procedure described above.

The permanganate solutions obtained by oxidation were filtered through an asbestos filter into a measuring flask usually containing 50 cc of liquid. The asbestos used was carefully purified by burning out and boiling with a permanganate solution, concentrated HCl and water. The Mn content of the filtered solutions was determined colorimetrically with an Autenrieth colorimeter.

The reference solution (1 cc = 0.05 mg Mn) was regularly treated, as well as the solution to be tested, with nitric acid and lead peroxide. In the case of samples, which presumably contained very little Mn, such as urine and some organ samples, a known amount of Mn (mostly 0.25 mg) was added prior to oxidation. Then the Mn present originally was determined by difference, since very small and very large amounts of Mn could be very poorly determined in the colorimeter (see above).

Since Mn is present in most cells, we also had to analyze the animal feed; animals were fed only turnips and oats, and we were unable to find any Mn in turnip pulp and only traces in the juice. Oats contained larger amounts of Mn: once, we found 30 mg Mn/1000 g, and another time 27.5 mg Mn/1000 g. These Mn amounts are irrelevant, however, and are excreted quantitatively in the feces. By the way, we also carried out tests to determine the average consumption of oats (and therefore Mn) of the animals in their food; we found that different animals ate, in 7 days, 435, 420, 415, 380, 360, 355 and 345 g oats, thus an average of 55 g/day. Since sick animals occasionally ate much less, we have weighed out and reweighed the amounts of oats consumed by two such animals.

The subcutaneous injections were carried out in the most aseptic

manner possible, namely in such a way that the first injection was made at the left top side, the second at the right lower side, the third at the right top side, the fourth at the left lower side, etc. Local reactions were never observed; during dissection, we always removed pieces (fragments) from the last and next-to-last injection site (pelt, subcutaneous tissue, muscles) and analyzed these pieces; we have not found, at the sites where 10 mg of Mn compound was injected, more than fractions of a milligram of Mn. Only in animal # 3, did we find 2.55 mg Mn in 23 g of moist substance in the vicinity of the injection site. The individual test results obtained are given below.

Rabbit # 1

Treated with the complex aromatic Mn salt; female, weight 2350 g at the beginning of the test, received 23 mg Mn dissolved in 2.3 cc with each injection.

Date	Weight in g	Injection	Remarks
21.I.	2350	Injection	--
29.I.	2300	Injection	--
5.II.	2250	Injection	--
9.II.	2200	Injection	Does not eat
15.II.	--	--	Dead

A total of 92 mg Mn was injected into the animal; the acute lethal dose would have been 115 mg Mn.

Results of organ analyses were as follows:

Organs	Wet Weight in g	Mn content in mg
Liver	54.4	0.025
Kidneys	13.7	0.012
Spleen	0.4	0.05
Heart	6.9	0.025
Brain	6.6	0.05
Blood	--	0.40
Pelt	230	0.875
Muscles	--	0
Lung	10.1	0.012
Stomach wall	28	0.075
Small intestine wall	39	0.175
	Total	1.65

Dissection gave no significant macroscopic findings.

Microscopic Findings

Liver: Light fatty degeneration of liver cells, occurring sometimes more frequently at the center, and sometimes more frequently at the periphery of the liver lobes. Clear fatty degeneration of Kupffer's stellate cells. Light increase of connective tissue and therefore widening (distention) of Glisson's sheaths, in which proliferations of small bile ducts are often seen, exhibiting a very strong fatty degeneration (Fig 1). No necroses. Brown, granular pigment in the Kupffer's cells (iron detected microchemically).

Kidney: Slight fatty degeneration of the tubuli contorti at the cortical marrow boundary.

Spleen: Hyperemia of the pulpa. In the wide sinus, large round cells, often containing clearly visible fat and pigment granules (iron

reaction).

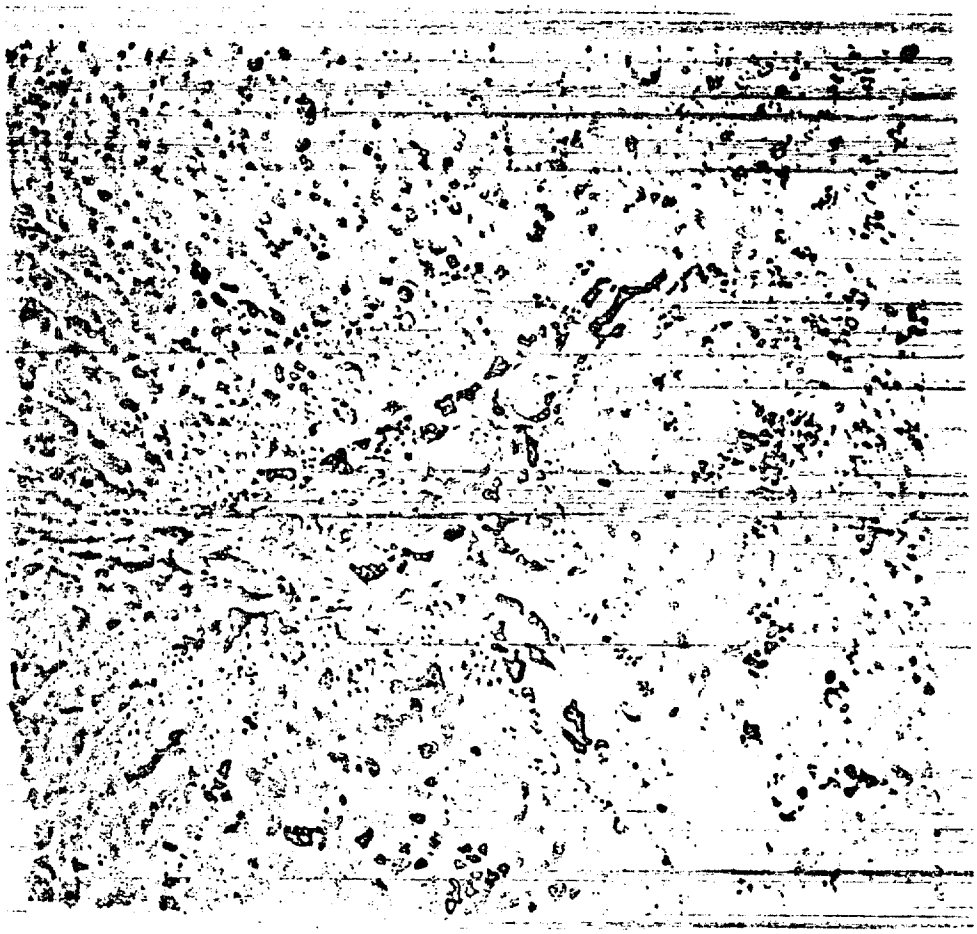

Fig. 1. Fatty degeneration of proliferated biliary ducts in the Glisson sheaths of the liver. Fatty degeneration of Kupffer cells and liver cells. Rabbit # 1. Sudan staining.

Thus, a total of 1.6 mg Mn was found in the organs; 0.48 mg in the stomach and small intestine, and 0.48 mg in the large intestine. If we calculate, by analogy with rabbits # 5 and 9 and simply to determine the order of magnitude, that about 54 mg Mn was excreted in the feces and 18 mg Mn was deposited in the bones, then we can account for 74.5 mg out of the 92 mg Mn injected; several milligrams Mn were

probably not resorbed.

Rabbit # 2

Treated with the complex aromatic Mn salt; female, weight 2820 g at the beginning of the test, received 28 mg Mn dissolved in 2.8 cc with each injection.

Date	Weight in g	Injection	Remarks
21.I.	2820	Injection	--
29.I.	2900	Injection	--
5.II.	2900	Injection	--
9.II.	2850	Injection	--
12.II.	2600	Injection	Killed by bleeding to death

A total of 112 mg Mn was injected into this animal.

Results of organ analyses are as follows:

Organs	Wet Weight in g	Mn content in mg
Liver	76.2	2.74
Kidney	16.4	0.225
Spleen	1.7	0.012
Heart	6.6	0.012
Brain	6.9	0.175
Blood	217	0.36
Pelt	280	0.975
Muscles	846	0
Lung	10.6	0.05
Intestinal wall	50.4	0.40
	Total	4.75

Analysis of the intestinal contents was unsuccessful. Dissection gave no special findings.

Microscopic Findings

Liver: Medium-strong fatty degeneration of liver cells occurring at the periphery of the lobes (see Fig. 2). Fatty degeneration of Kupffer's cells. No clearly visible pigment.

Kidney: Nothing particular.

Spleen: Nothing particular.

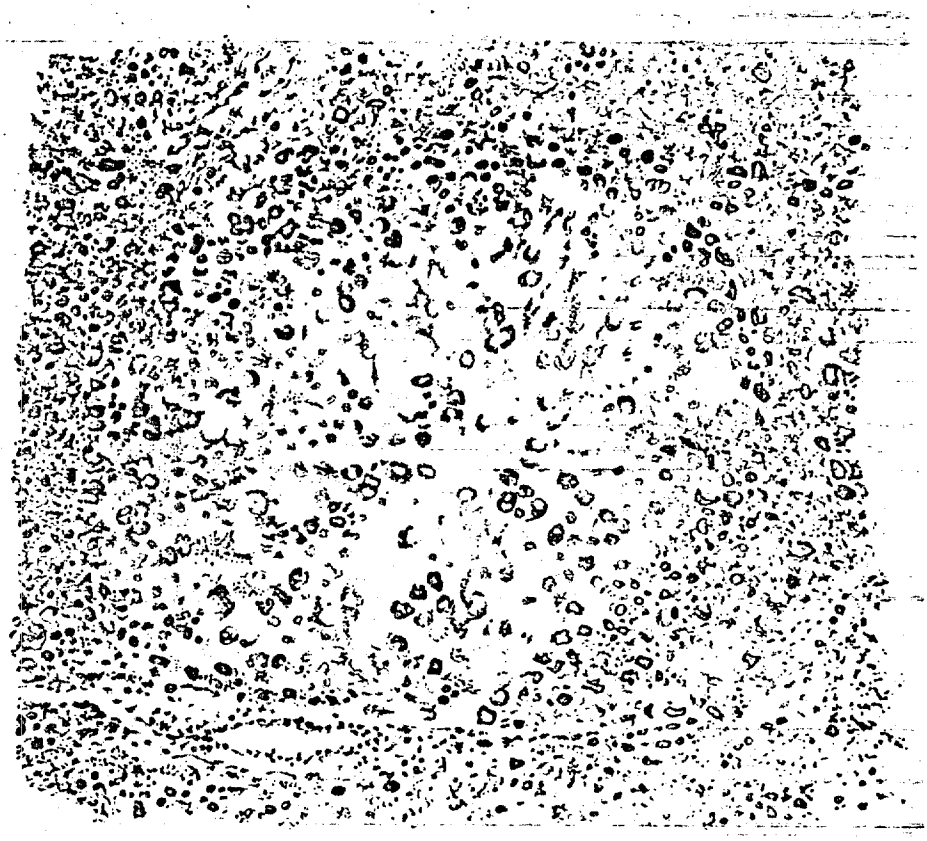

Fig. 2. Peripheral fatty degeneration of the liver and of Kupffer cells. Rabbit #2, Sudan staining.

Rabbit # 3

Treated with $MnCl_2$; female, weight 2350 g at the beginning of the test, received 23.5 mg Mn dissolved in 4.7 cc with each injection.

Date	Weight in g	Injection	Remarks
21.I.	2350	Injection	--
29.I.	--	Injection	--
5.II.	2150	Injection	--
9.II.	2200	Injection	--
12.II.	--	--	Killed by bleeding to death

A total of 94 mg Mn was injected into the animal.

Results of organ analyses are as follows:

Organs	Wet Weight in g	Mn content in mg
Liver	71.5	0.00
Kidney	11.6	0.05
Spleen	1.5	0.05
Heart	5.8	0.06
Brain	6.5	0.075
Blood	40	0.05
Pelt	--	2.07
Muscles	705	11.71
Lung	8.7	0.025
Fat	14	0.075
Colon wall	48	0.85
	Total	15.32

In the colon contents, 1.3 mg Mn was found. Dissection did not reveal any macrosopically significant findings.

Microscopic Findings

Liver: As a whole, low in fat, cells often surprisingly large and showing vacuole degeneration. Isolated, fatty cell complexes in the acini and in the Glisson sheaths. Strikingly clear

fatty degeneration of Kupffer's cells, no proliferation or fatty degeneration in biliary ducts. No increase of connective tissue.

Kidney: Small foci of inflammation here and there in the interstitial connective tissue, otherwise no findings.

Spleen: Strikingly blood-rich pulpa. In the lumen of sinuses, large-cells loaded with pigment granules (positive iron reaction).

Rabbit # 5

Treated with $MnCl_2$; female, weight 2120 g at the beginning of the test, received 21 mg Mn dissolved in 4.2 cc with each injection.

Date	Weight in g	Injection	Remarks
21.I.	2120	Injection	--
29.I.	1900	Injection	--
5.II.	2000	Injection	--
9.II.	1950	Injection	--
12.II.	1900	Injection	--
16.II.	1800	Injection	--
18.II.	1500	Injection	--
21.II.	--	--	Dead

A total of 147 mg Mn was injected into the animal.

Results of organ analyses are as follows:

Organs	Wet Weight in g	Mn content in mg
Liver	59.5	1.57
Kidney	16.8	0.625
Spleen	1.4	0.075
Heart	5.0	0.025
Muscles	640	4.14
Lung	13.1	0.175
Bones	--	17.27
	Total	23.88

In the intestinal contents 2.15 mg Mn was found, and in the stomach contents 0.97 mg Mn. At that time, the animal had eaten 720 g oats. A very striking feature is the high Mn content of bones; since large pieces of bone yield a very voluminous ash, we were able to analyze only small pieces. Analysis was done on 1.1 and 1.0 g tubular bone material, from which bone marrow was carefully removed.

Dissection did not give any macroscopically significant findings.

Microscopic Findings

Liver: Moderately strong fatty degeneration of liver cells, accompanied by hyaline drop-shaped degeneration and necroses, distributed in spots, of liver cells (see Fig. 3). Very clear fatty degeneration of newly formed biliary ducts in the Glisson sheaths. Fatty degeneration of Kupffer cells. Increase in connective tissue, starting from the Glisson sheaths. In the better preserved portions of liver lobes, strikingly numerous mitotic karyokinetic figures of liver cells (see Fig. 4). Brown pigment in the Kupffer cells (positive iron reaction).

Kidney: No findings.

Spleen: Very dense pulpa, not blood-rich. No pigment.

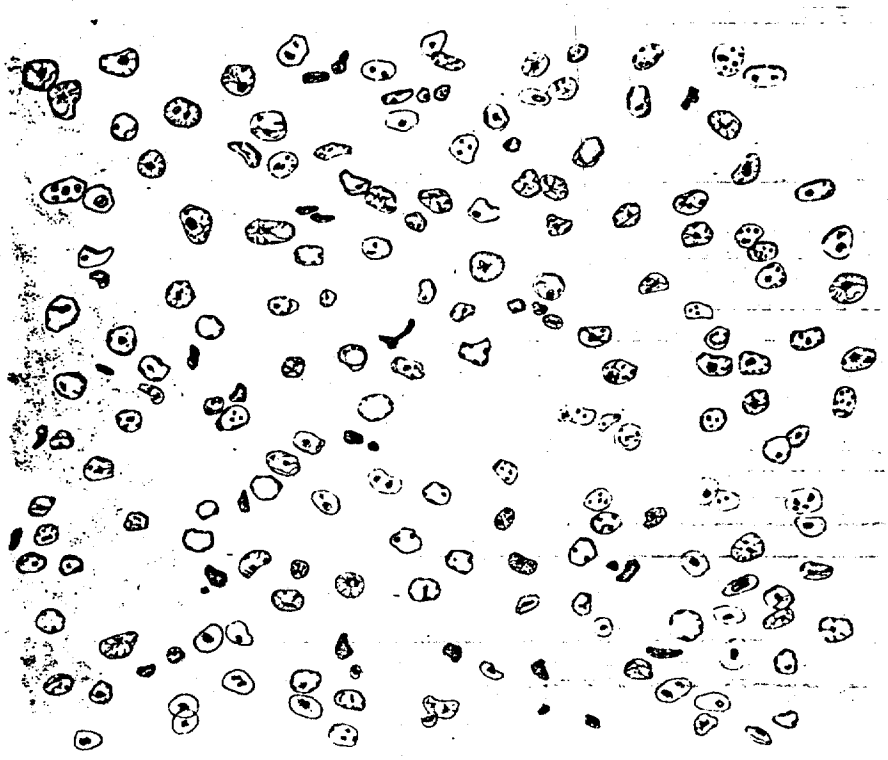

Fig. 3. Degeneration focus in the liver of rabbit # 5. Liver cells without a nucleus, partially disintegrated. Swelling, pale coloring (staining) of many nuclei. Strong magnification.

Fig. 4. Liver mitoses in rabbit # 5, in the vicinity of a degeneration focus. Strong magnification.

Rabbit # 4

Treated with Mn citrate; female, weight 2050 g at the beginning of the test, received 20.5 mg Mn dissolved in 4.1 cc with each injection.

Date	Weight in g	Injection	Remarks
21.I.	2050	Injection	--
29.I.	--	Injection	--
31.I.	--	--	Dead

A total of 41 mg Mn was injected into the animal.

Analysis of organs gave 0.32 mg Mn in the liver, 0.05 mg Mn in the brain, heart and lungs, and only unmeasurable traces of Mn in the kidney and spleen. Dissection showed a macroscopic enlargement and yellow coloration of the liver.

Microscopic Findings

Liver: Very strong fatty degeneration of liver cells affecting the entire lobes; where fat accumulation is not so extensive, participation of Kupffer cells can again be seen. No pigment. No increase in connective tissue.

Kidney: No findings.

Spleen: No findings.

Rabbit # 6

Treated with Mn citrate; female, weight 1750 g at beginning of the test, received 17.5 mg Mn dissolved in 3.5 cc with each injection.

Date	Weight in g	Injection	Remarks
21.I.	1750	Injection	--
23.I.	--	--	Dead

A total of 17.5 mg Mn was thus injected into the animal, whereas the acute lethal dose (causing death within a few hours) would have amounted to 87.5 mg Mn. Organ analyses revealed unmeasurable traces of Mn in the kidneys, brain, spleen and heart. This animal was not dissected.

Rabbit # 9

Treated with the complex aromatic Mn salt; female, weight 1600 g at the beginning of the test, received 16 mg Mn dissolved in 1.6 cc with each injection. The excretions of this animal were investigated.

Date	Oats consumed	Weight in g	Mn injected mg	Feces excreted	Mn in feces mg	Urine excreted in cc	Mn in urine mg
23.II.	from 23.II to	1600	16	from 23.II to	13.5	270	0.15
26.II.	2.III 250 g	--	16	2.III 57 g		--	--
2.III.	from 2.III to	1600	16	from 2.III to	26.9	230	0.10
5.III.	9.III 321 g	1550	16	9.III 117 g		--	--
9.III.	from 9.III to	1550	16	from 9.III to	17.4	180	0.10
12.III.	16.III 220 g	1540	16	16.III 105 g		--	--
16.III.			Died				

Amounts of Mn taken up (absorbed): by injection, 96 mg; in feed 23.7 mg. The amounts of Mn excreted in the feces and urine were 57.8 mg and 0.35 mg, respectively.

Organ analyses were not performed on this animal. Dissection did not reveal any significant macroscopic findings.

Microscopic Findings

Liver: Liver cells proper in general free of fat, but very clear fatty degeneration of Kupffer cells, which contain large amounts of pigment (positive iron reaction). Glisson sheaths in part slightly thickened and infiltrated with lymphocytes.

Spleen: Pulpa very rich in blood. In the wide sinuses, numerous large and round cells, containing brown pigment granules.

Kidneys: Small shrinkage foci of inflammatory nature.

Heart: No findings.

Rabbit # 10

Treated with $MnCl_2$; female, weight 1600 g at the beginning of the test, received 16 mg Mn dissolved in 3.2 cc with each injection. Organ analyses were also not performed with this animal, only analyses of the excreta.

Date	Oats consumed	Weight in g	Mn injected mg	Feces excreted	Mn in feces mg	Urine excreted in cc	Mn in urine mg
23.II.	from 23.II to	1600	16	from 23.II to	20.4	400	0.15
26.II.	2.III 337 g	--	16	2.III 96 g		--	--
2.III.	from 2.III to	1600	16	from 2.III to	25.5	625	0.08
5.III.	10.III 265 g	1520	16	10.III 103 g		--	--
9.III.	--	1490	--	--		--	--
10.III.			Died				

Amounts of Mn taken up (absorbed): by injection 64 mg, with feed 18.06 mg; amounts of Mn excreted: 45.9 mg in feces, 0.23 mg in urine. Dissection showed macroscopically a clearly enlarged fatty liver.

Microscopic Findings

Liver: Very strong, quite diffuse fatty degeneration of liver cells. Here and there, a clearly detectable participation of Kupffer cells, which contained little pigment.

Kidney: Clearly visible fatty degeneration of the basal portions of the epithelium of tubuli contorti and recti.

Heart: Slight but definite fatty degeneration of the heart muscle fibers.

Spleen: Very rich in blood. Between the wide sinuses, a strikingly large-cellular, apparently hyperplastic pulp tissue; no pigment.

The above information summarizes the test results. For the sake of comparison, the following table gives the amounts of Mn found in different organs, the figures representing milligrams of Mn per 1000 g wet (moist) organ.

Organs	Complex aromatic Mn salt		$MnCl_2$	
	Rabbit 1 died	Rabbit 2 killed	Rabbit 3 killed	Rabbit 5 died
Liver	0.458	35.82	0.0	26.42
Kidney ...	9.63	13.72	4.31	37.20
Spleen ...	125	7.36	33.33	52.60
Heart	3.62	1.89	10.76	5.0
Brain	15.14	25.38	11.54	—
Blood	—	1.67	1.25	—
Pelt	3.64	3.48	7.35	—
Muscle ...	0	0	16.68	6.56
Lung	1.24	4.72	2.87	13.35
Bones	—	—	—	68.17
Colon wall	—	8.0	17.7	—
Bile	75	25	92.5	—
Ascites ..	—	40	—	40
Pus	37.5	—	—	—

From these chronic poisoning tests we wish to point out the following facts:

1. In order to kill the animals in a chronic test, in general, smaller Mn amounts are mostly required than the acute dosage causing death; thus, rabbit # 1 died after receiving 40 mg/kg, rabbit # 5 after 70 mg/kg, rabbit # 9 after 40 mg/kg, rabbit # 10 after 30 mg/kg, while the acute lethal dose is 50 mg/kg.

2. In the chronic test, Mn citrate is considerably more toxic, which is probably due to its faster resorption, and is confirmed by the fact that rabbit # 4 exhibits the greatest pathological changes, although it lived only 10 days. The dose in this case was not sufficient to cause an acute effect, but the chronic effect was accelerated.

3. The injected Mn is very rapidly excreted in the feces, to the extent of about 50%; only traces of Mn are detected in the urine during long-term tests, and the kidney is thus Mn-proof. The same thing was observed by Reimann, Clarence and Minot (J. Biol. Chem. 1920, Vol. 42, p. 329; Vol. 45, p. 133) in people suffering from Mn poisoning.

4. A striking factor is the large amount of Mn found in bile, ascites and pus. The body thus rids itself of the foreign metal by this route much more easily than through the kidneys. Changes in the permeability of vascular capillaries during pathological processes make it more easy for heavy metals to penetrate into vessels. The amounts of Mn found in bone substance are very large; here also, the large amounts of carbonate and phosphate present promote the

deposition (precipitation) of metal, and greater attention should be given to the study of bones in cases of metal poisoning than has been done before.

5. The amount of Mn deposited in individual organs does not appear to play a significant role in the onset of death and the anatomical changes of the corresponding organ; undoubtedly, there are organs in which large amounts of Mn are deposited, primarily the spleen and brain. Then there are organs in which the Mn content varies greatly, such as the liver, and finally organs containing very little Mn, such as lungs. We were unable to determine why Mn is deposited in a larger amount in one liver and less in another; this must be due to local reasons or causes found in individual organs. The only differnece, observed by us, between organs which have stored Mn and those that have not is the different behavior of the sectioned and fixed preparations in a dark field; in those livers where no Mn could be found, we were unable to observe in a dark field the otherwise regularly visible flashing of the cell nuclei outlines. Also, of the animals treated in the same manner, we have killed one animal and left another alive until its spontaneous death, in order to find out if a migration of Mn could perhaps be detected in this manner; the amounts of Mn found in the organs of killed animals were different from those found in the organs of animals dying from natural death. However, these changes are not synonymous (i.e. they do not follow the same direction), so that they do not allow us to draw any kind of conclusions. It is striking that only Mn injected as the chloride could be found in muscles, whereas no Mn could be detected in muscles after injection of the complex organic Mn salt.

A relation between the amount of poison and the damage done to individual organs can also be hardly deduced from the figures published by Lomholts (Biochem. J. 1924, Vol. 18, p. 69), who studied the distribution of various Hg, Bi and Pb compounds in the rabbit organism after subcutaneous injection and found a much more uniform distribution of these metals than we did with Mn. Thus, the above author found a considerable accumulation in the kidney of two Bi compounds, and, among Hg compounds, only the presence of large amounts of Hg benzoate in the kidney, whereas this was not the case for Hg salicylate, HgCl and a 50% Hg oil preparation. The amount of Mn found in individual organs at the time of death (and this should also apply to other heavy metals) thus depends on the local conditions found in these organs and is of secondary importance for the overall poisoning pattern. Of interest is the fact that our findings agree with those reported by the above-mentioned American researchers Reimann, Clarence and Minot on human patients suffering from manganese poisoning; these authors have carried out, as others have done occasionally, large-scale studies on workers engaged in manganese mining and processing. They observed, in this connection, that the Mn content of normal human blood, averaging 1 mg/1000 g, was twice as high in some people with strong Mn intoxication symptoms, but only in individual cases; only traces of Mn were always found in the urine, but in patients with biliary fistulas, which received 0.3 - 0.5 mg Mn per os, the Mn content of the bile, which normally is equal to about 0.3 mg/1000 g, increased up to 1 mg/1000 g. When large amounts of the Mn ore franklinite, containing about 10% Mn, were fed to people ; cases of poisoning were observed only in persons

appearing to have a special predisposition to intoxication. In our systematic poisoning tests, the amounts of Mn found were naturally considerably higher, and we were never able to detect 0.01 mg Mn in normal rabbit organs; in any case, only a few grams of wet (moist) organs from one animal were available to us in our study.

6. A certain uniformity can be seen in the pathological histological findings: 1) the fatty degeneration starting in Kupffer cells and then extending to other liver cells, in some cases combined with fatty degeneration of the kidneys and heart. In one case (rabbit # 5), fat deposition was accompanied by clearly visible degenerative processes in the liver. Mitoses in the cells obtained indicate the presence of vigorous regenerative processes. 2) Destruction of blood corpuscles, expressing itself in hemosiderin formation. Of course, we must point out that these findings do not agree with those reported by G. M. Findlay (Brit. J. Exp. Pathol. 1924, Vol. 5, p. 92), which came to our attention only a short time ago. Findlay injected 3-5 mg $MnCl_2$ every other day into rabbits, guinea pigs and rats; the rabbits lived for 5-6 weeks and did not exhibit any fatty degeneration but rather a very extensive cirrhosis of the liver. The difference between our tests and Findlay's can perhaps be explained by the fact that our animals were poisoned more quickly and therefore also died much earlier; only our rabbit # 5 exhibited histological changes which perhaps agree with those observed by Findlay or at least represent a preliminary stage of such changes.

7. We can also report tests on guinea pigs which were poisoned with sublethal doses of Mn. These animals occasionally lived for

several months but showed a greatly reduced weight and changes in the liver similar to those observed in chronically poisoned animals. As an example, the following results can be reported:

Guinea pig # 13 ($MnCl_2$): 15.I, weight 320 g, received 14.5 mg Mn; 20.III: weight 300 g, found dead (untreated animals of the same age weighed 500-600 g).

Guinea pig # 17 (complex aromatic Mn salt): 21.I: weight 350 g, received 17.5 mg Mn; 8.IV: found dead, weight 375 g.

8. Thus, the action mechanism of Mn is radically different in acute and chronic poisoning, and the same can be said mutatis mutandis about the action of other heavy metals, as we were able to observe on the basis of other tests. In acute poisoning, an increasing paralysis of the central nervous system takes place, and such a paralysis is involved in the acute poisoning action exerted by all heavy metals; in the same way, the isolated heart stands still after treatment with all heavy metals. Acute poisoning takes place only when a definite, very high Mn concentration is suddenly present in the blood. If this high concentration is not reached, we were unable to observe any nervous disturbances in our test animals, and poisoning symptoms in the liver are then observed; this poisoning follows a chronic course and is irreversible, as we observed with our guinea pigs, which received sublethal doses of Mn. The rate (velocity) of the chronic poisoning depends on the amount of metal resorbed per unit time, as we were able to determine particularly in tests with other metals; this rate (velocity) of resorption is again dependent on the way in which the metal is bound in the overall molecule of the toxic compound. The residual

presence of the metal in individual organs appears to depend on local conditions and is irrelevant for the course or end result of the toxic effects; the main issue here is namely a functional disturbance.

Within the liver, chronic poisoning affects primarily the Kupffer cells. Serological observations during the use of Mn for the treatment of infectious diseases can perhaps be related to this fact. Walbum (Zeitschr. f. Immunitatsforsch. u. exp. Therapie, Orig. 1925, Vol. 42, p. 32) and his associates have observed that $MnCl_2$ injection into horses immunized against diphtheria resulted in an antitoxin concentration of the blood serum which was higher than the one originally achieved by the usual immunization with toxin alone. Amboceptor formation is also promoted most by $MnCl_2$, as compared to all other heavy metals. The occasionally described therapeutic effect of Mn compounds in furuncle formation and other infectious diseases, recently tuberculosis was also mentioned (Walbum, Deutsch. Med. Wochenschr. 1925), may also be possibly related to the increased antibody formation or the washing out of antibodies from the stellate cells of the liver.

Archiv für Experimentelle Pathologie und Pharmakologie, Vol. 10, pp. 265–80, 1925/26 (No. 5/6)

XVI.

Aus dem Pharmakologischen und dem Pathologischen Institut der Universität Göttingen.

40. Über akute und chronische Schwermetallvergiftungen.

I. Mitteilung: Manganovergiftung[1]).

Von

H. Handovsky, H. Schulz und M. Staemmler.

(Mit 3 Tafeln.)

(Eingegangen am 10. VIII. 1925.)

Zweck der vorliegenden Untersuchungen[1]) war es, zu prüfen, ob die Wirkungen von Schwermetallverbindungen mit der Art der Bindung des Metalls im Molekül der Substanzen zusammenhängen. Es wurden folgende Mn-Verbindungen geprüft: $MnCl_2$, Manganozitrat und ein Komplexsalz des Mangans mit einem Brenzkatechinderivat (Heyden Nr. 730, welch letzteres uns von der chemischen Fabrik von Heyden zur Verfügung gestellt und an anderer Stelle chemisch definiert werden soll); es handelt sich also um ein anorganisches Salz, eine aliphatische und eine aromatische Komplexverbindung. Die Manganochloridlösung haben wir selbst aus einzelnen Präparaten hergestellt, und zwar als genaue 0,5%ige Lösung. Das Manganozitrat enthielt nach unseren Analysen 12,45% zweiwertiges Mangan; die aromatische Komplexverbindung enthielt ebenfalls nach eigenen Analysen 8,2% Mangan; vom Zitrat wurde stets eine ½% Mn, von der aromatischen Verbindung eine 1% Mn enthaltende Lösung verwendet.

In den akuten Wirkungen zeigten die drei Verbindungen keine Unterschiede, weder was Dosis noch was Vergiftungsbild anlangt. Mäuse, Meerschweinchen und Kaninchen starben innerhalb längstens 12 Stunden, wenn man ihnen 50 mg pro Kilogramm subkutan injiziert. Sie zeigten dabei stets Lähmungserscheinungen, die an den

1) Diese Untersuchung ist die erste einer Serie, die sich mit der Wirkung der Schwermetalle beschäftigt.

hinteren Extremitäten beginnen, diese werden nur schwer bewegt, zum Teil nachgeschleift; die Lähmung steigt dann auf, ergreift die vorderen Extremitäten, und die Tiere gehen unter asphyktischen Krämpfen zugrunde. Unterschiede in der Wirkung der drei Verbindungen stellten sich jedoch in chronischen Vergiftungsversuchen heraus. Bei diesen wurde den Tieren in bestimmten Intervallen je 10 mg Mangan pro Kilogramm Tier injiziert. Hierbei erwies sich unter sonst gleichen Bedingungen das Zitrat, also das aliphatische Komplexsalz, als weitaus am giftigsten, dann kam in weitem Abstand das Chlorid, das wiederum etwas giftiger war als die aromatische Komplexverbindung. Besondere augenfällige Erscheinungen konnten bei den chronischen Vergiftungen nicht beobachtet werden; die Intervalle wählten wir so, daß wir mit den Injektionen aussetzten, wenn sich Freßunlust einstellte.

Um einen Einblick in den Wirkungsmechanismus gewinnen zu können, mußten wir untersuchen, ob Zusammenhänge zwischen der Verteilung des Giftes im Organismus, der Giftwirkung und pathologisch-anatomischer Veränderungen einzelner Organe bestehen.

Dazu mußten wir zunächst eine chemische Methode ausarbeiten, die es gestattet, größere Serienuntersuchungen zu machen.

Wir verfuhren so, daß wir die übliche Methode zum qualitativen Mn-Nachweis zu einer quantitativen ausbauten, wir oxydierten zu diesem Zweck das Mn der Asche zu Permanganat und bestimmten dasselbe kolorimetrisch.

Die Organe wurden, soweit sie nicht zu groß waren, in konzentrierter Salpetersäure gelöst bzw. verascht, bis die Lösung einigermaßen klar war. Das erfolgte in schräg gestellten Veraschungskolben (Inhalt 100—150 ccm) aus Jenaer Glas mit langem, weitem Hals. Die Säure wurde durch Tropftrichter zugegeben, die so weit hinter den Kolben angebracht waren, daß von den den Säuredämpfen ausgesetzten eisernen Befestigungsringen nichts in die Kolben gelangen konnte; die Trichter waren mit einem dementsprechenden langen Ausflußrohr versehen. Anfangs verwendeten wir zur Veraschung Gemische mit konzentrierter Schwefelsäure; dies hat sich aber als unnötig erwiesen. Besondere Vorsicht beim Veraschen erfordern Kot-, Harn-, Blut-, Fell- und Fettproben wegen der stürmischen Reaktion der Salpetersäure. Größere Organmassen, z. B. Magen, Darm + Inhalt wurden in einer Schale mit konzentrierter Salpetersäure übergossen, so daß nach einigem Stehen ein vollkommen homogener konsistenter Brei entstanden war, von dem dann aliquote Teile abgewogen und verarbeitet wurden.

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Von den Kotproben wurden gewöhnlich 10 g in konzentrierter Salpetersäure gelöst. Dabei ist zu beachten, daß die Reaktion nicht zu stürmisch ist, da sonst die Masse zu schäumen beginnt und leicht überläuft. Nach längerem Erhitzen mit konzentrierter Salpetersäure wird auch hier eine klare, gefärbte Lösung erhalten.

Die Harnproben wurden in ihrer gesamten Menge verarbeitet. Der Harn wurde mit 50—100 ccm konzentrierter Salpetersäure versetzt. Dieses hat sehr vorsichtig zu erfolgen, da dann und wann sehr starkes Schäumen auftritt; wir ließen darum den Harn in kleinen Portionen zu der Säure zufließen. Das Säure-Harngemisch wurde dann in den Tropftrichter gebracht und nach und nach in die Kolben fließen gelassen, die mit etwa 5 ccm konzentrierter Schwefelsäure beschickt waren. Auch hier wurde mit dem Erhitzen aufgehört, wenn der gesamte Harn etwa auf ein Volumen von 10—15 ccm eingedampft war; das Eindampfen muß ständig beobachtet werden, da oft ohne ersichtlichen äußeren Grund heftiges Schäumen auftritt, das sich durch Zutropfen von etwas Wasser besänftigen ließ. Zu starkes Stoßen beim Kochen wurde durch einige wenige Siedesteine vermieden.

Das Fell reagierte sehr heftig mit Salpetersäure, indem der Übergang in Stickoxyde sehr plötzlich, mitunter unter Feuererscheinung vor sich ging; wir haben darum auch das Fell immer in größeren Schalen mit der Säure behandelt.

Das Fettgewebe mußte zuerst mit Schwefelsäure verkohlt werden, da bei der heftigen Nitrierung leicht Explosionen auftreten können.

Die auf die oben beschriebene Weise erhaltenen salpetersauren Lösungen, meist 100—150 ccm, wurden in größere Porzellantigel (hohe Form), aus denen sich mit Säuren keine Spuren Mn extrahieren ließen, übergeführt und auf dem Wasserbade möglichst weit eingeengt.

Der Rückstand wurde auf offener Flamme zur Trockne eingedampft und zur endgültigen Zerstörung der organischen Substanz verglüht; soweit die zu untersuchenden Substanzen mit Schwefelsäure versetzt waren, mußte diese natürlich vorsichtig abgeraucht werden. Bei sehr konsistentem Material, z. B. Darminhalt, blieben beim Verkohlen große Rückstände zurück, deren Bearbeitung Schwierigkeiten machte, darum wurden von diesen Proben nur kleine Teile zur Analyse verwendet.

Der geglühte Rückstand wurde mit konzentrierter Salzsäure ein wenig erwärmt, um etwa entstandenes Mangandioxyd wieder in Lösung zu bringen. Nach Zugabe von etwas Wasser wurde die salzsaure Lösung abermals auf dem Wasserbade zur Trockne eingedampft.

Dieser Rückstand der salzsauren Lösung wurde zur Entfernung des Chlorions mit konzentrierter Schwefelsäure (rund 2 ccm) versetzt und bis zum Auftreten der weißen Schwefelsäuredämpfe erwärmt. Es liegt nunmehr alles etwa vorhandene Mangan als Manganosulfat vor. Die Chlorionen müssen entfernt werden, da sie bei der folgenden Oxydation mit Bleisuperoxyd störend wirken.

Die schwefelsaure Lösung wurde schließlich in einen Erlenmeyer überführt, auf rund 50 ccm verdünnt, mit 5 ccm konzentrierter Salpetersäure versetzt und zum Sieden erhitzt. Dann wird vorsichtig, unter Wegnahme der Flamme, etwa 1 g Bleisuperoxyd zugesetzt und noch einige Minuten im Sieden erhalten. Hierbei erfolgt die Oxydation des Mangansulfats zum Permanganat. Das verwandte Bleisuperoxyd muß selbstverständlich manganfrei sein.

Bei den ersten Versuchen hat sich herausgestellt, daß die Veraschung mit Schwefelsäure und Salpetersäure — es wurden höchstens 5 ccm Schwefelsäure und sehr große Mengen Salpetersäure verwandt — keine vollständige ist. Größere Schwefelsäuremengen hätten vor der Oxydation des Mangans zum Permanganat entfernt werden müssen, da stark schwefelsaure Lösungen Permanganat unter Sauerstoffbildung zersetzen. Die Behandlung mit Salpetersäure war so lange fortgesetzt worden, bis die Lösung wasserklar war und beim Eindampfen bis zur starken Entwicklung von Schwefelsäuredämpfen keine Verfärbung (Verkohlung) eintrat. Wurde diese Lösung stark (auf das Zehnfache mindestens) verdünnt, etwa entstandene Nitrosylschwefelsäure und noch vorhandene Stickstoffoxyde durch Kochen entfernt und mit Permanganatlösung versetzt, so trat sehr oft sofort oder nach ganz kurzer Zeit Entfärbung ein. Eine Permanganatlösung, die die gleiche Schwefelsäuremenge enthält, behält ihre Farbe.

Wir konnten daher bei diesem einfachen Verfahren nicht bleiben und mußten zu den oben beschriebenen komplizierteren übergehen.

Die durch Oxydation erhaltenen Permanganatlösungen wurden durch ein Asbestfilter in einen gewöhnlich 50 ccm enthaltenden Meßkolben abfiltriert. Der verwendete Asbest wurde sorgfältig durch Ausglühen und Kochen mit Permanganatlösung, konzentrierter Salzsäure und Wasser gereinigt. Der Mangangehalt der filtrierten Lösungen wurde kolorimetrisch mittels des Autenriethschen Kolorimeters bestimmt.

Die Vergleichslösung (1 ccm = 0,05 mg Mn) wurde regelmäßig ebenso wie die zu untersuchende Lösung mit Salpetersäure und Bleisuperoxyd behandelt. Bei Proben, die sehr wenig Mn vermuten ließen, z. B. Harn und einigen Organproben, wurde vor der Oxydation eine bestimmte Menge Mn zugesetzt (meistens 0,25 mg Mn) und nachher das ursprünglich vorhandene Mn durch Differenzbestimmung fest-

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gestellt, da sich sehr geringe sowie sehr große Mengen Mn nur sehr schlecht im Kolorimeter bestimmen lassen[1]).

Da in den meisten Zellen Mangan vorhanden ist, mußten wir auch das Futter der Tiere analysieren; die Tiere wurden ausschließlich mit Rüben und Hafer gefüttert; in dem Rübenmark konnten wir kein Mn finden, im Saft kleine Spuren. Der Hafer enthielt etwas mehr, und zwar auf 1000 g einmal 30 mg, ein anderes Mal 27,5 mg Mn; diese Manganmengen sind aber belanglos, sie werden quantitativ mit den Fäzes ausgeschieden. Übrigens haben wir auch Versuche gemacht, wieviel Hafer und daher Mn die Tiere durchschnittlich fressen; es fraßen in 7 Tagen verschiedene Tiere 435, 420, 415, 380, 360, 355 und 345 g, also durchschnittlich 55 g pro Tag; da aber die kranken Tiere gelegentlich viel weniger fraßen, haben wir auch bei zweien die Hafermengen zu- und zurückgewogen.

Die subkutanen Injektionen wurden möglichst aseptisch vorgenommen, und zwar so, daß die erste links oben, die zweite rechts unten, die dritte rechts oben, die vierte links unten usw. vorgenommen wurde. Lokale Reaktionen haben wir nie beobachtet; wir haben auch stets bei der Sektion von der letzten und vorletzten Injektionsstelle Stücke (Fell, Unterhautzellgewebe, Muskulatur) entnommen und analysiert, ohne indes an den Stellen, an denen wir 10 mg injiziert hatten, mehr als Bruchteile eines Milligramms gefunden zu haben. Nur bei Tier 3 fanden wir in 23 g feuchter Substanz der Umgebung der Injektionsstelle 2,55 mg Mn. Es folgen nun zunächst die einzelnen Protokolle:

Kaninchen 1.

Behandelt mit dem aromatischen Komplexsalz; das Tier, weiblich, wiegt zu Beginn des Versuches 2350 g und erhält mit jeder Injektion 23 mg Mn in 2,3 ccm gelöst.

Datum	Gewicht in g	Injektion	Bemerkungen
21. I.	2350	Injektion	—
29. I.	2300		—
5. II.	2250		—
9. II.	2200		Frißt nicht.
15. II.	—	—	Gestorben.

Im ganzen wurden dem Tier also 92 mg Mn injiziert; die akut tödliche Dosis hätte 115 mg Mn betragen.

1) Vgl. S. 268.

Die Organanalysen ergaben folgendes:

Organe	Feuchtgewicht in g	Enthält Mn in mg
Leber	54,4	0,025
Nieren	13,7	0,012
Milz	0,4	0,05
Herz	6,9	0,025
Gehirn	6,6	0,05
Blut	—	0,40
Fell	230	0,875
Muskel	—	0
Lunge	10,1	0,012
Magenwand	28	0,075
Dünndarmwand	39	0,175
		Im ganzen 1,65

Die Sektion ergab makroskopisch keine wesentlichen Befunde.

Mikroskopische Befunde.

Leber: Leichte, bald mehr zentral, bald mehr peripher in dem Leberläppchen auftretende Verfettung der Leberzellen. Deutliche Verfettung der Kupfferschen Sternzellen. Leichte Bindegewebsvermehrung und dadurch Verbreiterung der Glissonschen Scheiden. In diesen sieht man vielfach Wucherungen kleiner Gallengänge, die eine sehr ausgesprochene Verfettung zeigen (Bild 1). Keine Nekrosen. Braunes, körniges Pigment in den Kupfferschen Sternzellen (mikrochemisch Eisen nachgewiesen).

Niere: Leichte Verfettung der Tubuli contorti an der Rindenmarkgrenze.

Milz: Hyperämie der Pulpa. In den weiten Sinus große runde Zellen, die vielfach deutlich Fettkörnchen und Pigmentkörnchen enthalten (Eisenreaktion).

Es wurden also in den Organen 1,6 mg Mn gefunden. Im Magen und Dünndarm 0,48 mg, im Dickdarm 0,48 mg; berechnen wir nach Analogie mit Kaninchen 5 bzw. 9, bloß um die Größenordnung festzustellen, daß im Kot etwa 54 mg ausgeschieden, in den Knochen 18 mg abgelagert wurden, dann können wir uns über 74,5 mg von den injizierten 92 Rechenschaft geben; einige Milligramme dürften ja auch nicht resorbiert worden sein.

Kaninchen 2.

Behandelt mit dem aromatischen Komplexsalz; das Tier, weiblich, wiegt zu Beginn des Versuchs 2820 g und erhält mit jeder Injektion 28 mg Mn in 2,8 ccm gelöst.

Datum	Gewicht in g	Injektion	Bemerkungen
21. I.	2820	Injektion	—
29. I.	2900		—
5. II.	2900		—
9. II.	2850		—
12. II.	2600		Durch Verbluten getötet.

Im ganzen wurden dem Tier also 112 mg Mn injiziert.

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Die Organanalysen ergaben folgendes:

Organe	Feuchtgewicht in g	Enthält Mn in mg
Leber . .	76,2	2.74
Nieren . .	16,4	0,225
Milz . . .	1,7	0,012
Herz . . .	6,6	0,012
Gehirn . .	6,9	0,175
Blut . . .	217	0,36
Fell . . .	280	0,975
Muskel . .	846	0
Lunge . .	10,6	0,05
Darmwand	50,4	0,40
	Im ganzen:	4,75

Die Analyse des Darminhalts ist mißlungen.
Der Sektionsbefund ergab nichts Besonderes.

Mikroskopischer Befund.

Leber: Mittelstarke, in der Läppchenperipherie angeordnete Verfettung der Leberzellen (vgl. Bild 2). Verfettung der Kupfferschen Sternzellen. Kein deutliches Pigment.
Niere: Nichts Wesentliches.
Milz: Nichts Wesentliches.

Kaninchen 3.

Behandelt mit Manganochlorid; das Tier, weiblich, wiegt zu Beginn des Versuchs 2350 g und erhält mit jeder Injektion 23,5 mg Mn in 4,7 ccm gelöst.

Datum	Gewicht in g	Injektion	Bemerkungen
21. I.	2350	Injektion	—
29. I.	—	»	—
5. II.	2150	»	—
9. II.	2200	»	—
12. II.	—	—	Durch Verbluten getötet.

Im ganzen wurden dem Tier also 94 mg Mn injiziert.

Die Organanalysen ergaben folgendes:

Organe	Feuchtgewicht in g	Enthält Mn in mg
Leber	71,5	0,00
Niere. . . .	11,6	0,05
Milz	1,5	0,05
Herz	5,8	0,06
Gehirn	6,5	0,075
Blut	40	0,05
Fell	—	2,07
Muskel	705	11,71
Lunge	8,7	0,025
Fett	14	0,075
Dickdarmwand	48	0,85
	Im ganzen:	15,32,

davon im Muskel 11,71 mg.
Im Dickdarminhalt wurden 1,3 mg Mn gefunden.
Die Sektion ergab makroskopisch nichts Wesentliches.

Mikroskopischer Befund.

Leber: Im ganzen fettarm, Zellen vielfach auffallend groß, vakuolig degeneriert. Einzelne verfettete Zellkomplexe in den Acini und in den Glissonschen Scheiden. Auffallend deutliche Verfettung der Kupfferschen Sternzellen. Keine gewucherten oder verfetteten Gallengänge. Keine Bindegewebsvermehrung.
Niere: Hier und da kleine Entzündungsherde im interstitiellen Bindegewebe. Sonst ohne Befund.
Milz: Auffallend blutreiche Pulpa. Im Lumen der Sinus große mit Pigmentkörnchen beladene Zellen (Eisenreaktion positiv).

Kaninchen 5.

Behandelt mit Manganochlorid; das Tier, weiblich, wiegt zu Beginn des Versuches 2120 g und erhält mit jeder Injektion 21 mg Mn in 4,2 ccm gelöst.

Datum	Gewicht in g	Injektion	Bemerkungen
21. I.	2120	Injektion	—
29. I.	1900	—	—
5. II.	2000	»	—
9. II.	1950	»	—
12. II.	1900	»	—
16. II.	1800	»	—
18. II.	1500	»	—
21. II.	—	—	Tot.

Im ganzen wurden dem Tier also 147 mg Mn injiziert.

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Die Organanalysen ergaben folgendes:

Organe	Feuchtgewicht in g	Enthält Mn in mg
Leber . .	59,5	1,57
Niere . .	16,8	0,625
Milz . .	1,4	0,075
Herz . .	5,0	0,025
Muskel .	640	4,14
Lunge .	13,1	0,175
Knochen	—	17,27
	Im ganzen:	23,88,

davon 17,27 mg im Knochen und 4,14 mg im Muskel.

Im Darminhalt wurden 2,15 mg, im Mageninhalt 0,97 mg Mn gefunden. Das Tier hat in dieser Zeit 720 g Hafer gefressen.

Sehr auffallend ist der hohe Mn-Gehalt des Knochens; da große Knochenstücke eine sehr massige Asche ergeben, konnten wir nur kleine Stücke analysieren. Es wurde 1,1 bzw. 1,0 g Röhrenknochen analysiert, der sorgfältig vom Knochenmark befreit war.

Der Sektionsbefund ergab makroskopisch nichts Wesentliches.

Mikroskopischer Befund.

Leber: Mäßig starke Verfettung der Leberzellen. Daneben fleckförmig verteilt hyalintropfige Degeneration und Nekrosen der Leberzellen (Bild 3). Sehr deutliche Verfettung neu gebildeter Gallengänge in den Glissonschen Scheiden. Verfettung der Kupfferschen Sternzellen. Bindegewebsvermehrung, ausgehend von den Glissonschen Scheiden. In den besser erhaltenen Teilen der Leberläppchen auffallend zahlreiche mitotische Kernteilungsfiguren der Leberzellen (Bild 4). Braunes Pigment in den Kupfferschen Sternzellen (Eisenreaktion positiv).

Niere: Ohne Befund.

Milz: Pulpa sehr dicht, nicht blutreich. Kein Pigment.

Kaninchen 4.

Behandelt mit Manganzitrat. Das Tier, weiblich, wiegt zu Beginn des Versuchs 2050 g und erhält mit jeder Injektion 20,5 mg Mn in 4,1 ccm gelöst.

Datum	Gewicht in g	Injektion	Bemerkungen
21. I.	2050	Injektion	—
29. I.	—	—	—
31. I.	—	—	Gestorben.

Im ganzen wurden dem Tier 41 mg Mn injiziert. Die Organanalyse ergab in der Leber 0,32 mg Mn, im Gehirn, Herz und Lungen je 0,05 mg

Mn, in der Niere und Milz nur unmeßbare Spuren. — Die Sektion ergab makroskopisch Vergrößerung und gelbe Verfärbung der Leber.

Mikroskopischer Befund.

Leber: Sehr starke die ganzen Läppchen betreffende Verfettung der Leberzellen. Wo die Fettansammlung nicht so extrem ist, sieht man wieder die Beteiligung der Kupfferschen Sternzellen. Kein Pigment. Keine Bindegewebsvermehrung.

Niere: Ohne Befund.

Milz: Ohne Befund.

Kaninchen 6.

Behandelt mit Manganzitrat. Das Tier, weiblich, wiegt zu Beginn des Versuchs 1750 g und erhält mit jeder Injektion 17,5 mg Mn in 3,5 ccm gelöst.

Datum	Gewicht in g	Injektion	Bemerkungen
21. I.	1750	Injektion	—
23. I.	—	—	Gestorben.

Im ganzen wurden dem Tier also 17,5 mg injiziert. Während die akut innerhalb weniger Stunden tödliche Dosis 87,5 mg betragen hätte. Bei den Organanalysen wurden in den Nieren, Gehirn, Milz und Herz unmeßbare Spuren von Mn nachgewiesen. Das Tier wurde nicht obduziert.

Kaninchen 9.

Behandelt mit dem aromatischen Komplexsalz. Das Tier, weiblich, wiegt zu Beginn des Versuchs 1600 g und erhält mit jeder Injektion 16 mg Mn in 1,6 ccm gelöst. Von diesem Tiere wurden die Ausscheidungen untersucht.

Datum	(2) Fraß Hafer	(3) Gewicht in g	(4) Injektion mg Mn	(5) Schied Kot aus	(6) Im Kot mg Mn	Schied. Harn aus in ccm	Im Harn mg Mn
23. II.	vom 23. II. bis	1600	16	vom 23. II. bis	13,5	270	0,15
23. II.	2. III. 250 g	—	16	2. III. 57 g		—	—
2. III.	vom 2. III. bis	1600	16	vom 2. III. bis	26,9	230	0,10
5. III.	9. III. 321 g	1550	16	9. III. 117 g		—	—
9. III.	vom 9. III. bis	1550	16	vom 9. III. bis	17,4	180	0,10
12. III.	16. III. 220 g	1540	16	16. III. 105 g		—	—
16. III.				gestorben (9)			

Aufgenommen wurden durch Injektion 96 mg Mn, im Futter 23,7 mg Mn; ausgeschieden wurden im Kot 57,8 mg, im Harn 0,35 mg Mn.

Organanalysen wurden bei diesem Tier nicht gemacht.

Die Sektion ergab makroskopisch nichts Wesentliches.

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Mikroskopischer Befund.

Leber: Leberzellen selbst im allgemeinen fettfrei, aber sehr deutliche Verfettung der Kupfferschen Sternzellen. Reichlich Pigment in den Kupfferschen Sternzellen (Eisenreaktion positiv). Glissonsche Scheiden zum Teil leicht verdickt und mit Lymphocyten infiltriert.
Milz: Pulpa sehr blutreich. In den weiten Sinus zahlreiche große runde Zellen, die braune Pigmentkörnchen enthalten.
Nieren: Kleine Schrumpfungsherdchen entzündlicher Natur.
Herz: Ohne Befund.

Kaninchen 10.

Behandelt mit Manganochlorid. Das Tier, weiblich, wiegt zu Beginn des Versuchs 1600 g und erhält mit jeder Injektion 16 mg Mn in 3,2 ccm gelöst. Organanalysen wurden auch bei diesem Tiere nicht gemacht, nur Analysen der Ausscheidungen.

Datum	Fraß Hafer	Gewicht in g	Injektion mg Mn	Schied Kot aus	Im Kot mg Mn	Schied Harn aus in ccm	Im Harn mg Mn
23. II.	vom 23. II. bis	1600	16	vom 23. II. bis	20,4	400	0,15
26. II.	2. III. 337 g	—	16	2. III. 96 g		—	—
2. III.	vom 2. III. bis	1600	16	vom 2. III. bis	25,5	625	0,08
5. III.	10. III. 265 g	1520	16	10. III. 103 g		—	—
9. III.	—	1490	—	—		—	—
10. III.				gestorben			

Aufgenommen wurden durch Injektion 64 mg Mn, im Futter 18,06 mg Mn; ausgeschieden wurden im Kot 45,9 mg, im Harn 0,23 mg.

Die Sektion ergab makroskopisch eine deutlich vergrößerte Fettleber.

Mikroskopischer Befund.

Leber: Sehr starke, ganz diffuse Verfettung der Leberzellen. Stellenweise deutlich nachweisbare Beteiligung der Kupfferschen Sternzellen. Wenig Pigment in den Kupfferschen Sternzellen.
Niere: Deutliche Verfettung der basalen Teile der Epithelien der Tubuli contorti und recti.
Herz: Geringe, aber deutliche Verfettung der Herzmuskelfasern.
Milz: Sehr blutreich. Zwischen den weiten Sinus ein auffallend großzelliges, anscheinend hyperplastisches Pulpagewebe. Kein Pigment.

Dies die Versuchsprotokolle. Vergleichsweise seien nun in der folgenden Tabelle die Mengen Mangan zusammengestellt, die in den verschiedenen Organen gefunden wurden, und zwar bedeuten die Zahlen Milligramm Mn auf 1000 g feuchtes Organ.

Organe	Aromatisches Komplexsalz Kaninchen 1 gestorben	Kaninchen 2 getötet	$MnCl_2$ Kaninchen 3 getötet	Kaninchen 5 gestorben
Leber	0,458	35,82	0,0	26,42
Niere	9,63	13,72	4,31	37,20
Milz	125	7,36	33,33	52,60
Herz	3,62	1,89	10,76	5,0
Gehirn	15,14	25,38	11,54	—
Blut	—	1,67	1,25	—
Fell	3,64	3,48	7,35	—
Muskel	0	0	16,68	6,56
Lunge	1,24	4,72	2,87	13,35
Knochen	—	—	—	68,17
Dickdarmwand	—	8,0	17,7	—
Galle	75	25	92,5	—
Ascites	—	40	—	40
Eiter	37,5	—	—	—

Aus diesen chronischen Vergiftungsversuchen wollen wir folgendes hervorheben:

1. Um Tiere im chronischen Versuch zu töten, sind insgesamt meist geringere Mn-Mengen nötig, als um sie akut zu töten; so stirbt Kaninchen 1 nach 40 mg/kg, Kaninchen 5 nach 70 mg/kg, Kaninchen 9 nach 40 mg/kg, Kaninchen 10 nach 30 mg/kg, während die akut tödliche Dosis 50 mg/kg beträgt.

2. Das Zitrat ist im chronischen Versuch wesentlich stärker giftig; das hängt wohl mit der schnelleren Resorption zusammen; dafür spricht auch, daß Kaninchen 4 die stärksten pathologischen Veränderungen aufweist, obwohl es nur noch 10 Tage gelebt hat; es genügte die Dosis nicht, um eine akute Wirkung hervorzurufen, aber die chronische wurde beschleunigt.

3. Das injizierte Mangan wird sehr schnell zu ungefähr 50% im Kot ausgeschieden; im Harn erscheinen auch bei langandauernden Versuchen nur Spuren, die Niere ist also mangandicht. Das gleiche beobachteten Reimann, Clarence und Minot[1]) an manganvergifteten Menschen (vgl. unten).

4. Auffallend ist zunächst die große Menge Mangan, die man in Galle, Ascites und Eiter findet. Der Körper entledigt sich also des fremden Metalls auf diesen Wegen wesentlich leichter als durch die Nieren. Permeabilitätsänderungen der Gefäßkapillaren bei pathologischen Vorgängen machen die Gefäße für die Schwermetalle leichter

1) Journ. of biol. chem. 1920, Bd. 42, S. 329; Bd. 45, S. 133.

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durchgängig. Sehr groß sind die Manganmengen, die in der Knochensubstanz gefunden wurden. Hier disponiert ja auch die große Menge Karbonat und Phosphat zur Niederschlagung von Metall, und man müßte der Untersuchung der Knochen bei Metallvergiftungen größeres Augenmerk zuwenden als bisher.

5. Die Menge des in den einzelnen Organen deponierten Mangans scheint für den Eintritt des Todes und die anatomischen Veränderungen des entsprechenden Organs nicht wesentlich zu sein; unzweifelhaft gibt es Organe, in denen viel Mangan abgelagert wird, vor allem die Milz und das Gehirn; dann gibt es solche, in denen der Mangangehalt sehr wechselt, z. B. die Leber, und schließlich Organe, in denen sehr wenig vorkommt, z. B. die Lunge. Warum in der einen Leber mehr, in der anderen weniger abgelagert ist, konnten wir nicht ergründen; es muß sich um lokale Ursachen in den einzelnen Organen handeln; der einzige Unterschied zwischen Organen, die Mn gespeichert haben, und solchen, die nicht, den wir beobachten konnten, ist ein verschiedenes Verhalten der geschnittenen fixierten Präparate im Dunkelfeld; bei den Lebern, bei denen kein Mangan vorgefunden wurde, konnten wir im Dunkelfeld das sonst regelmäßig zu sehende Aufleuchten der Konturen der Zellkerne nicht beobachten. Wir haben auch von gleichbehandelten Tieren jeweils ein Tier getötet und eines bis zum spontanen Tod am Leben gelassen, um zu sehen, ob auf diese Weise vielleicht eine Wanderung des Mangans nachweisbar wäre; es sind nun wohl die vorgefundenen Mengen in den Organen der getöteten Tiere andere als in denen der gestorbenen. Aber diese Veränderungen sind nicht gleichsinnig, so daß sie keinerlei Schlüsse gestatten. Auffallend ist, daß im Muskel nur das als Chlorid injizierte Mn wiedergefunden wurde, bei Injektionen des organischen Komplexsalzes wurde im Muskel kein Mn gefunden.

Auch aus Lomholts[1]) Zahlen, der die Verteilung von verschiedenen Hg-, Bi-, und Pb-Verbindungen im Kaninchenorganismus nach subkutanen Injektionen untersucht und eine viel regelmäßigere Verteilung gefunden hat, als wir beim Mn, kann man kaum eine Beziehung zwischen Giftmenge und Schädigung der einzelnen Organe erkennen. So fand er bei zwei Bi-Verbindungen eine wesentliche Anhäufung in der Niere, von Hg-Verbindungen war aber bloß benzoesaures Hg in der Niere in größeren Mengen zu finden, während das bei dem salizylsauren Hg ebensowenig ausgesprochen war wie beim HgCl und beim 50%igen Hg-Öl. Die Menge des in den einzelnen

1) Biochem. journ. 1924, Bd. 18, S. 69.

Organen beim Tode vorgefundenen Mangans — und dasselbe dürfte auch für andere Schwermetalle gelten — ist also wohl von lokalen Bedingungen in den einzelnen Organen abhängig und für das gesamte Vergiftungsbild von untergeordneter Bedeutung. Von Interesse ist die Übereinstimmung unserer Befunde mit denen, die die erwähnten amerikanischen Forscher Reimann, Clarence und Minot an manganvergifteten Menschen gefunden haben; diese Forscher haben, wie gelegentlich andere auch, großzügige Untersuchungen an Arbeitern in Hüttenwerken angestellt; sie beobachteten dabei, daß der Mangangehalt des normalen menschlichen Blutes, der im Durchschnitt 1 mg pro 1000 g beträgt, bei einzelnen stark vergifteten Personen bis auf das Doppelte vermehrt ist, aber nur bei einzelnen; im Harn fanden auch sie stets nur Spuren; bei Patienten mit Gallenfisteln, denen sie 0,3—0,5 g Mn per os eingaben, stieg der Mn-Gehalt der Galle, der normalerweise etwa 0,3 mg pro 1000 g beträgt, bis zu 1 mg pro 1000 g. Bei Verfütterung von größeren Mengen des etwa 10% Mn enthaltenden Erzes Franklinit traten nur bei besonders disponiert erscheinenden Personen Vergiftungen auf. Bei unseren systematischen Vergiftungen waren die gefundenen Mengen natürlich wesentlich größer; in normalen Kaninchenorganen konnten wir niemals 0,01 mg Mn nachweisen; allerdings stehen ja von einem Tier immer nur wenige Gramme feuchte Organe zur Verfügung.

6. Eine gewisse Einheitlichkeit kommt in den pathologisch-histologischen Befunden zum Ausdruck: 1. Die in den Kupfferschen Sternzellen beginnende und sich dann auf die übrigen Leberzellen erstreckende Verfettung, in einzelnen Fällen mit Verfettung in Niere und Herz kombiniert. In einem Falle (5) war die Fettablagerung von deutlichen degenerativen Prozessen in der Leber begleitet. Mitosen in den erhaltenen Zellen deuteten auf lebhafte regenerative Prozesse hin. 2. Die Blutkörperchenzerstörung, die sich in der Hämosiderinbildung äußert. Allerdings müssen wir darauf hinweisen, daß diese Befunde mit denen von G. M. Findlay[1]), die uns erst vor kurzem zu Gesicht kamen, nicht übereinstimmen. Findlay injizierte Kaninchen, Meerschweinchen und Ratten jeden zweiten Tag 3—5 mg Manganochlorid. Die Kaninchen lebten 5—9 Wochen und zeigten keine Verfettung, wohl aber äußerst intensive Cirrhosen der Leber. Der Unterschied läßt sich vielleicht dadurch erklären, daß unsere Tiere schneller vergiftet wurden und darum auch viel früher starben; nur bei Kaninchen 5 liegen histologische Veränderungen vor, die mit den Be-

1) Brit. journ. of exp. pathol. 1924, Bd. 5, S. 92.

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funden von Findlay vielleicht übereinstimmen oder wenigstens Vorstufen von ihnen sind.

7. Wir können auch über Versuche an Meerschweinchen berichten, die mit subletalen Dosen vergiftet wurden. Diese Tiere lebten mitunter einige Monate, blieben im Gewicht stark zurück und zeigten in der Leber ähnliche Veränderungen wie die chronisch vergifteten. Als Beispiele seien folgende Protokolle angeführt:

Meerschweinchen 13 ($MnCl_2$).

15. I.: 320 g Gewicht, erhält 14,5 mg Mn.
20. III.: 300 g Gewicht, tot aufgefunden (gleichalterige unbehandelte Tiere wogen 500—600 g).

Meerschweinchen 17 (aromatisches Komplexsalz).

21. I.: 350 g Gewicht, erhält 17,5 mg Mn.
8. IV.: tot aufgefunden, 375 g Gewicht.

8. Der Mechanismus der Wirkung des Mangans ist also bei der akuten und bei der chronischen Vergiftung grundsätzlich verschieden, und dasselbe gilt mutatis mutandis, wie wir aus anderen Versuchen vorwegnehmen können, auch für die Wirkung anderer Schwermetalle. Bei der akuten Vergiftung entsteht eine aufsteigende Lähmung des Zentralnervensystems; eine solche ist an den akuten Giftwirkungen aller Schwermetalle beteiligt; ebenso steht das isolierte Herz nach Behandlung mit allen Schwermetallen still. Die akute Vergiftung kommt nur zustande, wenn im Blute auf einmal eine bestimmte sehr hohe Mangankonzentration vorhanden ist. Wird diese hohe Konzentration nicht erreicht, dann konnten wir an unserem Tiermaterial nervöse Störungen nicht beobachten; es kommt vielmehr dann gleich zu Vergiftungen in der Leber; der Verlauf dieser Vergiftungen ist ein chronischer und, wie wir an unseren Meerschweinchen, die subletale Dosen bekamen, beobachteten, irreversibel. Die Geschwindigkeit der chronischen Vergiftung hängt, wie wir insbesondere in Versuchen mit anderen Metallen feststellen konnten, von der in der Zeiteinheit resorbierten Metallmenge ab; diese Geschwindigkeit der Resorption ist wieder von der Art der Bindung des Metalls im Gesamtmolekül des Giftes abhängig. Das Verbleiben des Metalls in den einzelnen Organen scheint von lokalen Bedingungen abzuhängen und ist für den Ausfall der Giftwirkungen belanglos; es kommt eben auf die Störung der Funktion an.

Die chronische Vergiftung greift innerhalb der Leber zuerst in den Kupfferschen Sternzellen an. Damit können vielleicht die sero-

logischen Beobachtungen bei der Anwendung des Mangans zur Behandlung von Infektionskrankheiten in Zusammenhang gebracht werden. Walbum[1]) und seine Mitarbeiter haben beobachtet, daß gegen Diphtherie immunisierte Pferde sich durch Injektion von Manganochlorid auf eine Antitoxinkonzentration des Blutserums bringen lassen, die größer war als die, welche ursprünglich durch die gewöhnliche Immunisierung mit Toxin allein erzielt worden war. Auch die Ambozeptorbildung läßt sich von allen Schwermetallen am meisten durch Manganochlorid befördern. Mit der gesteigerten Antikörperbildung oder doch Ausschwemmung der Antikörper aus den Sternzellen der Leber dürfte vielleicht die gelegentlich beschriebene therapeutische Wirkung von Mn-Verbindungen bei Furunkulose und anderen infektiösen Erkrankungen — in letzter Zeit wird ja auch die Tuberkulose[2]) genannt — zusammenhängen.

1) Zeitschr. f. Immunitätsforsch. u. exp. Therapie, Orig. 1925, Bd. 42, S. 32.
2) Walbum, Dtsch. med. Wochenschr. 1925.

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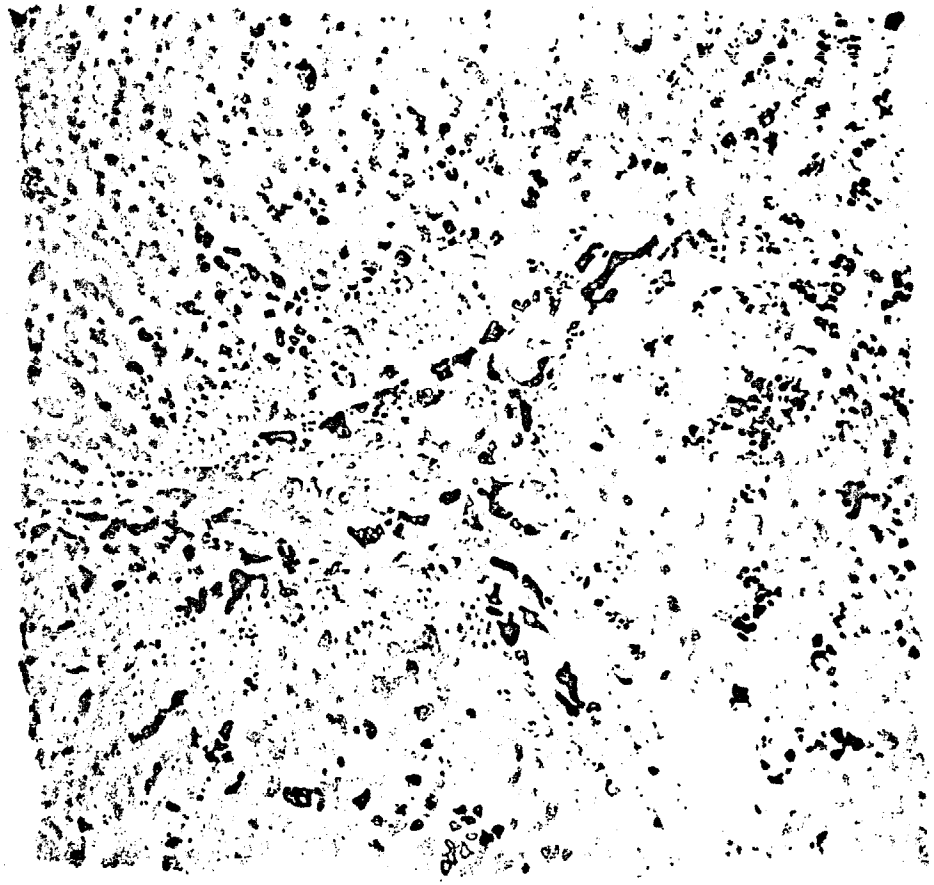

Fig. 1.

Abb. 1. Verteilung gewucherter Gallengänge in der [illegible] Schichten der Leber. Verteilung von Kupfferschen Sternzellen [illegible] Einzelzellen. Kaninchen 1. Sudan Fuchsin.

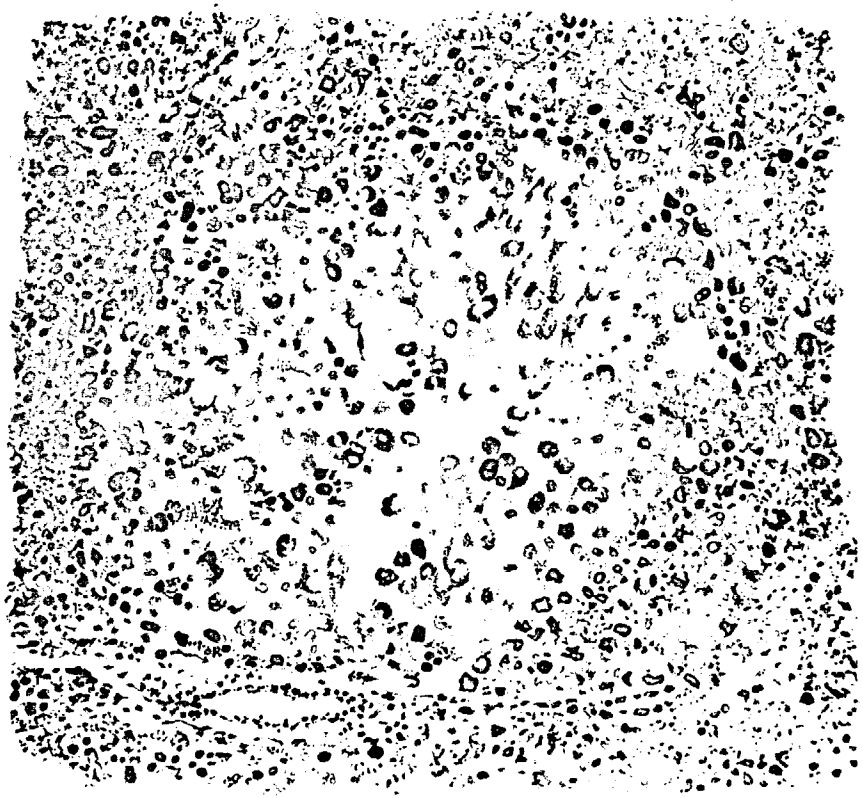

Fig. 2.

Abb. 2. Periphere Verteilung der Leber mit Verteilung der Kupfferschen Sternzellen. Kaninchen 2. Sudan Färbung.

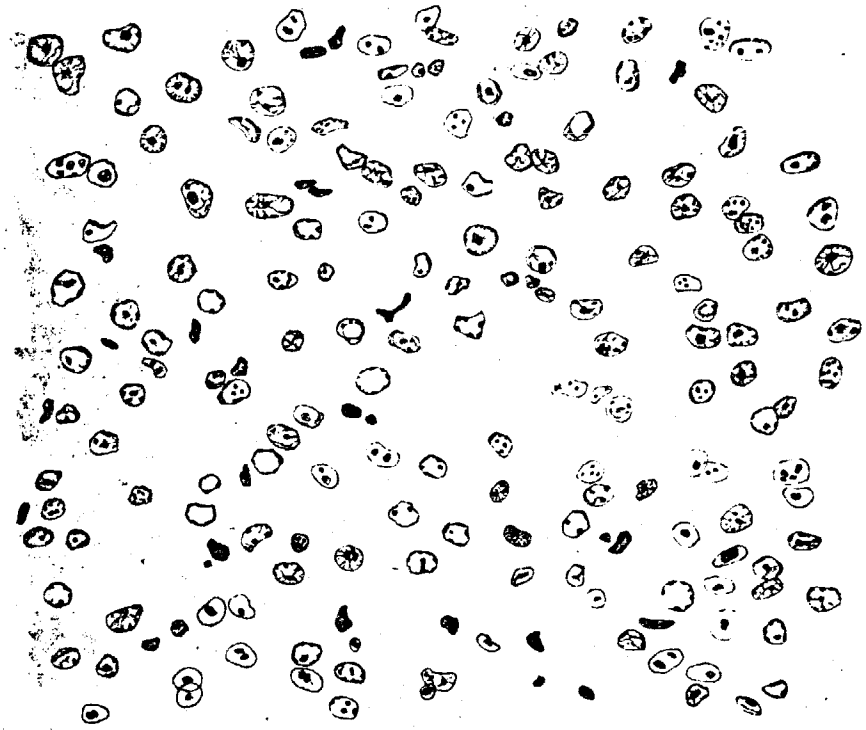

Fig. 3. *Abb. 3. Degenerationsherd aus der Leber bei Kaninchen 5. Kernlose, z. T. in Zerfall begriffene Leberzellen. Quellung, blasse Färbung vieler Kerne. Starke Vergrösserung.*

Fig. 4. *Abb. 4. Mitosen aus der Leber bei Kaninchen 5. Aus der Umgebung eines Degenerationsherdes. Starke Vergrösserung.*

Handovsl f. Schulz, Stämmler. Verlag von F. C. W. Vogel in Leipzig.

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EFFECT OF HIGH DIETARY MANGANESE ON HEMOGLOBIN FORMATION [1, 2]

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J. of Nutr. 57:429-437, 1955

FOUR FIGURES

(Received for publication July 2, 1955)

Effects of different dietary levels of manganese are of interest because of the evidence of antagonisms existing between this mineral and other elements and because of the reports of extreme and variable concentrations of manganese in forages (Beeson and Matrone, '50; Beeson, '54; Blakemore et al., '37; Russell, '44; Stuart et al., '50). Previous work (Hawkins et al., '55) indicated that feeding high levels of manganese to the calf affected the concentration of serum magnesium and interfered with calcium and phosphorus metabolism. Subsequent studies, in which lambs were used, revealed an apparent interference of manganese with iron utilization. Data from these studies form the basis for the present report.

EXPERIMENTAL

The effects of manganese on hemoglobin formation were investigated in two experiments. The first was conducted with lambs restricted to a fortified milk diet and the second, with lambs fed a roughage diet.

[1] Published with the approval of the Director of Research, N. C. Agricultural Experiment Station, as paper No. 650 of the Journal Series. The data were taken from a thesis submitted by the senior author in partial fulfillment of the requirements for the Master of Science degree.

[2] Supported in part by the Tennessee Valley Authority, Knoxville, Tennessee.

Experiment 1

Procedure. Eight ewe and 8 wether lambs, ranging in age from 7 to 19 days, were the test subjects. The lambs were assigned to individual wooden pens having elevated floors of aluminum-painted wire screen. During a preliminary period of one month, these lambs were fed fresh cow's milk, from nippled bottles, at a rate of one pound of milk per 5 pounds of body weight daily. Additional vitamins, A and D, were administered in capsules (5000 I.U. of vitamin A and 500 I.U. of vitamin D per capsule [3]) on alternate days.

TABLE 1

Levels of manganese added to the basal diet at different periods in the experiment

PERIODS		MANGANESE SUPPLEMENTATION [1]			
No.	Weeks	Mn_0	Mn_1	Mn_2	Mn_3
		ppm	*ppm*	*ppm*	*ppm*
1	0 – 12	0	5	15	45
2	12 – 20	0	5	30	90
3	20 – 24	0	–	450	900
4	24 – 35	0	–	2500	5000

[1] Supplements of manganese ($MnSO_4 \cdot H_2O$) were added to the whole milk diets on the basis of the total solids content.

The principal component of the experimental diets was whole milk, which was fortified with 50 ppm of iron,[4] 5 ppm of copper,[4] and 0.1 ppm of cobalt [4] on a dry matter basis. As shown in table 1, the dietary treatments consisted of 4 levels of manganese, designated as Mn_0, Mn_1, Mn_2, and Mn_3, during periods 1 and 2, and three levels, Mn_0, Mn_2, and Mn_3, during periods 3 and 4. The supplements of manganese were increased gradually in successive periods. Two ewe and two wether lambs were randomly assigned to each experimental diet. Because of the death of three test lambs (two on Mn_0 and one on Mn_1) during periods 1 and 2, the Mn_1 treatment

[3] Code No. 200 capsules supplied through the courtesy of the Gelatin Products Division of R. P. Scherer Corporation, Detroit, Mich.

[4] $FeSO_4.H_2O$, $CuSO_4.5H_2O$ and $Co(C_2H_3O_2)_2.4H_2O$ were the compounds used.

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was discontinued after period 2. The lambs on this treatment were reassigned to the remaining diets. The amount of the diet offered was adjusted to body weight at weekly intervals until the maximum intake was reached. From this time until the end of the experiment the lambs were fed as much as they would consume at each feeding.

Responses to the treatment were evaluated from data on the hemoglobin concentrations of the whole blood and the iron and copper concentrations in the blood serum. Samples of blood were collected from the jugular vein of the lambs before the experimental regimen was initiated and at regular two-week intervals thereafter. Hemoglobin concentrations were determined by the method described by Shenk et al. ('34). Iron and copper were measured by a method described by Matrone et al., ('47). At the end of the experiment, 6 wether lambs, two from each treatment, were slaughtered. Samples of the liver, kidney, spleen, and heart were analyzed for iron and copper.

RESULTS

Hemoglobin. As shown in figure 1, the mean biweekly concentrations of hemoglobin of the test lambs receiving the Mn_3 level of manganese were lower than those of the lambs fed the other levels. An analysis of variance of these data indicated that the difference between the highest level, Mn_3, and the other levels was significant ($P \leq 0.01$) in all periods. A further analysis of the data indicated that there was no significant difference among treatments Mn_0, Mn_1 and Mn_2 in any of the periods.

Iron. The serum iron concentrations for the lambs on treatment Mn_3 decreased during period 1 and had the lowest mean concentration during period 2, as shown in figure 2. An analysis of the linear regression coefficients, measuring the change in serum iron with time, revealed a significant ($P \leq 0.05$) linear difference among diets for period 1. This difference is interpreted to mean that the level of serum iron of the animals on treatments Mn_0, Mn_1, and Mn_2 increased

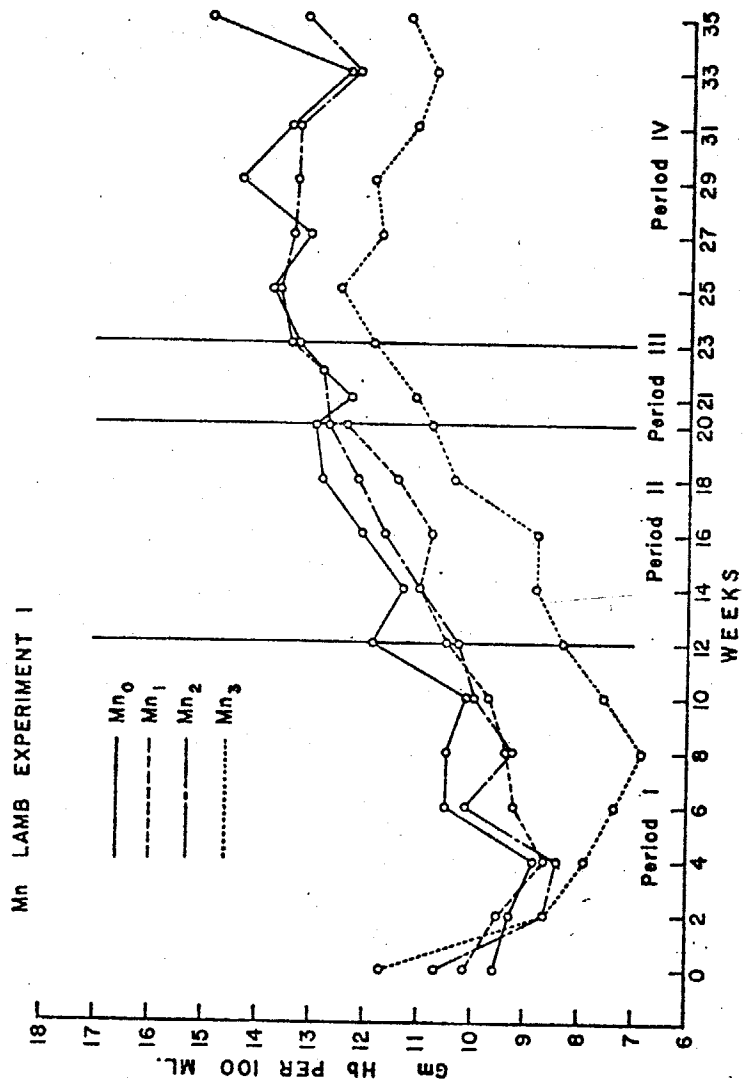

Fig. 1 Mean values of hemoglobin of the lambs fed various levels of manganese during the 4 periods.

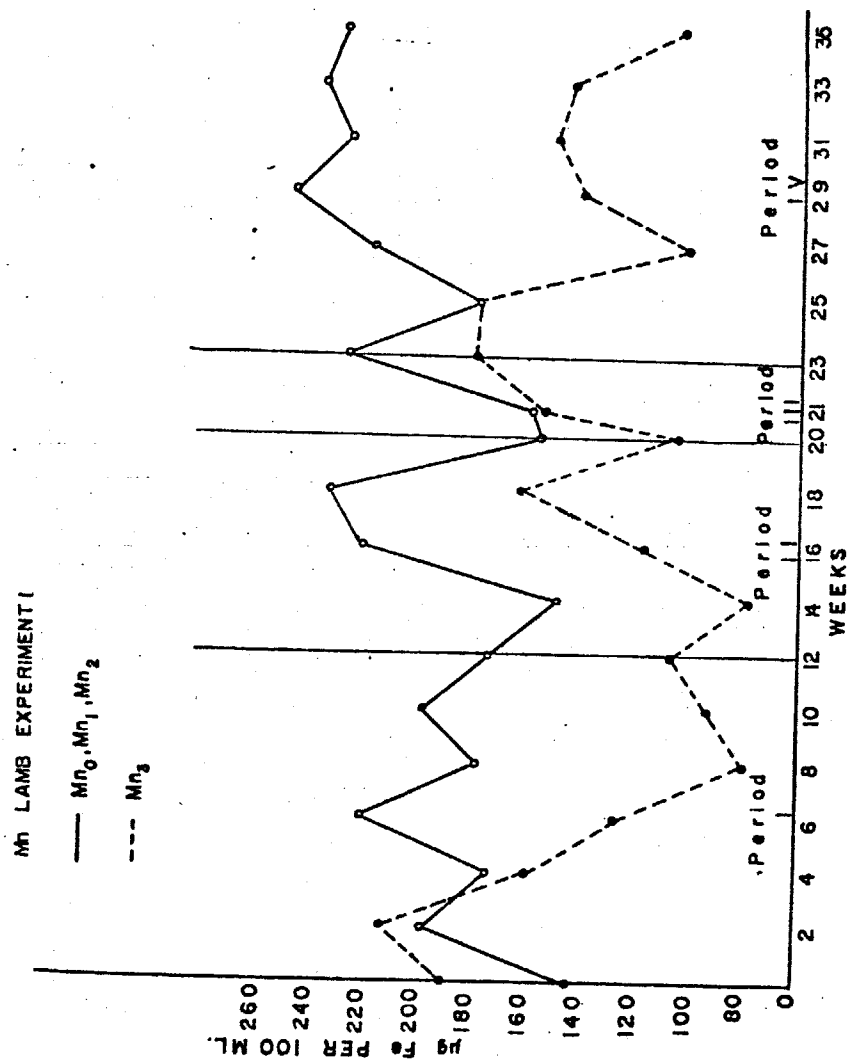

Fig. 2 Mean values of serum iron of the lambs fed various levels of manganese during the 4 periods. Since the responses to the Mn_0, Mn_1 and Mn_2 were not significantly different, the serum iron values were combined into one curve.

with time, whereas the serum iron of animals on Mn_3 decreased. Thus, the highest intake of manganese was associated with the lowest serum iron value.

Copper. The experimental treatments had no significant effect on the serum copper concentrations of the lambs in any of the periods. The mean concentration of serum copper for all experimental lambs increased with each successive period; the mean values during periods 1, 2, 3 and 4 were 69, 112, 123 and 142 µg per 100 ml of serum, respectively.

TABLE 2

Concentrations of iron and copper in the liver, kidney, spleen and heart of the lambs (experiment 1)

DIETARY TREATMENT [1]	CONCENTRATION OF IRON AND COPPER IN ORGANS							
	Liver		Kidney		Spleen		Heart	
	Fe	Cu	Fe	Cu	Fe	Cu	Fe	Cu
	µg per gm dry weight							
Mn_0	178	622	173	29	803	11	156	29
Mn_0	152	734	187	39	879	8	122	14
Mn_2	110	1185	110	27	198	8	125	18
Mn_1	94	1251	125	19	202	3	109	18
Mn_2	94	2751	23	22	171	9	65	7
Mn_3	99	2521	174	28	236	18	123	13

[1] Each treatment represents the values obtained from one lamb.

Composition of organs. The results of the analysis of the organs are shown in table 2. From these data it is apparent that the high dietary levels of manganese were related to decreased concentrations of iron in the organs. In the liver the decrease in the concentration of iron was associated with an increase in the concentration of copper.

Experiment 2

Procedure. Eight lambs from 6 to 9 days of age were nursed on unsupplemented cow's milk for approximately two months prior to feeding of the experimental diets. During the second month of this preliminary period, the lambs were bled periodically until the concentration of hemoglobin de-

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creased to 4 to 6 gm per 100 ml of whole blood, at which stage they were placed on experiment.

The basal diet of the experiment consisted of 60% excellent quality chopped soybean hay, 32% glucose,[5] 4% casein, and 4% cottonseed oil.[6] The three manganese treatments under study were: (1) basal, (2) basal + 1000 ppm of Mn, and (3) basal + 2000 ppm Mn (hereafter referred to as Mn'_0, Mn'_2 and Mn'_3). The lambs were randomly assigned to the diets. Three lambs received the Mn'_0, three the Mn'_3 and two the Mn'_2 diet.

As the animals increased their consumption of the experimental diets, the whole milk was reduced gradually to 500 ml per day and the roughage diets were increased to the level of one pound per day per lamb. One thousand parts per million of manganese, based on the dry matter content, were added to the milk supplement of these lambs receiving the Mn'_2 and Mn'_3 diets. Because of this procedure the Mn'_3 diet contained somewhat less (1986 ppm) than 2000 ppm of manganese. This small discrepancy was ignored in the results presented.

Each lamb remained on the experimental treatments for 11 weeks. Observations on the concentrations of blood hemoglobin, serum iron, and serum copper were made immediately before feeding the test diets, throughout the feeding period and for a period of three weeks after the manganese additions were discontinued.

Results

Hemoglobin. As shown in figure 3, the weekly mean concentrations of hemoglobin in blood of lambs fed the basal diet increased more rapidly than in animals receiving either the 1000 ppm of manganese or the 2000 ppm. These data indicate that feeding high levels of manganese retarded the regeneration of hemoglobin. It is apparent, however, that

[5] "Cerelose," Corn Products Refining Company, New York, N. Y.
[6] Wesson Oil and Snowdrift Sales Company, New Orleans, La.

the difference between the treatments of 1000 ppm and of 2000 ppm manganese was negligible.

Iron. The biweekly mean values of serum iron from the second through the 14th week are shown in figure 4. The lambs receiving 1000 ppm and those receiving 2000 ppm of manganese had lower concentrations of serum iron than those fed the basal diet. There was no significant difference, however,

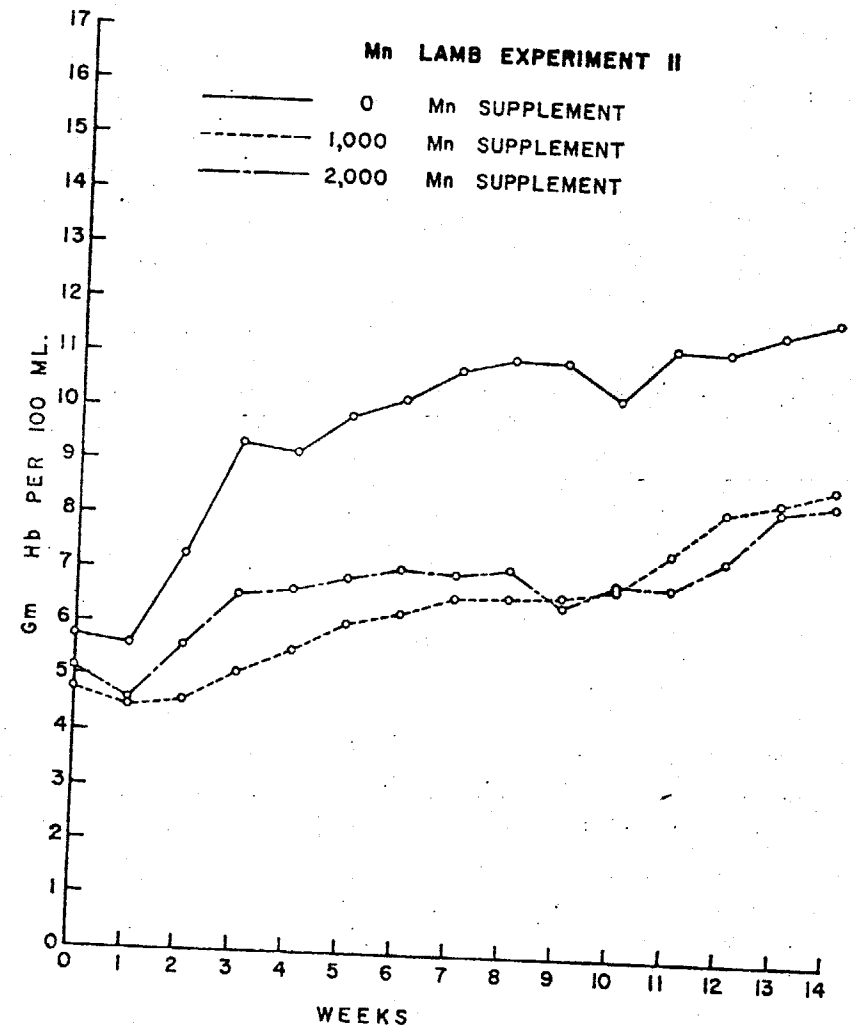

Fig. 3 Mean values of hemoglobin of the lambs fed three levels of manganese.

between the concentrations of serum iron from the 1000 and the 2000 ppm manganese treatments. The removal of supplemental manganese from the diets (11th week) was followed by a rise in the level of serum iron of the lambs previously fed manganese, but this response occurred earlier in the group fed 1000 ppm manganese than in the group fed 2000 ppm.

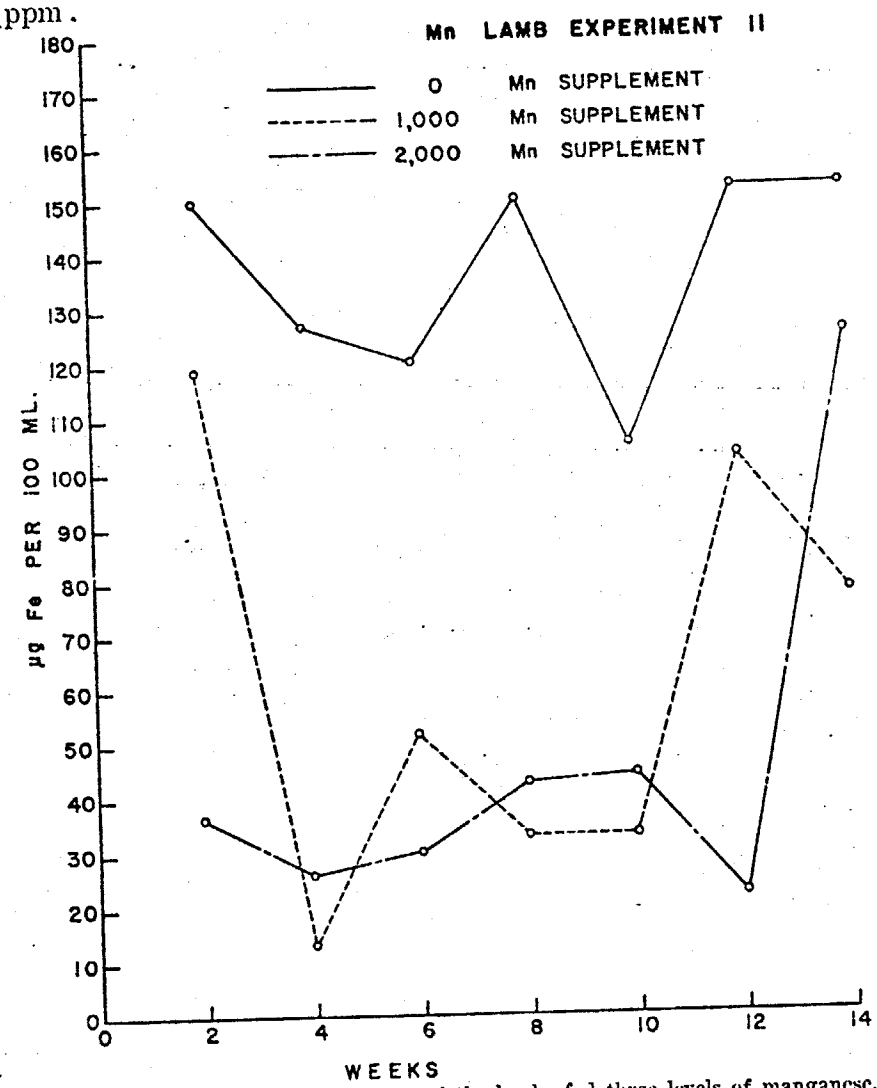

Fig. 4 Mean values of serum iron of the lambs fed three levels of manganese.

Copper. The concentrations of serum copper showed no effects ascribable to the manganese treatments. The mean serum copper concentration of all the lambs decreased from 84 µg per 100 ml of serum at the beginning of the experiment to 55 µg per 100 ml of serum on the 12th week.

DISCUSSION

When lambs depleted of iron were fed this element either as an inorganic salt in the milk diet or as a natural constituent of a roughage diet, the presence of excessive manganese depressed or retarded hemoglobin formation but had no perceptible effect on productive functions, including growth. The adverse hematopoietic responses may be explained in several ways: the excessive manganese may interfere with (1) the absorption of iron, (2) the formation of hemoglobin, and (3) the combination of (1) and (2).

Since the concentrations of iron in the sera, livers, and spleens of lambs fed appreciable quantities of manganese were low, the probabilities are that manganese interfered with iron absorption rather than with hemoglobin formation *per se.* A further indication that iron absorption was reduced in lambs fed manganese was the decreased concentration of iron in the liver accompanied by an increase in copper stores, a relationship synptomatic of iron deficiency (Hahn and Fairman, '36). It is suggested that excessive manganese either converts iron to a form physiologically unavailable or in some manner antagonizes the enzyme systems that oxidize or reduce iron at the site of absorption. Further studies on the mode of action of excessive manganese in the utilization of iron are being conducted.

SUMMARY

In two experiments young growing lambs were depleted of iron either by an iron deficient diet or by a combination of the diet and phlebotomy. In the first experiment, a whole milk diet fortified with iron, copper, cobalt and vitamins A

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and D was supplemented with various levels of manganese. A level as low as 45 ppm of manganese fed to young lambs brought about a decrease in the concentration of hemoglobin and of serum iron. Higher levels of manganese, up to 5000 ppm, were associated with decreased concentrations of iron in the liver, spleen and kidney.

In the second experiment anemic lambs were fed a roughage diet supplemented with three levels of manganese, 0, 1000, and 2000 ppm. Hemoglobin regeneration was markedly retarded and serum iron depressed in lambs fed diets containing either 1000 ppm or 2000 ppm of manganese.

The data indicate that manganese interferes with iron absorption rather than with hematopoiesis. It is postulated that excessive manganese antagonizes the enzyme systems that oxidize or reduce iron at the site of absorption.

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Am. J. Physiol., 211: 207-210, 1966

Tissue concentrations of manganese and adrenal function[1]

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HUGHES, E. R., S. T. MILLER, AND G. C. COTZIAS. *Tissue concentrations of manganese and adrenal function.* Am. J. Physiol. 211(1): 207–210. 1966.—The possible endocrine regulation of manganese metabolism was investigated by studying the effects of ACTH or cortisol administration, and those of adrenalectomy, on the excretion and tissue distribution of radioactive and stable manganese (Mn^{54} and Mn^{55}). Adrenal cortical stimulation with ACTH led to a shift of radioisotope from the liver to the carcass, similar to that described after administration of glucocorticoid hormones. The concentration of stable manganese (Mn^{55}) was measured as a function of dietary intake by means of neutron activation. This showed a considerable stability in the liver and diaphragm over a wide range of manganese intakes. Adrenalectomy did not alter these tissue concentrations, except in animals receiving high manganese intakes. In these, the two organs contained higher concentrations of the metal following adrenalectomy. The conclusions drawn from these studies are that the concentrations of manganese in tissues are constant and quite stable but that their stability cannot be ascribed as yet solely to the function of the adrenal cortical hormones.

neutron activation analysis; ACTH; glucocorticoid hormones; adrenalectomy

THE PRECEDING PAPER (1) indicated that the turnover of manganese in mice depends extensively on the concentration of this metal in the diet consumed by these animals. This raised two questions regarding *1*) the constancy of this metal's concentration in animal tissues, and *2*) the stability of these concentrations when the intake of the metal is varied. Both questions were deemed pertinent to the search for hitherto unidentified mechanisms which might be regulating the metabolism of this essential nutrient (2).

An earlier study (7) demonstrated that administration of exogenous glucocorticoid hormones markedly affected the tissue distribution of the artificial radioisotope Mn^{54} in the mouse. This effect might have been a pharmacological one rather than indicative of physiological regulation. Hence it was investigated first whether or not stimulation of the animals' own adrenal cortices with ACTH results in changes similar to those described earlier (7). Affirmative evidence was followed by a demonstration of the constancy of tissue manganese concentrations from one animal to the next. Subsequent experiments were designed to perturb this constancy. The variables investigated in this regard included administration of cortisol, adrenalectomy, and imposition of various manganese loads.

MATERIALS AND METHODS

Animals. Most of these were Hale-Stoner Swiss albino, adult male mice, reared at Brookhaven National Laboratory. In studying the constancy of manganese concentrations, some of the mice used were C3H Bar Harbor adult males, reared at Little Rock. A total of 150 animals is reported on in this communication.

Diets. In the initial experiments groups of animals were given a low-manganese diet (Nutritional Biochemical Corp., 2 μg/g; estimated intake: 10 μg Mn/mouse per day). Other groups were given Purina laboratory chow, which contained 32.5 μg Mn/g by neutron activation analysis (estimated intake about 200 μg Mn/mouse per day). This was supplemented with reagent grade $MnCl_2$ as indicated. All animals received triple-distilled water ad lib. In order to impose gross metabolic loads of manganese, some animals received 0.02 M $MnCl_2$ instead of drinking water (estimated daily intake 4,000 μg Mn).

In another experiment 50 BNL mice were divided into two groups: the members of the first group were adrenalectomized, those of the second were sham operated. Each group was then divided into nine subgroups receiving different amounts of manganous chloride in their diets; each subgroup consisted of two to four animals, the larger number being placed among the adrenalectomized ones receiving the smaller concentrations of metal. The diets were made up with one part of Borden's evaporated milk and one part of 2% NaCl solution containing the manganese (Fig. 3). Prior to the addition of the metal

Received for publication 8 October 1965.
[1] This work was supported by the U.S. Atomic Energy Commission and by National Institutes of Health Grant A5683.
[2] Recipient of National Institutes of Health Career Development Award (AM31841).

TABLE 1. *Carcass-to-liver ratio 30 days after Mn^{54}*

Treatment	NBC Low-Manganese Diet	Purina Laboratory Chow Diet
Control	3.9±.5	9.2±.7
ACTH, 1.0 U/day	5.1±.4	15.7±2.3
Adrenalectomy	5.6±.4	7.7±.6

Values are means ±SD. *P* values are given in text.

this mixture contained 42.5 μg manganese/liter, slightly more than double that present in the basal diet used in the accompanying report (1). During the first postoperative week, this diet contained 1 mg tetracycline/ml. During the 2 months on this diet one control and seven adrenalectomized mice died and were excluded from this study. These latter animals were receiving the largest amounts of manganese. The remaining animals were sacrificed and two samples of liver and as much of the diaphragm as possible were secured for neutron activation analysis with nonmetallic instruments (4, 5, 8).

At the completion of these experiments some of the mice on the basal diet were seen to eat their corn-cob bedding, as was reported for manganese-deficient animals (9). Therefore a supplementary experiment was carried out as follows: 12 animals were adrenalectomized and 12 were used as sham-operated controls. Groups of three were placed in plastic cages without bedding and offered the basal diet discussed above. Two groups received only the mixture of evaporated milk and NaCl; in two groups there were added 1.1×10^2 μg Mn/liter and in two other groups 2×10^4 μg Mn/liter. The distilled water was again offered ad lib. This regimen remained unchanged for 54 days.

Isotobes. 1) Carrier-free $Mn^{54}Cl_2$ was obtained from the Nuclear Science and Engineering Corporation in 20% HCl containing 500 μc/ml. This was diluted 50- or 100-fold with 0.9% NaCl solution; 0.1-ml aliquots were injected intraperitoneally (0.5–1.0 μc/mouse). 2) Stable Mn^{55} was supplied in the form of $MnSO_4 \cdot H_2O$ (Baker).

Measurement of Mn^{54}. Total-body radioactivity was assayed either in the total-body counter previously described (1) or by placing the mice 6 cm above a 3-inch sodium iodide-scintillation crystal using a Styrofoam mold to maintain constant geometry. This crystal was connected to a Nuclear Chicago 8000 series gamma-ray spectrometer. The respective isolated organs were counted on the corresponding devices. Standard error of counting did not exceed ±3%. The convention used previously (7) of expressing shifts of the radioisotope as "carcass-to-liver ratios" was used here also.

Measurement of Mn^{55} of tissues and diets. Tissues were obtained by means of polyethylene-tipped forceps and quartz knives. All samples were inserted into tared polyethylene sample holders, dried, weighed, and analyzed by means of the neutron activation procedure described earlier (3, 5, 8), which employs the Mn^{55} (n, γ) Mn^{56} reaction. The photopeak used for the measurement of Mn^{56} is identical to that used for assaying the long-lived radioisotope, Mn^{54}. The error of quantifying these isotopes (by difference, while both varied) has not been assessed. Therefore specific activities were not determined.

Hormones. One milligram per day (≈50 mg/kg) cortisone acetate (Upjohn) or 5 mg every 5 days of cortisol acetate (Upjohn) were injected subcutaneously into the treated groups as indicated. ACTH in gelatin (Armour) was administered in a like manner in doses of 1 USP U/day.

RESULTS

Observations with Mn^{54}. Two groups of six animals each, tagged with Mn^{54}, were injected daily with gelatin or ACTH for 30 days (Table 1) while eating Purina. The total-body radioactivity did not differ significantly between these two groups during 30 days of observation, but the internal distribution of the isotope was significantly affected by this hormone. The mean ratio of carcass-to-liver radioactivity was 9.2 in the case of the control animals and 15.7 in the ACTH-treated group, a significant elevation, without overlap of the data ($P < .05$). After eating the low-manganese diet for 2 weeks prior to, as well as during, the ACTH administration, the carcass-to-liver ratio of a control group was 3.9 and an ACTH group 5.1, an elevation of questionable significance ($0.1 > P > 0.05$).

In five adrenalectomized animals studied while on the low-manganese diet the distribution of Mn^{54} was not impressively different from that of the corresponding five control animals (mean carcass-to-liver ratio 3.9 vs. 5.6 ($0.1 > P > 0.05$). Similarly, in the adrenalectomized group of five animals maintained on Purina chow, the mean carcass-to-liver ratio was 7.7 as compared to 9.2 in the five controls, namely, not significantly different ($P > .1$).

Observations of Mn^{55} concentrations. The preceding experiments as well as those published earlier (7) might be suspected as pertaining only to the metabolism of the artificial tracer rather than to that of the essential metal. Therefore, in the experiments which follow, there was quantified the concentration of the natural manganese isotope, Mn^{55}, indigenous to tissues.

The range of manganese concentration was measured in tissues of two different strains of mice reared in two different centers. No differences could be detected be-

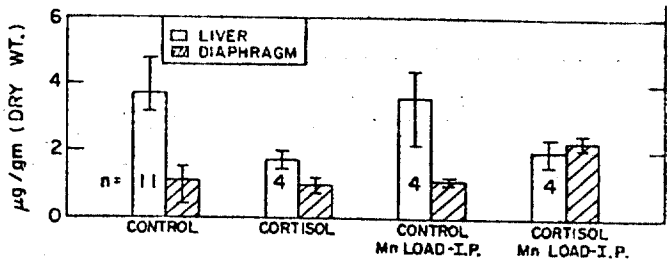

FIG. 1. Manganese (Mn^{55}) concentration in livers and diaphragms are shown for cortisol-treated (1 mg/day for 10 days) and saline-injected controls. Columns = mean for the data and bars enclose the range of all data. The manganese load was given intraperitoneally, 20 μg Mn^{++} as the chloride. n = number of animals.

tween the tissue concentrations of the two strains, both of which were consuming Purina chow: the mean concentration of manganese in the livers of 11 animals was 3.73 μg (SD ± 0.49)/g dry wt; in the diaphragms it was 1.11 μg (SD ± 0.35)/g dry wt.

Administration of cortisol to four animals for 10 days resulted in diminution of these values in the liver to 1.70 (1.48–2.00) and in the diaphragm to .98 (0.69–1.15) μg/g dry wt (Fig. 1). In an effort to replete the manganese in these tissues, 20 μg Mn^{++} as the chloride were injected intraperitoneally into four control and four cortisol-treated animals on the 6th day of hormone administration, 4 days prior to sacrifice. In the control animals of this series, the ensuing mean concentrations in liver and diaphragm were 3.58 (2.17–4.39) and 1.07 μg (.98–1.15)/g dry tissue, respectively. In the cortisol-treated animals the mean concentration of manganese per gram of dry liver was 1.98 μg/g (range 1.55–2.39), but that of the diagram rose to 2.29 μg/g (range 2.12–2.46).

Four animals that had been adrenalectomized 40 days prior to sacrifice and were fed the Purina chow showed a mean liver concentration of 4.37 (range 3.55–5.49) μg Mn/g dry wt. Their diaphragms contained 1.05 (range .84–1.17) μg/g dry wt (Fig. 2).

The foregoing indicated a possible relationship between manganese intake and these hormonal effects: the latter seemed more striking when the manganese intake was high. Hence, the question arose whether the effects of adrenalectomy would conform to this pattern. Therefore, four intact and four adrenalectomized mice were given 0.02 M $MnCl_2$ in their respective drinking fluids for 20 days prior to sacrifice. The concentration of manganese in the livers of the controls became 7.30 (range 6.69–7.53) μg Mn/g dry wt and 9.91 (range 8.58–11.50) μg Mn/g dry wt in the adrenalectomized mice. The respective figures for the diaphragm were 2.18 (1.74–2.60) and 2.66 (1.49–3.96) μg Mn/g dry wt. Thus it appeared that the tissue concentration of manganese could be affected by adrenalectomy and cortisol, but significantly, the changes were in opposite directions. Furthermore, administration of massive quantities of this manganous salt had increased the tissue concentration of manganese, but this effect was less when the adrenals were intact.

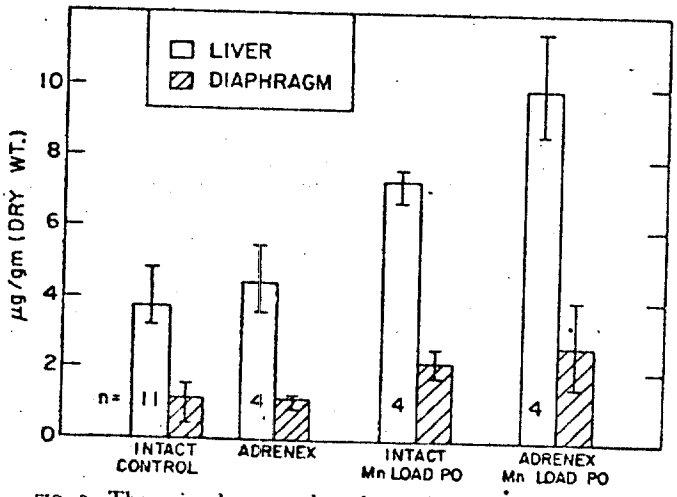

FIG. 2. The animals were adrenalectomized for 40 days and the oral loads were imposed in the form of 0.02 M manganese chloride as the drinking water. Symbols same as in Fig. 1.

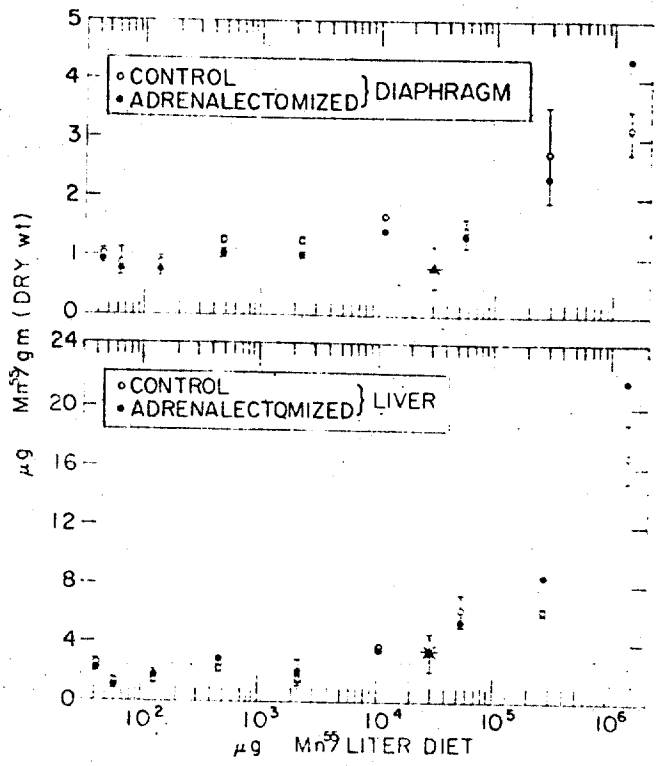

FIG. 3. *Abscissae* represent manganese content of the milk diet. *Ordinates* represent concentrations found in the liver and diaphragm.

The behavior of the tissue concentration of manganese was investigated next on intact and adrenalectomized animals by means of adding graded amounts of manganous sulfate to their milk for 2 months prior to sacrifice. The results of these experiments are summarized in Fig. 3, where the concentration of the element in two tissues is plotted as a function of dietary concentration. In order to include the Purina-fed animals in these calculations, their estimated daily manganese intake was matched with the estimated daily intake of the milk-fed mice. These data were also plotted on log-log scale to estimate roughly an over-all change in dietary concentration which might double the tissue concentration. It appeared that if the dietary concentration were increased at least 150 times, the tissue concentration of the metal doubled. It is evident from Fig. 3 that the effect of adrenalectomy was apparent in the one adrenalectomized mouse on high manganese that had survived for the 54 days of this experiment. The remaining data do not reflect the absence of the adrenal gland.

The manganese concentrations in these tissues was investigated also in groups of four animals each, which were kept for 54 days on milk containing 1×10^2 and 2×10^4 μg Mn^{++}/liter. The respective values for the livers of these animals were 2.23 (2.14–2.50) and 5.39 (5.12–5.61) μg Mn/g dry tissue. The respective values

for the diaphragms were 0.92 (0.88–0.99) and 1.63 (1.52–1.74) μg Mn/g dry wt. These findings indicated that a 200-fold increase of the level of the element in the diet had resulted only in doubling of the manganese concentration in both tissues tested. Four adrenalectomized and four intact mice, kept on the milk diet alone for 54 days showed the following manganese concentrations: adrenalectomized: liver 0.81 (0.75–0.87), diaphragm 0.64 (0.48–0.73); intact: liver 0.88 (0.71–1.03); diaphragm 0.70 (0.60–0.80) μg/g dry tissue. The sum of these latter data confirmed that adrenalectomy did not affect the tissue manganese concentration under the dietary conditions tested.

DISCUSSION

The effect of ACTH on the distribution of Mn^{54} suggested the existence of an adrenal regulatory mechanism in the metabolism of manganese. This argued against the assumption that the glucocorticoid hormones studied earlier (7) had induced merely a pharmacological response. Yet, adrenalectomy did not evoke the opposite effect on the radioisotope's distribution.

In our hands, the natural, stable isotope of this metal was shown to occur in constant concentrations in the livers of two strains of mice. However, other workers (6, 10) encountered in one strain of rabbits a higher range of values. Also, the two mouse tissues studied by us showed a fairly marked stability of their manganese concentrations over a considerable dietary range, whereas recalculation of the results quoted above (6, 10) indicate a lesser stability in bone and liver of rabbits receiving much higher intakes than those reported here. The differences between our data and those quoted above might well be due to radically different analytical approaches: neutron activation analysis permits minimal contamination provided that minimal handling of the samples is practiced (4). Indeed, contamination was proved to constitute an important analytical variable in microanalysis (3).

Regardless of the reasons for these differences, the following appears noteworthy: a very wide range of dietary manganese induced comparatively minor changes in tissue concentration, whereas the preceding paper indicates that manganese was probably being absorbed in rough proportion to the amounts presented for absorption (1). Indeed, such a wide range of dietary concentrations could be investigated only because of the low toxicity of this metal by contrast to other essential minerals. Consequently it was not possible to compare the stability of manganese with that of minerals obeying rigorous homeostatic control.

The tissue concentrations of Mn^{55} were changed by cortisol in the same direction as those of the radioisotope Mn^{54} (7). This proved that the glucocorticoid hormone affected the metabolism of the essential metal itself and not merely that of its artificial tracer. It should be noted that glucocorticoid hormones are being administered to man in large amounts, over long periods of time and for many reasons. Hence these hormones should be studied as to whether they induce in man syndromes kindred to experimental manganese deficiency (2, 6, 10).

The effect of adrenalectomy was in the opposite direction from that which followed administration of the ACTH and cortisol, but it became manifest only after large amounts of metal were fed to the mice. The sum of the present experiments argues in favor of the postulate that the metabolism of this essential trace mineral is under regulatory control but the precise role of the adrenal in this control remains uncertain.

Miss Carol A. Rozanski and Mrs. Patricia Warrick rendered expert assistance with the dietary experiments and some of the analyses, respectively.

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Katasato Ach. Exp. Med., 18: 145-149, 1941

INTOXICATION BY MANGANESE IN WELL WATER

By

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Intoxication by manganese is usually caused by inhalation of its powder or fume for a long duration of time. Such intoxication is not infrequent throughout western and eastern countries. But intoxication caused by manganese dissolved in drinking water is rare. In Europe and America since the first report of Couper, 1837, and later confirmation by von Jaksch and Emden, 1901, 187 cases were tabulated up to 1939 by Voss. The majority of them were isolated cases of intoxication, but cases of mass intoxication were reported by Casamajar (1919–20), Meyer (1930), Flintzer (1931) and Nazif (1936). In Japan instances of manganese intoxication were reported by Miura (1), Sato (3), Ochi (3), Kamada (2), Tsuneyoshi (1), Nishino (1), Ashizawa (1), Oe (3), Kitaoka (1), Inada (1), Ishihara (1) and Hayashi (2), but all these 20 were isolated cases. In the fall of 1939 we came across an epidemic of such intoxication and had occasion to make autopsy on one of the two fatal cases, and detailed laboratory tests were carried out with all possible materials. These studies gave finally conclusive evidence of maganese intoxication. The present paper includes the results of the studies.

Brief Summary of the Epidemic Study.

On December 19th, 1939 we received a report informing us that there was an outbreak of encephalitis-like disease in a family, all the six members presenting the same symptoms.

There was some doubt as to certainty of the cases being true encephalitis for two reasons. One is that encephalitis is rare in winter in this country and the other, that encephalitis usually breaks out sporadically and it is exceedingly rare to see all the members of a family taken down with it. It, therefore, became necessary for us to investigate carefully. All necessary materials were taken with us to study the cases including a number of mice. On arriving at the spot we found one case had terminated fatally at a hospital, two were in bed and the remaining three, though ill, were strong enough to be up. Blood and spinal fluid were taken from each of the patients and portions of them were used to inoculate mice, while the other portions were used for bacteriological and microscopical examinations. Autopsy was immediately carried out on the dead case and materials were taken for further studies.

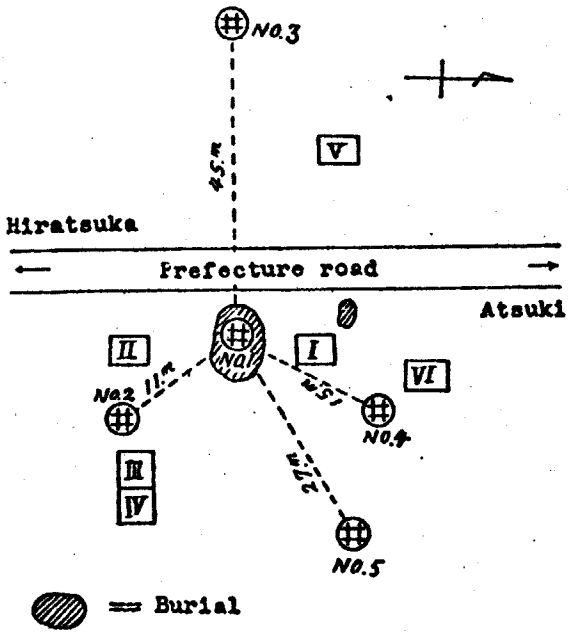
Examinations of these materials showed the blood and spinal fluid to be sterile. Spinal fluid were all clear with normal cellular counts. The freezing preparation of fixed brain and spinal cord of the autopsy case showed no histological pictures of encephalitis, such as perivascular cellular infiltrations, changes of nerve cells, increase of glia cells and neuronophagy. Mice inoculated with blood and spinal fluid failed to show any symptoms though watched for three weeks.

Clinical pictures of the cases and laboratory examinations of the materials indicated that the epidemic was not encephalitis. The mass outbreak and the symptoms due to disturbances of the extrapyramidal tract pointed strongly to some form of intoxication. Carbon monoxide intoxication could be discounted, but no other toxic cause was discovered. A hint was soon found. The family maintained a bicycle repair shop and many old dry cells for bicycle lamps had been buried near a well, from which water was supplied to the family. A large number of decayed dry cells were dug out and it was presumed that intoxication was caused by manganese, which is a principal constituent of the cells. This

chemically dissolved and permeated into the well water. Ten more patients having similar symptoms were discovered near by the original family. Some of them used wells belonging to their own families, but all of the wells were not very far distant from the toxic well. Chemical analysis of the well waters showed an unusually large quantity of manganese in all of them. From this fact it became clear that the lethargic epidemic was due to intoxication caused by manganese dissolved in well waters.

Survey of the Epidemic Vicinity.

The outbreak of the disease occurred in a small agricultural town of 2.000 population about 100 kilometers southwest of



Tokyo in the environs of Hiratsuka city. Five families were involved in the outbreak. The relation between the wells and families were as follows: The family No. 1, which we have

already mentioned, has its own well No. 1, around which the dry cells had been buried—the cause of the present mass intoxication. The families No. III and No. IV used well, No. 2, which is situated 11 meters south east of well, No. 1. The family No. V has its own well, No. 3, some 45 meters north of well, No. 1. There were two other wells near by, one 15 meters and one 27 meters and one 27 meters south west of well No. 1, but the two families using these wells suffered no ill effects. The family No. VII came to nurse the family No. 1 during their illness and used the water from well, No. 1 and two members became ill. Thus toxic substance permeated into three wells while the other two, though situated fairly near by, remained uncontaminated.

The Courses of the Outbreak and General Symptoms of the Cases.

Family No. V.—The grandmother of the family, aged 79, became lethargic on November 1st and soon became severely ill. This case died on Nov. 20th. Her son, aged 55 and wife, aged 41, and grandson, aged, 27, became ill about Nov. 2nd. The last three cases recovered completely by the end of December. The wife of the grandson, aged 25, did not become ill.

Family No. I.—Man aged 46, suffered the onset of intoxication on Nov. 15th, which soon became severe causing death on the 17th. His wife, aged 45, first son, aged 18, and second son, aged 16 became ill Nov. 10th and 18th, respectively. The third son, age 14, and first daughter, aged 11, became ill on Nov. 20th and Dec. 10th respectively. Thus the whole family suffered from the intoxication.

Family No. VII.—Man, aged 71 and wife, aged 70, came to nurse the family No. 1, and became ill on Dec. 27th and 20th respectively. The man gradually became melancholic and took his life by drowning in a river.

Family No. III.—Man, aged 32, and wife, aged 27, became ill on Jan. 5th. Their children aged 4 and 2, remained healthy.

Family No. IV.—Man, aged 44, and wife, aged 40, became ill on Jan. 8th. Their 6 children all remained healthy.

Thus there were 16 cases of intoxicatoin, two of which resulted in death and one which committed suicide. All of these had used the three wells, into which manganese had permeated into the water. An interesting fact was that the greater the ages of the cases the more severe the symptoms, while the children escaped the effect of the intoxication almost entirely. These relations according to age are shown in Table I.

Table I.

Relation of Severity of Intoxication and Ages.

Age	51-80	31-50	11-30	1-10	Total
Severe	2	2	1	0	5
Moderate	1	0	1	0	2
Mild	1	4	3	0	8
Not toxicated	0	0	4	6	10
Total	4	6	9	6	25

General Symptoms Common to the Cases: The initial symptom is lethargy with loss of appetite and constipation. Expression of face is mask-like with running saliva. Tonicity of muscles increased. Joints of lower extremities rigid with rheumatic pains. There was edema from the beginning. With increased tonicity of arm muscles there were marked tremours, which made eating and writing difficult. There was temporary double vision. Senses were somewhat disturbed. Tendon reflexes were markedly increased so that walking was difficult. Sometimes there was marked mental disturbances and unbalances. Memory was also lost and the cases became melancholic. A few cases had halucination. There was no fever unless complications set in. There was no change of the spinal fluid,

but the pressure was somewhat increased. Two died of emaciation, one committed suicide due to melancholy. Others recovered gradually.

Examination of blood smears: Blood smears were taken from five cases and stained with Giemsa. The differential counts of leucocytes are shown in Table II.

Table II. (%)

Cases of Family No. I	Lymphocytes Large	Lymphocytes Small	Leucocytes 1	Leucocytes 2	Leucocytes 3	Leucocytes 4	Mononuc.	Acidoph.	Basoph.
♀ 15	13.0	35.3	5.0	20.3	14.4	2.0	7.6	2.0	0.4
	48.3		41.7						
♂ 13	15.0	33.5	2.0	15.0	17.0	3.0	11.0	3.0	0.5
	48.5		37.0						
♂ 18	10.0	9.7	2.0	31.6	10.6	2.0	14.1	0	—
	19.7		66.2						
♂ 16	24.6	10.4	1.6	12.4	29.4	9.0	11.0	1.6	—
	35.0		52.4						
♀ 45	17.0	10.0	6.0	19.0	28.0	6.0	14.0	0	—
	27.0		59.0						

Thus there were no marked changes in leucocytes.

Summary of the Autopsy Findings.

The case was male, age 46. Onset of illness November 15, died on December 17th and autopsy was performed on the 19th, 45 hours after death.

Autopsy diagnosis

1. Hyperemia of cerebrospinal meninges.
2. Edema of brain meninges.
3. Increased blood contents in brain and spinal cord.
4. Decreased coagulation of blood.
5. Moderate putridity.

Moderate degree of congestion was noted in brain, spinal cord and meninges. Meningeal edema prominent in the occipital part.

Cerebrospinal liquid transparent. Venous congestion in brain basis. Basilar arteries were not atherosclerized. Brain 1460 gms. in weight. A little tendered. At the horizontal section of brain blood points eminent, otherwise no abnormity. No change at section of pedunclus cerebri and substantia nigra. Brain chambers were not dilated. No increase of clear cerebrospinal liquor.

Histological examination of thalamus, nucleus caudatus and Louis' body showed no changes in nerve cells as well as nerve fibers. There were also no changes in lobus insulate, claustrum and inner and external capsules. On the other hand there were distinct changes in the globus pallidus. Fatty droplets and granules appeared in nerve cells. These were heavily injured and even necrosed. Nissl's bodies were disturbed markedly. At these places there appeared fatty substances colored brownish-yellow or yellow in Kawamura and Yasaki's new fat staining method. They were found in granular, clotted or globular forms. They could be differentiated easily from the granular lipochrom in normal nerve cells which stained yellowish-brown or brownish-yellow red. But we could find in the degenerated nerve cells transitional stages of both substances. It was distinguished in staining with nile blue sulphate. Namely lipochrom was colored greenish, other lipoid substances violet blue. In staining with hematoxylin singly or hematoxylin and eosin the latter appeared brown or golden red as degenerated lipochrom. Further there was a marked increase of fatty granules of brownish red color in the glia cells by Kawamura and Yasaki's method. The above fatty substances showed no double fraction in nile blue stained section.

Besides the above mentioned granulous and droplike fatty substance there were distributed in several places at the periphery with a Sudan III stainable tubulous substances. On examination of the cross section it appeared as circulous fat. It was presumed to be degenerated myelin sheath. In the globus

pallidus there more multiplicated perivascular fatty granules than in other portions. No increase of glia cells and no infiltration of round cells appeared in the parenchym.

Above changes were observed in almost the same grade in both hemispheres. Blood vessels were congested, but there was no proliferation of the endotherial cells. In this case, as to the changes in the central nervous system, degeneration and destruction of nerve cells in the globus pallidus were considered most important, but for the fact that these findings were isolated, it was difficult to find the changes by the routine method.

Chemical Analysis of the Well Waters.

As we have mentioned before the present epidemic of intoxication by manganese was caused by dry cells buried around the well No. 1. Over 300 dry cells were dug out at the spot 1 to 2.5 meters from the well and another 100 cells from a spot 10 meters from it. They were buried 5 cm. to 2 meters deep. These dry cells were removed completely from the spots.

Well waters were examined bacteriologically as well as chemically. The formed proved nothing significant. The samples were taken on Dec. 18, Jan. 8 and 18 and Feb. 5. The results of the chemical analysis are shown in Table III.

Thus examinations of the water of well No. 1 on December 18 showed clearly a large amount of manganese, which gradually decreased in subsequent examinations. Water of Nos. 2 and 3 showed also large amounts of manganese. While Nos. 4 and 5 showed no metal. The users of the latter two wells, though they lived very close to the other people, had not suffer from manganese intoxication.

Manganese and Zinc in Viscera, Blood and Urine.

Chemical analysis of liver and kidney of the autopsy case and blood and urine of an intoxicated patient were made and

Table III.

Chemical analyses of well water.

	Methods of qualitative and quantitative analyses	No. 1 well 18/XII	No. 1 well 8/I	No. 1 well 18/I	No. 1 well 5/II	No. 2 well 18/I	No. 3 well 18/I	No. 4 well 18/I	No. 5 well 18/I
$Mn^{\cdot\cdot}$	Mn_3O_4 wt. mg/l	14.34	9.80	8.75	4.20	10.65	7.63	0	0
	$KMnO_4$ color. ,,	14.25	10.25	9.25	4.50	11.05	7.86	0	0
$Zn^{\cdot\cdot}$	ZnS wt. ,,	16.33	12.29	10.38	9.82	12.37	8.25	0.031	0.082
	Di hizon col. ,,	17.00	11.89	10.70	9.52	13.08	8.13	0.032	0.080
HCO_3'	$Ba(OH)_2$ oxyalic ,	37.12	36.35	34.21	35.30	34.87	32.04	18.69	21.32
Cl'	$AgNO_3$ titr. ,,	30.2	29.9	29.9	28.2	14.90	14.91	17.04	20.82
SO_4''	$BaSO_4$ wt. ,,	308.6	240.0	238.1	240.0	240.0	241.2	239.80	241.02
Organic	$KMnO_4$ titr. ,,	32.16	28.42	21.06	29.16	29.16	28.67	31.34	15.95
Sed.		427.21	421.98	422.20	412.02	396.50	423.01	450.72	401.97
Hardness	German m.	7.61°	7.56°	6.25°	3.47°	10.47°	6.22°	7.56°	9.00°
PH	Elect. m.	7.41	7.50	7.42	7.35	7.21	7.21	7.32	7.22
NO_3'	Diphenylamin	+	+	+	+	+	+	+	+
NO_2'	I_2Zn-starch	+	+	−	−	−	−	−	−
NO_4'	Nessler	+	+	+	+	+	+	+	+
Na	Spectroscop.	+	+	+	+	+	+	+	+
K	,,	+	+	+	+	+	+	+	+
Mg	,,	+	+	+	+	+	+	+	+
Ca	,,	+	+	+	+	+	+	+	+
Si	,,	+	+	+	+	+	+	+	+
Cu	,,	+	+	+	+	+	+	+	+
Fe	,,	+	+	+	+	+	+	+	+
Pb	,,	−	−	−	−	−	−	−	−
Hg	,,	−	−	−	−	−	−	−	−

also similar analysis made with other materials for control. Results are shown in Table IV:

Thus manganese of the liver of the affected case was greatly increased in comparison with the control liver, while the zinc in the same organ was also somewhat increased. Manganese in the blood of a patient was also greatly increased. Zinc in the liver and kidney was also greatly increased. In the literature the content of manganese in the livers and kidneys of normal

Table IV.

Mn and Zn contents.

		Mn (mg %) Liver	Mn Kidney	Mn Blood	Mn Urine	Zn (mg %) Liver	Zn Kidney	Zn Blood	Zn Urine
Intoxicated cases	Autopsy Patient	0.385	0.210	0.183	0.031	8.61	4.23	0.862	0.610
Control	No. 7 autopsy	0.132	0.153			3.84	2.82		
	No.14 ,,	0.186	0.165			4.63	3.71		
	No. 1 healthy person			0.062	0.023			0.631	0.463
	No. 2 ,,			0.095	0.025			0.730	0.459

human cases is 0.087–0.392 mg% and 0.17 mg% respectively. In the blood of a healthy man its content is 0.03–0.05 mg%, in urine Ca 0.02 mg%.

Discussion.

There had to be considered in these cases, poisoning by manganese or zinc. Both of these metals were found in the well water and the organs of the autopsy case contained more of these metals than were to be found in the control cases. Also an abnormal increase of these metals was found in the blood and urine of all the cases. It was necessary to determine whether both of these metals were responsible for the intoxication in these cases under observation.

As to zinc intoxication it has long been known and commonly called "pouring fever." It is caused by the inhalation of zinc oxid fog produced by the melting of the metal. The symptoms are high fever, dull sense and dizziness. Safir supposed it to be due to the absorption of protein produced through the injury and ulceration of the mucous membrane of the trachea by the inhalation of the zinc oxid powder. Following the recent

great development of electric metal cement engineering, especially in electric work with zinc amalgamated steel plate, the acute zinc intoxication has been often observed. Cases from our navy docks were reported by Junnai, Kamitate, Tashiro etc., while making studies on blood. Sakai and Ohne reported cases of acute pneumonia following acute zinc poisoning. Schlockow (1878) observed the case of chronic poisoning in a zinc miner, the symptoms exhibited being convulsion, paresthesia, taste anomalia, and unharmonic movement, but it seems probable at present that this was a case of poisoning by manganese which was associated with the zinc. But, however, when a small amount of zinc was given experimentally, there resulted a deminution of motor power and muscle convulsion followed by complete paralysis due to peripheral injury. According to the experiment of Vernick and Michaelis and others, there were observed nausea, vomiting, fever, thirst, tetanic or spasmodic pulse and pain in the extremities. After vomiting, diarrhea and sweating, these symptoms became reduced or disappeared altogether. But upon again taking zinc oxid, these symptoms reappeared.

Drinker, gave cats and dogs zinc oxid over a long period of time with the result that their body weight was markedly reduced. This was supposedly due to loss of appetite due to the sweet taste. Giving a large quantity caused vomiting, but no anemia or pathological changes in the blood cells. However elimination of protein in the urine and fibrosis of the pancreas was observed. Also in zinc poisoning, there is not noted any change of the corpus striatum in the central nervous system.

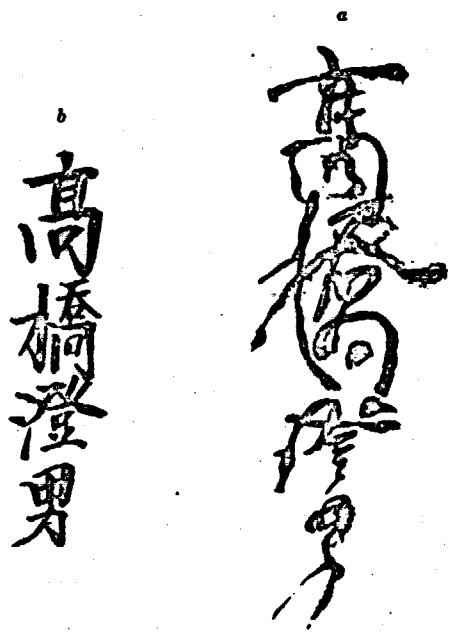
In manganese poisoning, the corpus striatum of the central nervous system is chiefly attacked. Besides changes in vegetative and vasomotor centers, the medulla oblongata and spinal cord are affected. In consequence, the symptoms are varied and complicated and therefore, patho-anatomical findings of the different observers and the results of their experimental works

do not always coincide. The clarification of this question is dependent upon further research based upon clear cases and experimental facts.

From the clinical observations, these cases were considered subacute manganese mass poisoning which broke out during the first part of November, 1939 and terminated early in January, 1940. Since the latter part of December the use of the well-waters for drinking was strictly prohibited, as a marked manganese intoxication was suspected. Owing to certain circumstances, the thorough investigation of all the cases could not be made. However, the present investigation was based chiefly upon the clinical observation of 6 cases in the original intoxicated family, to which a few other cases were added.

In these cases under our observation, lethargy and edema were the first symptoms to be observed. According to McNally, 12 cases of lethargy and 6 cases of leg edema were noted out of the 68 cases he investigated. Inada observed leg edema in one third of all the cases he investigated. In our cases these two symptoms were the first to appear and therefore, encephalitis epidemica and beriberi were suspected. Also muscular rigidity and weakness or paralysis were observed. The muscular rigidity was noticed in the face, neck and extremities. The facial expression became mask-like. The knee and cubital joints were felt resistant to a passive movement. The reflex action of the tendon was accelerated and muscular pain was complained of. As to the muscular weakness, rough power was affected. The accommodation of the eye muscles having been disturbed, diplopia was observed. Regarding motor motion, the rigidity of fingers became noticeable and their movement clumsy, making writing difficult. In writing when a certain amount of strength and power of accommodation were required, a symptom of micrographia was markedly noticed, distinctly exhibiting a tremor. Also, when the body was kept in the same position for a certain length of time, coarseness in writing was observed; namely, dynamic tremor. Latero- and retropulsion, which were

held as characteristic of this illness, were not marked in our cases, but the accommodating movement of the extremities was affected, and walking was disturbed. According to McNally there was forced laughter in 70% and compulsive weeping in



Explanation { a : 7/1 Autograph by patient.
b : 2/3 " " after recovery.

20%. In our cases both were observed. Affection of speaking was observed in severe cases. The psychical state became excitant or depressive. Concentration and memory became impaired. Salivation was sometimes seen. Sexual disorder was not examined, but in the autopsy case spermatogenia was clearly disturbed. Pressure of cerebrospinal liquid was not certain, high or low. In smear blood specimens there was no quantitative and qualitative change. The above signs correspond in general to manganese poisoning as described in the literatures.

As to patho-anatomical findings the autopsy cases were very rare. There has been published up to the present time only five cases (Casamajor, Ashizawa, Canavan, Cobb and Drinker, Stadler and Voss). Of these cases the examination of the case of Casamajor 1913, (male, age 49) was not sufficient and the case of Stadler and Voss 1939, (male, age 37) was complicated with amyotrophic lateral sclerosis. In the other 3 cases Ashizawa, 1927, (male, age 33), Canavan etc., 1934, (69 years old man) and Stadler, 1936, (male, age 69(?)) brain stem, namely corpus striatum and globus pallidus were affected. There was present in every case disappearance of nerve cells. Of the nerve fibres, Ashizawa noted their reduction, but not greatly. Canavan and Stadler confirmed a loss of nerve cells in the cortical part of the brain, but the nerve fibres were only slightly disturbed or not at all. As to glia proliferation Ashizawa found it very slight in the degenerated portion of the nerve cells, but in the cases of Canavan and Stadler glia proliferation occurred in a diffuse way, also the case of Stadler was complicated by a strong atherosclerosis. The majority of human cases are complicated by other diseases and it is, therefore, necessary to do experimental research on cases of pure manganese poisoning to clarify the symptoms.

Regarding experimental manganese poisoning, there are many publications abroad, such as by Levy, Tiefenbach (1921), Schwarz, Pagen (1923), Mella (1924), Grünstein, Papowa (1922), Jötten-Reploh, and Hegemann (1939), and in Japan, by Yonenaga (1927–28), Watanabe (1929), Matsumura (1931) and Okada (1937). Levy and Tiefenbach gave rabbits brownstone mixed with food. The animals exhibited epileptic attacks and opistotonus after about 3 weeks and characteristic rigidity in over two months. Histologically the central nervous system was changed generally, but most especially in some places like the corpus striatum, cortical substance, ammon horn and corpora quadrigenia in descending grade. The grey matter was chiefly

affected, degeneration of nerve cells and proliferation of glia tissue, but the reaction of satellite cells was slight. These changes were about the same as exhibited in lead poisoning, etc. Further, proliferation of the endothelium of the blood vessels was observed. Schwarz and Pegal used cats as experimental animals, which were given brownstone with food. Paralysis was observed in the posterior extremities, but was not histologically examined. Mella used monkeys in his experiment. His method of induction was by the injection of chloric manganese liquid into the peritoneal cavity. Chorea or chorea-athetosis like movements, then rigidity of muscles and movement disturbances resulted. Histologically, there was a slight but diffuse degeneration of nerve cells, which was not accompanied by glial cells and degeneration of marrow sheath. Meninges and blood vessels were not changed. By scarlet red staining there were no fatty droplets found in nerve cells of cortical zones. In putamen and nucleus caudatus a great many of the nerve cells were degenerated having lost their nuclei, sometimes neuronophagia was observed. Large striatum cells were reduced in number and swollen, hydropic and with eccentrically placed nuclei. Glial tissue was increased in spots and the marrow sheath disappeared. Cerebellum, pons, spinal cord, and peripheral nerve were not changed.

In the cases of Grünstein and Popowa, rabbits were given manganese dioxid. One group of the experimental animals showed transitional paralysis. Histologically, in the corpus striatum heavy injury to the nervous system was observed. Also degeneration of nerve cells was found in the spinal cord, medulla oblongata, pons, cerebellum (Pulkinje's cells), thalamus, nucleus caudatus, putamen, globus pallidus and cortical substance. Especially prominent was the change in the corpus striatum.

Jötten, Reploh and Hagemann giving brownstone powder by inhalation succeeded to produce in rabbits artificial pneumonia which was like Thomaschlackenpneumonia of Gundel, but

not a heavy interstitial pneumonia as in silcosis. Further the authors have injected brownstone powder and calculus powder into the peritoneal cavtiy, the latter acting as a harmless foreign body it produced simple exudation of leucocytes, while the former caused fibrinous, apostomatous, haemorrhagical peritonitis. This experiment was sufficient to prove that brownstone applied to the lung tissue could produce exudative pneumonia. It was their opinion that manganese powder could chiefly attack the epithelial tissue producing necrosis. Through these small defects the finest powder was absorbed and passed into the body liquid. They have not described any changes of the central nervous system.

In Japan, Yonenaga injected 2% aseptic potassium permanganate suspension liquid into the ear veins of rabbits. Histologically there were found local inflammatory changes in the blood-vessel-walls and nervous system. In the latter, the gray matter, especially the cortical substance of the brain, was attacked followed by the changes in the thalamus, corpus striatum, globus pallidus and ammon horn. Further changes were disseminated in the pons, medulla and spinal cord, and appeared slightly in the cerebellum. The meninges were affected in spots. Perivascular cell infiltration was one of the chief changes, especially in the small arteries, subcapillary and capillary, and also appeared slightly in the veins.

Watanabe fed rabbits brownstone powder with food. He obtained the same results as Levy and Tiefenbach. In the brain the gray matter was changed. Changes were especially marked in the cortical substance of the brain, corpus striatum and globus pallidus followed by the changes in the thalamus and ammon horn. Due to the changes of the central nervous system, productive inflammatory conditions of the blood vessel were observed and also so-called inflammatory foci in the brain parenchym, glia cell nodules and necrotic spots. In the corpus

striatum and globus pallidus many polypolar great nerve cells were degenerated.

Matsumura inoculated rabbits and dogs with chloric manganese, subcutaneously. In rabbits, there occurred in the central nervous system, hyperemia, hemorrhage and atrophy of the nerve cells. These changes were distributed throughout the whole brain but were not especially severe in the diencephalon, corpus striatum and their neighborhood. Further he found so-called annular cirrhosis in the liver. In dogs, reticular degeneration of neurons, edematous change with hydropic melting, enlargement and proliferation of glia cells and blood vessel cells were observed. Changes of the nerve cells were observed chiefly in the superficial zone of the brain cortica, thalamus, and corpus striatum, followed by the changes of the glia cells in the corpus striatum, thalamus and neighborhood of the base of the IV ventricle. Changes of the blood vessels in the thalamus and corpus striatum were quite marked but in general in a diffuse way. Therefore, it is not clear that in dogs manganese intoxication could affect a special part of the brain, namely corpus striatum.

Okada used rats which were given either food mixed with manganese oxid and potassium permanganate solution, or injection of the latter. Clinically, there were observed severe signs of extrapyramidal syndroms (slowing and reduction of movement), gastrointestinal symptoms (reduction and loss of appetite), psychical signs of hyperaesthesia, terror, escape movement to external stimulants. Especially should be mentioned exophthalmia and ulcer of corea. There were described histologically degeneration of the parenchym of the central nervous system and slight progressive changes of the connective tissue.

Considering the above findings on human and animal manganese poisoning, it can be seen that the central nervous system, especially the gray matter, was affected. It is agreed by all the investigators that in the case of the animal, the

changes were generalized, although they were more marked in the superficial zone. As to whether the grade of affection differed in relation to locality, it can be stated either that it was more pronounced in the brain center, especially, corpus striatum and globus pallidus, or that there was no difference. It depends partly upon the method of investigation, but chiefly upon the animal used for the experiment. For example, in monkeys the local changes were very distinct, as in the case of humans. It is worthy of mention that changes obtained by intestinal, intravenous and subcuteneous introduction of manganese were the same . In the human cases, the chief changes were located in the corpus striatum and pallidus system. In the cortical part of the brain, and in other gray matter, the changes were slighter. As to the histological changes, degeneration of the neurons was prominent, while the marrow sheath was not much affected. Changes in the glial tissue were observed in the experimental animals, but in the human cases, they were seen by some investigators in the cortical substance, and not by others. Regarding changes in the blood vessel system, they were confirmed in the cases of animals, but not in the human cases, since in the majority of human cases other complicating changes were involved. Nevertheless, it is certain that in both human and animal cases of manganese poisoning, the central nervous system is affected, and, especially, the brain center in humans.

In our findings the changes of the central nervous system was chiefly located in the globus pallidus and, especially, in the inner system. These showed neurons in several stages of degeneration and finally demolished. The polypolar great neurons were especially injured, the marrow sheath being attacked at points, and swollen, but there was no grouped degeneration of the neuron fibres. Glial cell proliferation and multiplication of the perivascular connective tissue and endothelial cells was not observed. As our case showed no other complications it will therefore be considered as a subacute manganese intoxication.

In regard to absorption, Dargero and Pucinini found manganese in the urine of dogs which had been fed with combined citral-manganese. Reisman and Minot examined in cases of manganese intoxication, the resorption, distribution and elimination of manganese oxid and silicate in the tissues. No intoxication was observed. but absorption was proved. Franklinit was given to a patient having gall bladder fistula, and manganese was excreted in the gall and urine. After giving 3–5 g. of Franklinit, the amount of manganese in the gall rose to 0.12 mg/100 ccm, the normal manganese content of the human gall being 0.003–0.001 mg/100 ccm. According to Reisman and Minot, ore with a manganese content, or maganese peroxid could come to solution in good quantity, depending on the acidity of the stomach fluid and the time of retention, resulting in the production of manganese chloride. That is, hard soluble carbonate and phosphate may be changed into soluble $MnCl_2$ by the chloric acid or acidity of the digestive and intestinal tract.

Illustrating how manganese can enter into the organism through the inhalation of brownstone powder, Schopper found manganese as a foreign body in the lung alveoles of pneumonia cases of men engaged in the crushing of brownstone. Baarder remarked often of changes in the lung caused by the inhalation of brownstone powder. This condition being designated, "brownstone lung."

Jötten, Reploh and Hagemann produced inhalation-pneumonia in rabbits and rats with brownstone powder, which took an amalgamated form. It may be assumed that in these cases manganese acted as a toxic upon the covering epithelium producing a heavy inflammation. Through the injuries thus produced, very fine manganese powder was able to penetrate into the body fluid of the lung. In these cases inhalation of the brownstone powder was the important factor but some of the inhaled brownstone powder becoming mixed with the saliva

could have passed through the esophagus into the digestive canal and been resorbed through the mucous membrane of the digestive tract. When, as in our cases, water soluble bicarbonate of manganese reaches the stomach, by chloric-acid manganese-oxid is produced which can, presumably, be easily absorbed through the gastrointestinal mucous membrane.

Regarding the deposition of manganese in the viscera, it is observed first in the liver, then in the spleen and brain. In the cases of monkeys having manganese intoxication, Mella found manganese ten times in the brain, the content of liver being 30–40 times more than normal. The experimental animals, both monkeys and rabbits, showed the liver to be heavily necrotic with productive changes. Throughout the whole brain, especially in the gray matter, there were seen degeneration and disappearance of neurons and proliferation of glia cells. These changes were most prominent, particularly, in monkeys, in the stem ganglion, namely, corpus striatum and globus pallidus.

There was also found reactive changes in the adventitia, and endothelium of the blood vessels, accompanied by perivascular cellinfiltration. Therefore, in cases of manganese intoxication, the selective points of attack in the brain and nerve cells and the venous changes may be regarded as secondary, although some authors consider them of primary importance.

In the human cases, due to the rareness of autopsy material, long duration of the course of illness, and other complications, the pathological changes were very involved, so that a summary conclusion may be difficult. However, the chief changes were found to be those occurring in the ganglion cells and the proliferation of the glia cells, the changes in the blood vessels not being dominant except in the case of Stadler which showed cardio-vascular change. In all autopsy cases, nervous changes of the extrapyramidal motoric system were present. In our case, in which death occurred in a relative short time after the development and diagnosis of the illness, no complication was observed,

and intracerebral changes were located in the globus pallidus. Here the ganglion cells were degenerated and destroyed, but the nerve fibers were only isolatedly affected, their condition being detected only through fat staining. This showed the fatty granular cells to be perivascularly multiplied as proof of the fact that degeneration took place in the brain parenchym. In this, our pure case of manganese intoxication, the chief changes were in the neurons of the globus pallidus (large polypolar and small ganglion cells) which were isolatedly affected.

Concerning changes in other organs of experimental animals, (monkeys and rabbits), there was found in the livers necrotic productive changes and fatty degeneration. It may be presumed, therefore, that a large amount of manganese could be deposited in the liver. Charles, also proved disturbance of the liver, in human cases, by lactose-test. In our cases, the liver showed no change except a slight fatty degeneration. It is difficult to determine that changes in the liver are intrinsic to manganese poisoning. Furthermore a moderate proliferation of connective tissue does not point conclusively to manganese poisoning, but depends upon the age of the person. In the lung, no specific change was found. Clinically, sex-disorder may be complained of, but in our case this circumstance was not made clear. The stopping of spermatogenesis in the histological findings may indicate such a disturbance. No changes were marked in any other organs. The cause of a clinically eminent edema was not found.

As to the etiological causes, the individual predisposition is given consideration by authors. The symptoms of manganese intoxication may appear in manganese crushers who are working in insufficiently ventilated rooms relatively earlier. In Japan, Kodama's first case began to show evidence of intoxication in 3 months. Ohe's third case, in 5 months, Ashizawa's in 6 months, and others from 10–18 months. Abroad, Bickert reported cases showing evidence of the ailment after 3–6 months

and chronic cases were recognized after several to 10 years, but the poisonings all occurred under individual circumstances in the same room. Baader reported one case among 12 men engaged in transporting manganese from a ship. In such cases over-work and predilection must be considered (Sato). In our cases, the intoxication appeared in the beginning of November and as the dry-cells had been buried in August, September and October, symptoms were manifest in 2–3 months. From the latter part of December the drinking of the suspected well-waters was prohibited and the intoxication ceased to appear in the beginning of January, 5–6 months after the burial of the dry-cells. No doubt can be retained as to the relation of the outbreak of the disease and the manganese content of the well-waters. Regarding sex and age, in relation to manganese intoxication, since it occurred generally among the crushers of brown-stone and workers with manganese material the majority of workers were men and the cases were therefore predominantly masculine. According to Bickert persons over the age of 30 were most susceptable to the intoxication. In our cases there is no age or sex disposition. However, it is interesting that children were affected much less severely and after a longer interval, than the adults. Whether the age-disposition may have a like relation with poisoning by other heavy metals such as lead, mercury, etc. is necessary of further study.

Conclusion.

1. We have encountered a mass intoxication of manganese in drinking water from wells. There were a total of 16 cases, of which 5 were severe, 2 moderate and 9 mild cases. Two of the severe cases died and one of the moderate cases committed suicide from melancholy.

2. Symptoms corresponded to disturbance of the extra-pyramidal motor tract. An autopsy case showed atrophy and

disappearance of the nerve cells of the globus pallidus. The changes corresponded to the changes produced by manganese intoxication.

3. From animal tests and histological examinations encephalitis could be excluded. Search for the cause resulted in the finding that the intoxication was due to manganese dissolved in well water from old dry cells buried around a well. Manganese and zinc were found in large quantities in the well water used by the affected cases, while adjoining well water which did not cause intoxication contained no metals. These metals were also found in large quantities in the viscera of the autopsy case and in the urine and blood of intoxicated patients.

4. It is noticeable that the symptoms of intoxication began with lethargy and edema.

5. Intoxication was more severe in older persons than young ones. There was no difference due to sexes.

6. Zinc found in water appears to have no relation to symptoms produced and pathological changes found in tissues.

We wish to thank Dr. Nomura, chief of the Kamakura Insane Hospital for research on the mental states of the patients, and Prof. Nagaumi and Dr. Yamamura of the Tokyo Technical University for technical and chemical analysis.

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Explanation of Figures.

Fig. 1–3. Paraffin sections stained singly with hematoxylin.
Fig. 4–6. Freezing sections stained with hematoxylin and Sudan III (Kawamura and Yasaki).
Fig. I. Showing degeneration of large nerve cells; cells having lost polypolar processes and shrunken slightly; nucleus atrophic.
Fig. II. Ringformed swollen marrow sheath, brown-red; in neighborhood, some reddish-brown stained pigment, granules.
Fig. III. Showing degeneration of small nerve cells. Nerve cells slightly swollen with atrophic nucleus; loss of nucleus, with red-brownish pigment granules.
Fig. IV. Multiplied brown red pigments in degenrated neuron. Dead nerve and brown-red pigment cluster remaining.
Fig. V. Swollen and red stained marrow sheath. In environment, freed fatty granules.
Fig. VI. Disappearance of nerve cells; multiplication of red-brown pigment granules. In the neighborhood, multiplied perivascular fatty granular cells.

Fig. 1.

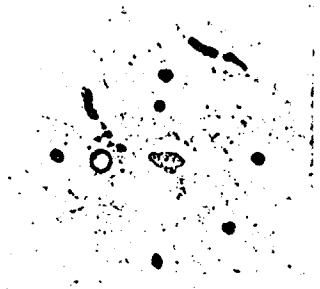

Fig. 2.

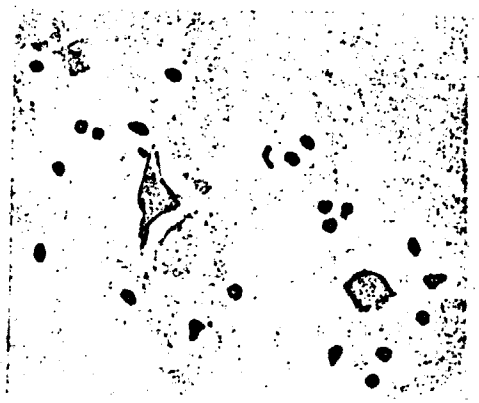

Fig. 3.

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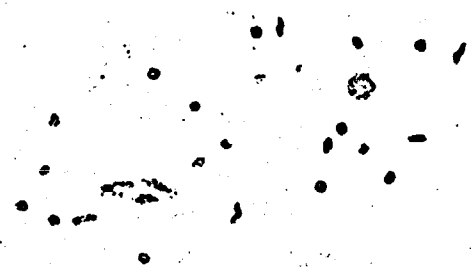

Fig. 4.

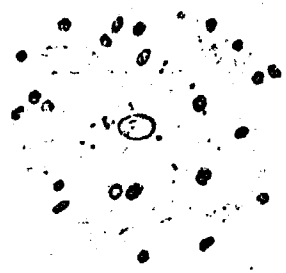

Fig. 5.

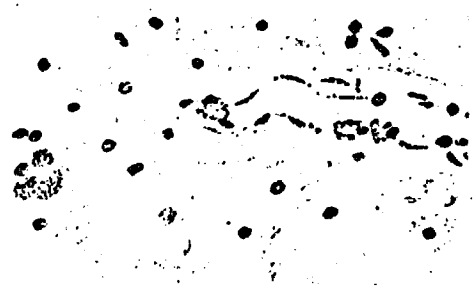

Fig. 6.

R. Kawamura, H. Ikuta, S. Fukuzumi, R. Yamada and S. Tsubaki.

Archives des Maladies Professionnelles, I:119 1938

Work of the Toxicology Laboratory of the Pharmacy Faculty of Paris, Professor Rene Fabre

Experimental Toxicological Research on Manganese

by A. C. Lemos*

Industrial usage of manganese has caused serious occupational poisonings among workers, and in France since 1935, accidents caused by pyrolusite and manganese dioxide must be reported. This has led us to study experimentally in the rabbit the distribution of manganese after the administration of various products of manganese content. In order to accomplish this, we measured the quantity of the metal in samples of organs at the time of autopsy of the animals by means of a method derived from the one which we described in collaboration with P. Cheramy (2). This technique was inspired by the work of G. Bertrand (1), Travers (6), Korenman (4), Leroy (5), concerning the determination of manganese by transformation to a permanganate and involving the following operations: destruction of the organic material by the nitro-sulfo-perchloric method of E. Kahane (3); the residual liquid is dried up with sand in a conical Pyrex flask with a large opening where the destruction took place; the acid vapors which are released are sucked into a glass tube connected to a water pump .30 cmc of distilled water are added to the dry product, the solution is filtered through a smooth filter which is washed in the same way as the flask with a little distilled water in such a way so as to obtain a final volume of about 60 cmc. To this liquid is added 9 to 10 cmc of pure sulfuric acid, 4 drops of silver nitrate (10 p. 100) and 0.25 g pulverized potassium

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*Lemos, A. University Doctoral Thesis (Pharmacy), Paris 1938, "Contribution to the experimental toxocological study of manganese".

persulfate, and it is heated to boiling and kept at that temperature for 10 to 12 minutes. The permanganate which forms colors the liquid pink at the same time as the excess of persulfate becomes decomposed; some minutes later when the temperature of the liquid is about 60 to 70°, 10 cmc of a solution of oxalic acid with a strength of 0.6 p. 1000 determined precisely in proportion to the permanganate N/100 is added. There is discoloration, and in the still warm mixture the excess of oxalic acid is determined by means of a centinormal solution of permanganate. It is simple to calculate by differentiation the amount of permanganate corresponding to the manganese contained in the sample, 1 cmc of permanganate N/100 corresponds to 0.110 mg of manganese.

For certain substances rich in calcium (bones, teeth, etc.), it is necessary to eliminate the calcium sulfate contained in the destruction residue in the form of a voluminous precipitate; to do this, 50 cmc of water, 10 cmc of sulfuric acid, and 40 cmc of alcohol are added to this residue; after 12 hours of standing, it is filtered, and the filter is washed with alcohol at 40°C. Then all the liquids are heated in a double boiler to get rid of the alcohol. Finally, the volume is brought to 60 cmc with distilled water and the determination is done as previously described.

This technique may be used for the quantitative determination of manganese in samples containing .005 to 1 mg of this metal; the absolute error will not exceed 2 to 3γ if a good microburette is used. Our control experiments were done using as the substance to be tested, human blood containing less than .003 mg of manganese per liter, as indicated by G. Bertrand (1). These experiments showed us that pure commercial reagents do not contain appreciable quantities of manganese.

The experimental substances comprised: 1-soluble derivatives: manganese chloride, manganese sulfate, potassium permanganate which were administered by the intra-muscular route, by esophagal tube, or simply by mixing it with the daily ration of food; 2-pulverized manganese dioxide which was absorbed in three ways: a) mixture with food which allows for prolonged experimentation; (b) an oily suspension injected into the stomach by means of an esophageal tube; (c) inhalation--the animal is kept for a given period of time in an atmosphere containing dioxide dusts; there is also important absorption by the digestive route in this case, from the animal licking his fur contaminated by the substance.

We used 13 rabbits and 1 guinea pig. Rabbit #1 served as a control and the manganese content was measured in the organs of this animal which received no manganese containing substance. Here, briefly summarized are the treatments given to the other animals:

Rabbit #2, weighing 1.885 g, received by an esophageal tube in 5 days, 5.50 g of manganese chloride in the form of a solution of 10 and 20 p. 100, death came after a comatose period of several hours.

Rabbit #3, weighing 1.720 g absorbed for three months, 2 g per day of manganese sulfate added in the form of a solution to his wheat bran; the animal was sacrificed.

Rabbit #4, weighing 1.620 g, seriously afflicted with coccidiosis, received an intra-muscular injection of 2 g of manganese sulfate in a solution of 10 p. 100, death followed in ten hours, but could not be attributed solely to the injected substance.

Rabbit #5, 1.290 g, received an intra-muscular injection of 20 cmc of a solution of 5 p. 100 potassium permanganate. Death came

after 27 hours, the animal showing a large abscess on the foot where the injection was given.

Three animals absorbed finely pulverized manganese dioxide mixed with their diet in amounts of 1 to 2 g per day. This treatment lasted two months for rabbit #6, three months for rabbit #7, and five months for rabbit #8. All these animals were sacrificed.

Rabbits #9 and 10 received manganese dioxide in the form of a suspension in olive oil titrated to about 2 p. 100 dioxide. This suspension was administered in doses of 10 to 15 cmc, by means of an esophageal tube. Unfortunately, the oil accidentally got into the lungs which caused the deaths of the animals after three doses of oil for #9 and six doses for #10.

Finally, animals #11, 12, 13, and 14 were placed in a container through which was sent a current of air filled with fine dust of manganese dioxide; the duration of their inhalation varied between 50 hours for #11 to 300 hours for #13, the rabbits absorbing as well through licking, a significant quantity of the product. Guinea pig #14 further received a diet containing manganese dioxide (he shared a cage with rabbit #8). All these animals were sacrificed.

The results of the quantitative determinations of manganese done on the animals' organs are shown in tables 1, 2, and 3; they are expressed in milligrams of manganese per 100 g of organ, which allows interesting localizations to be seen: finally, the figures for rabbit #1 who received no treatment can serve as points of comparison.

The results indicated in table 1 show that: (a) manganese chloride administered in large doses orally, causes death of the animals and is

localized principally in the heart, the kidneys, and the liver. But the elevated levels in the suprarenals, the bone marrow, the bile, and the skin should also be noted. The brain, on the other hand, shows a normal amount; (b) manganese sulfate absorbed daily in moderate doses brings about only a slight penetration of the entire organism; (c) after intra-muscular injections, on the other hand, high levels are found in several organs, particularly the suprarenals, the bone marrow and the spleen; (d) the injection of permanganate, having caused the death of the animal is characterized by high levels in the spleen, the heart and especially the suprarenals, which sometimes show at autopsy, a brownish coloration.

Table 2 summarizes the distribution of manganese in animals having absorbed the dioxide orally; one notices an increase in amount in the principal organs in relation to the duration of the treatment, this is particularly clear in the brain; the amounts in the muscles and the blood are by contrast obviously constant. The bile shows a high concentration, no doubt, in accord with its role as a route of elimination, and this is the same for the skin and the hair.

The figures in table 3 show the amounts of manganese in the organs of animals subjected to inhalations of dioxide dusts; here also there is a progressive increase in relation to the duration of the treatment, principally in the heat, the kidneys, the lungs, and the brain. The amounts found in the suprarenals and the bone marrow are significant as well.

To summarize, the experiments done showed the toxicity of soluble manganese salts injected or absorbed at high doses; on the other hand, manganese dioxide ingested or inhaled does not seem to have an immediate toxic effect; the animals were sacrificed or died accidentally, and they

often showed at autopsy, symptoms of hepatic or renal affliction. The study of the distribution of the metal in the organism has shown that a prolonged treatment leads to a penetration of the organs and especially the brain. But an important fact lies in the high levels frequently found in the small organs: the suprarenals, the bone marrow, the testicles, the spleen, which may with localization in nerve centers, explain accidents reported in workers exposed to prolonged absorption of significant quantities of manganese containing substances.

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Organes prélevés (2)	LAPINS N° (1) 1	2	3	4	5
	Normal. (3)	Cl_2Mn voie buccale. (4)	SO_4Mn voie buccale. (5)	SO_4Mn injecté. (6)	MnO_4K injecté. (7)
(8) Foie	0,08	6,3	0,31	11,12	0,02
(9) Coeur	0,03	61,9	0,46	4,75	5,1
(10) Reins	0,01	9,5	0,45	3,58	0,47
(11) Poumons	Traces	2,75	0,14	2,34	0,81
(12) Cerveau	0,67	0,74	1,36	1,10	0,13
(13) Rate	Traces		Traces	4,2	2,9
(14) Muscles	0,01	0,53	0,23		0,40
(15) Sang	Traces		0,18	2,8	0,19
(16) Peau et Poils	Traces	3,60	0,30		0,28
(17) Bile	0,30	10,8			
(18) Testicules ou ovaires	0,02	1,61	Traces	1,6	Traces
(19) Surrénales	Traces	50,6	Traces	5,0	10,9
(20) Os	Traces	0,46	Traces	0,96	Traces
(21) Moelle osseuse		10,3		6,33	

Table 1. Manganese in milligrams per 100 g fresh organ

1. Rabbit no.
2. Organs sampled
3. Normal
4. $MnCl_2$ oral route
5. $MnSO_2$ oral route
6. $MnSO_4$ injected
7. $MnKO_4$ injected
8. Liver
9. Heart
10. Kidneys
11. Lungs
12. Brain
13. Spleen
14. Muscles
15. Blood
16. Skin and hair
17. Bile
18. Testicles or ovaries
19. Suprarenals
20. Bone
21. Bone marrow

Organes prélevés. (2)	LAPINS N° (1) 6	7	8	9	10
	MnO_2 par voie buccale (3)			Suspension huileuse (4)	
	2 mois (5)	3 mois	5 mois	3 doses (6)	6 doses.
(7) Foie	3,92	0,94	0,95	1,26	1,17
(8) Cœur	0,27	1,87	2,5	3,8	4,38
(9) Reins	1,00	1,35	1,90	1,1	4,99
(10) Poumons	0,07	0,80	1,7		
(11) Cerveau	1,07	3,01	5,8	0,66	1,26
(12) Rate	Traces	Traces	Traces	0,23	8,12
(13) Muscles	0,54	0,17	0,45		0,38
(14) Sang		0,25	0,26		0,59
(15) Peau et Poils	1,49	2,20	3,9		1,13
(16) Bile	25,4		54		
(17) Testicules	0,71	0,41	11,9		2,12
(18) Surrénales	Traces		18,8		Traces
(19) Os	0,95	0,35			0,20
(20) Moelle osseuse	3,35				1,66

Table 2. Manganese in milligrams per 100 g fresh organ

1. Rabbit no.
2. Organs sampled
3. MnO_2 orally
4. Oil suspension of MnO_2 by esophageal tube
5. Months
6. Doses
7. Liver
8. Heart
9. Kidneys
10. Lungs
11. Brain
12. Spleen
13. Muscles
14. Blood
15. Skin and hair
16. Bile
17. Testicles
18. Suprarenals
19. Bone
20. Bone marrow

Organes prélevés.	LAPINS N°			COBAYE
	11	12	13	11
	Inhalation de MnO_2			[illegible]
	50 heures.	125 heures.	300 heures.	210 heures et alimentation chargée de MnO_2.
Foie	0,45	1,11	1,5	2,2
Cœur	1,00	2,84	3,06	3,1
Reins	0,71	1,35	3,05	3,4
Poumons	2,4	3,8	3,2	1,2
Cerveau	0,9	0,68	1,52	5
Rate	1,05	Traces	Traces	4,0
Muscles	0,38	0,39	0,67	0,86
Testicules	Traces	Traces	2,5	1,3
Surrénales				7,0
Os	1,2	0,78	1,7	4,0
Moelle osseuse	0,64	1,3	10,8	

Table 3. Manganese in milligrams per 100 g fresh organ

1. Rabit no.
2. Guinea pig
3. Organs sampled
4. Inhalation of MnO_2
5. Inhalation of MnO_2
6. Hours
7. 210 hours and food containing MnO_2
8. Liver
9. Heart
10. Kidneys
11. Lungs
12. Brain
13. Spleen
14. Muscles
15. Testicles
16. Suprarenals
17. Bone
18. Bone marrow

Arch. Mal. Prof. I: 119-122, 1938

TRAVAIL DU LABORATOIRE DE TOXICOLOGIE DE LA FACULTÉ DE PHARMACIE DE PARIS, PROFESSEUR RENÉ FABRE.

Recherches toxicologiques expérimentales sur le manganèse.

Par M.

Adamantios C. LEMOS (*).

Les emplois industriels du manganèse ont provoqué d'assez graves intoxications professionnelles chez les ouvriers et, depuis 1935, la déclaration des accidents causés par la pyrolusite et le bioxyde de manganèse est obligatoire en France. Ceci nous a incité à étudier expérimentalement, sur le lapin, la répartition du manganèse après administration de divers produits manganiques. Pour cela nous avons dosé le métal, dans les organes prélevés à l'autopsie des animaux, au moyen d'une méthode dérivée de celle que nous avons décrite en collaboration avec P. Chéramy (2). Cette technique est inspirée des travaux de G. Bertrand (1), Travers (6), Korenman (4), Leroy (5), concernant le dosage du manganèse par transformation en permanganate, elle comporte les opérations suivantes : destruction de la matière organique par la méthode nitro-sulfo-perchlorique de E. Kahane (3); le liquide résiduel est évaporé à sec, au bain de sable, dans la fiole conique en Pyrex à large ouverture, où s'est effectuée la destruction; les vapeurs acides qui se dégagent sont aspirées par un tube de verre relié à une trompe à eau. Le produit sec est repris par 30 cmc. d'eau distillée, la solution est filtrée sur un filtre sans plis qui est lavé, ainsi que la fiole, avec un peu d'eau distillée, de manière à obtenir un volume final de 60 cmc. environ. Ce liquide, additionné de 9 à 10 cmc. d'acide sulfurique pur, de 4 gouttes de nitrate d'argent à 10 p. 100 et de 0 g. 25 de persulfate de potassium pulvérisé, est chauffé jusqu'à l'ébullition qui est maintenue dix à douze minutes. Le permanganate qui se forme colore en rose le liquide en même temps que se décompose l'excès de persulfate; quelques minutes après, la température du liquide étant d'environ 60 à 70°, il est ajouté 10 cmc. de solution d'acide oxalique à 0,6 p. 1.000 dont le titre, par rapport au permanganate N/100 a été déterminé avec précision. Il y a décoloration et, dans le mélange encore chaud, l'excès d'acide oxalique est

(*) LEMOS, A., *Thèse de Doctorat de l'Université* (Pharmacie), Paris, 1938. « Contribution à l'étude toxicologique expérimentale du Manganèse. »

dosé au moyen de la solution centinormale de permanganate. Il est facile de calculer par différence la quantité de permanganate correspondant au manganèse contenu dans le prélèvement, 1 cmc. de permanganate N/100 correspond à 0 mg. 110 de manganèse.

Dans certains produits riches en calcium (os, dents, etc.) il est nécessaire d'éliminer le sulfate de calcium contenu dans le résidu de destruction sous forme de précipité volumineux; pour cela ce résidu est repris par un mélange de 50 cmc. d'eau, 10 cmc. d'acide sulfurique et 40 cmc. d'alcool; après douze heures de contact on filtre, le filtre est lavé à l'alcool à 40 C, puis les liqueurs réunies sont évaporées au bain-marie pour chasser l'alcool. Finalement le volume est amené à 60 cmc. avec de l'eau distillée et le dosage se termine comme précédemment.

La technique est applicable au dosage du manganèse dans des prélèvements contenant de 0 mg. 005 à 1 mg. de ce métal; l'erreur absolue ne dépassant pas 2 à 3 γ si l'on emploie une bonne microburette. Nos essais témoins ont été pratiqués en utilisant, comme substance à détruire, du sang humain qui contient, ainsi que l'a indiqué G. Bertrand (1), moins de 0 mg. 003 de manganèse par litre. Ces essais nous ont également montré que les réactifs purs du commerce ne contenaient pas de quantités appréciables de manganèse.

Les produits expérimentés ont compris : 1° des dérivés solubles : chlorure de manganèse, sulfate de manganèse, permanganate de potassium, qui ont été administrés soit par voie intra-musculaire, soit par une sonde œsophagienne ou, plus simplement, par mélange au son entrant dans la ration journalière; 2° du bioxyde de manganèse pulvérisé dont l'absorption a été réalisée de trois manières différentes : *a*) mélange aux aliments, ce qui permet une expérimentation prolongée; *b*) suspension huileuse injectée dans l'estomac au moyen d'une sonde œsophagienne; *c*) inhalation, l'animal étant maintenu pendant un temps donné dans une atmosphère chargée de poussières de bioxyde; il y avait également, dans ce cas, absorption importante par voie digestive, l'animal léchant son pelage souillé du produit.

Nous avons utilisé 13 lapins et 1 cobaye. Le lapin n° 1 asservi de témoin et le manganèse a été dosé dans les organes de cet animal n'ayant reçu aucun produit manganique. Voici, succinctement résumés, les traitements subis par les autres animaux :

Le lapin n° 2, pesant 1.885 g., reçoit, au moyen d'une sonde œsophagienne, en cinq jours, 5 g. 50 de chlorure de manganèse sous forme de solution à 10 et 20 p. 100, la mort survient après une période comateuse de plusieurs heures.

Le lapin n° 3, pesant 1.720 g., a absorbé, pendant trois mois, 2 g. par jour de sulfate de manganèse ajouté, sous forme de solution, à du son de blé; l'animal a été sacrifié.

Le lapin n° 4, pesant 1.620 g., gravement atteint de coccidiose, a reçu une injection intra-musculaire de 2 g. de sulfate de manganèse en solution à 10 p. 100, la mort est survenue en dix heures, mais ne peut être attribuée uniquement au produit injecté.

Le lapin n° 5, de 1.290 g., reçoit, en injection intra-musculaire 20 cmc. de solution à 5 p. 100 de permanganate de potassium. La mort survient au bout de vingt-sept heures, l'animal présentant un volumineux abcès de la patte injectée.

Trois animaux ont absorbé du bioxyde de manganèse finement pulvérisé,

mélangé à leur ration alimentaire à la dose de 1 à 2 g. par jour. Ce traitement a duré deux mois pour le lapin n° 6, trois mois pour le n° 7 et cinq mois pour le n° 8. Tous ces animaux ont été sacrifiés.

Les lapins n°s 9 et 10 ont reçu le bioxyde de manganèse sous forme d'une suspension dans l'huile d'olive titrant environ 2 p. 100 de bioxyde. Cette suspension était administrée, à la dose de 10 à 15 cmc., au moyen d'une sonde œsophagienne. Il s'est produit malheureusement des passages accidentels d'huile dans le poumon, ce qui a provoqué la mort des animaux, après trois doses d'huile pour le n° 9 et six doses pour le n° 10.

Enfin les animaux n°s 11, 12, 13 et 14 étaient placés dans une cloche traversée par un courant d'air chargé de fines poussières de bioxyde de manganèse, les durées de ces inhalations ont varié de cinquante heures pour le n° 11 à trois cents heures pour le n° 13, les lapins absorbaient également, par léchage, une quantité importante de produit. Le cobaye n° 14 a reçu de plus une alimentation chargée en bioxyde de manganèse (il partageait la cage du lapin n° 8). Tous ces animaux ont été sacrifiés.

Les résultats des dosages de manganèse pratiqués sur les organes des animaux sont rassemblés dans les tableaux n° 1, 2 et 3; ils sont exprimés en milligrammes de manganèse pour 100 g. d'organe, ce qui permet de mettre en évidence les localisations intéressantes; enfin les chiffres trouvés pour le lapin n° 1, n'ayant subi aucun traitement, peuvent servir de point de comparaison.

Les résultats mentionnés dans le tableau n° 1 montrent que : *a*) le chlorure de manganèse administré à hautes doses, par voie buccale, provoque la mort de l'animal et se localise principalement dans le cœur, les reins, le foie. Mais il faut signaler de plus la teneur élevée des surrénales, de la moelle osseuse, de la bile et de la peau. Le cerveau, par contre, a une teneur normale; *b*) le sulfate de manganèse absorbé journellement, à doses modérées, ne donne qu'une légère imprégnation de tout l'organisme; *c*) après injection intra-musculaire, au contraire, il se rencontre en dose élevée dans de nombreux organes, en particulier le foie, mais avec une prédominance marquée pour les surrénales, la moelle osseuse et la rate; *d*) l'injection de permanganate, ayant provoqué la mort de l'animal, est caractérisée par une teneur élevée de la rate, du cœur et surtout des surrénales, qui présentaient, d'ailleurs, à l'autopsie de l'animal, une coloration brunâtre.

Le tableau n° 2 résume la répartition du manganèse chez les animaux ayant absorbé le bioxyde par voie buccale, on remarque une augmentation de la teneur des principaux organes en rapport avec la durée du traitement, ceci est particulièrement net pour le cerveau; les teneurs du muscle et du sang sont, par contre, sensiblement constantes. La bile présente une concentration élevée, en rapport sans doute avec son rôle de voie d'élimination, il en est de même de la peau et des poils.

Dans le tableau n° 3 figurent les teneurs en manganèse des organes d'animaux soumis aux inhalations de poussières de bioxyde; cette fois encore il y a augmentation progressive, en rapport avec la durée du traitement, principalement dans le cœur, le rein, le poumon et le cerveau. La teneur des surrénales et de la moelle osseuse est, également, importante.

En résumé, les expériences effectuées ont montré la toxicité des sels solubles de manganèse injectés ou absorbés à hautes doses; par contre le

bioxyde de manganèse ingéré ou inhalé ne paraît pas avoir eu d'effet toxique immédiat, les animaux ont été sacrifiés ou sont morts accidentellement, ils présentaient souvent à l'autopsie des signes d'atteinte hépatique et rénale. L'étude de la répartition du métal dans l'organisme a montré qu'un traitement prolongé amenait une imprégnation des organes et surtout du cerveau. Mais un fait important réside dans les hautes teneurs rencontrées fréquemment dans de petits organes : surrénales, moelle osseuse, testicules, rate, qui peuvent, avec la localisation dans les centres nerveux, expliquer les accidents constatés chez les ouvriers exposés à l'absorption prolongée de quantités importantes de produits manganiques.

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Tableau n° 1. — *Manganèse en milligrammes pour 100 g. d'organe frais.*

Organes prélevés.	LAPINS N° 1	2	3	4	5
	Normal.	Cl_2Mn voie buccale.	SO_4Mn voie buccale.	SO_4Mn injecté.	MnO_4K injecté.
Foie	0,08	6,3	0,31	11,12	0,02
Cœur	0,03	61,9	0,46	4,75	5,1
Reins	0,01	9,5	0,45	3,58	0,47
Poumons	Traces	2,75	0,14	2,34	0,81
Cerveau	0,67	0,44	1,36	1,10	0,13
Rate	Traces		Traces	4,2	2,9
Muscles	0,01	0,43	0,23		0,40
Sang	Traces		0,18	2,8	0,19
Peau et Poils	Traces	3,60	0,30		0,28
Bile	0,30	10,8			
Testicules ou ovaires	0,02	1,61	Traces	1,6	Traces
Surrénales	Traces	50,6	Traces	5,0	10,9
Os	Traces	0,46	Traces	0,96	Traces
Moelle osseuse		10,3		6,33	

Tableau n° 2. — *Manganèse en milligrammes pour 100 g. d'organe frais.*

Organes prélevés.	LAPINS N°s 6	7	8	9	10
	MnO_2 par voie buccale.			Suspension huileuse de MnO_2 par sonde œsophagienne.	
	2 mois.	3 mois	5 mois.	3 doses.	6 doses.
Foie	3,92	0,94	0,95	1,26	1,17
Cœur	0,27	1,87	2,5	3,8	4,38
Reins	1,00	1,35	1,90	1,1	4,99
Poumons	0,07	0,80	1,7		
Cerveau	1,07	3,01	5,8	0,66	1,26
Rate	Traces	Traces	Traces	0,23	8,12
Muscles	0,54	0,47	0,45		0,38
Sang		0,25	0,26		0,59
Peau et Poils	1,49	2,20	3,9		1,13
Bile	25,4		54		
Testicules	0,71	0,41	11,9		2,12
Surrénales	Traces		18,8		Traces
Os	0,95	0,34			0,20
Moelle osseuse	3,35				1,66

Tableau n° 3. — *Manganèse en milligrammes pour 100 g. d'organe frais.*

Organes prélevés.	LAPINS N°s 11	12	13	COBAYE 14
	Inhalation de MnO_2.			Inhalation de MnO_2
	50 heures.	125 heures.	300 heures.	210 heures et alimentation chargée de MnO_2.
Foie	0,45	1,11	1,5	2,2
Cœur	1,00	2,84	3,06	3,1
Reins	0,71	1,35	3,04	3,4
Poumons	2,4	3,8	3,2	1,2
Cerveau	0,9	0,68	1,52	5
Rate	1,05	Traces	Traces	4,0
Muscles	0,38	0,39	0,67	0,86
Testicules	Traces	Traces	2,5	1,3
Surrénales				7,0
Os	1,2	0,78	1,7	4,0
Moelle osseuse	0,64	1,3	10,8	

Problemy Endokrinologii i Gormonoterapiya
No. 4, 1958: 25-30

CHANGES IN THE ADRENAL CORTEX OF WHITE RATS POISONED WITH MANGANESE OXIDES

E. N. Levina and N. A. Minkina

The effect of the state of the endocrine system or its individual elements on the sensitivity of the body to poisons and the changes that take place in the endocrine glands after exposure to poisons, including those used in industry, have not been adequately studied either in the clinic or under experimental conditions.

It is known from clinical observations as well as from some experiments that changes take place in thyroid function after exposure to such industrial poisons as carbon monoxide, lead, mercury, gasoline, and cobalt. For example, hyperthyroidism has been described as the result of acute carbon monoxide poisoning and enlargement of the thyroid without impairment of its function due to chronic carbon monoxide poisoning [12, 24, 30]. Symptoms of hyperthyroidism were observed in chronic mercury poisoning [3, 6, 17]. There are references to the development of exophthalmic goiter after chronic lead poisoning. Hyperthyroid has also been seen in animals poisoned with lead [7, 28]. Such occupational poisons as fluorine and cobalt are known to inhibit hormone production in the thyroid. The injection of cobalt also causes enlargement of the thyroid [10, 16, 22, and others].

The data on changes in the adrenals after exposure to industrial poisons are less concrete. There are very general and quite contradictory references to changes in both the glands proper and in their functions after poisoning by carbon monoxide, lead, cadmium, tetraethyl lead, silicon, etc. Some data are available on increased adrenal function as a result of acute and chronic carbon monoxide poisoning [15, 30, 24]. Enlargement

of the gland and functional changes in adrenocortical activity, judging by the excretion of 17-ketosteroids with urine, were also noted after exposure to silicon dioxide and tetraethyl lead [26, 23]. Enlargement of the adrenals and decrease in their ascorbic acid content followed the poisoning of animals with carbon tetrachloride [1]. Some investigators regard impairment of adrenal function as an early manifestation of benzonal (?) poisoning [7].

Study of the distribution of poisons revealed that they accumulate in large quantities in some endocrine glands, including the adrenals. This is known to be the case with such metals as mercury, lead, cadmium, tin, chrome and with several organic compounds [14, 19, 29].

The significance of such a distribution is still obscure. It may be due in part to the rich blood supply of the endocrine glands [20]. There are also indications that the poisons exert some specific influence on the function of certain endocrine glands. For example, fluorine, cobalt, and manganese may have an effect on the thyroid [16, 8, 10, 25, etc.].

Earlier studies of ours [8] showed that poisoning rats with manganese oxides results in a shrinking of the thyroid and depression of its function as well as a decrease in gas exchange. These findings together with the well-known fact that the adrenal cortex plays a role in the general adaptive and metabolic reactions to various external factors, including toxic ones, demonstrate the need for an investigation of the state of the adrenal cortex in animals poisoned with manganese compounds.

The external functional state of the adrenals may also be helpful in interpreting the beneficial effect of steroid hormones in the treatment of occupational poisonings. It is generally known that manganous chloride causes hyperemia, hemorrhages, degenerative and necrotic changes in the adrenals and an increased amount of lipids in the cortical layer in rabbits given the

the substance with food or injected with it subcutaneously. Prolonged inhalation of manganese dioxide produces the same effects in dogs [5, 9, 17]. Decreased excretion of 17-ketosteroids with urine was found in some persons chronically poisoned with manganese.

In our study, the use of certain histological and histochemical methods enabled us to detect morphological and functional changes in the adrenal cortex of rats chronically poisoned with manganese oxides. White rats were injected subcutaneously with one of two manganese oxides (MnO_2 or MnO) at a dose of 100 mg/kg of weight. Selected according to their original weight and of the same sex, the animals were divided into 3 groups of 15 each. One received MnO_2, the second MnO, and the third served as the control. Injections of suspensions of the manganese oxides in physiologic saline and of physiologic saline alone (control) were given every other day. The rats received a total of 18 injections. The experiments were run for a month, in April.

Intravitam symptoms of the toxicity of the manganese oxides were manifested by a loss of weight, adynamia, and development in some of the animals of central paresis of the hind legs. One of the 15 rats died after the 13th injection of mangahese dioxide. The ~~surviving~~ experimental and control animals alive 7 to 8 days after injection of the oxides was suspended were sacrificed. Autopsy revealed that the adrenals of the experimental animals was enlarged compared to the control. Weighing the gland and computation of the weight coefficient confirmed the marked increase.

In another series of experiments involving the inhalation of manganese dioxide aerosols by rabbits, the adrenals became enlarged as in the rats. The mean weight coefficient was 0.156 in the experimental rabbits and 0.094 in the control. In the same experiments, inhalation of a manganese dioxide aerosol resulted in an appreciable accumulation of manganese in the adrenals.

After 3½ months of inhaling MnO_2 dust, the manganese concentration in the adrenals was 1.4 mg% (~~weight~~ wet weight) and it was higher than in the liver, kidneys, brain, and other viscera of the same animals, but lower than in the thyroid.

Table 1

Weight Coefficients of the Right Adrenal in the Experimental and Control Rats

(1)	(2)															(3) (4)	(3) (5)
M 2	0.43	0.32	0.30	0.28	0.26	0.23	0.22	0.22	0.22	0.193	0.17	0.16	0.14	0.14	0.13	0.181	0.281
M 1	0.33	0.25	0.22	0.22	0.19	0.19	0.17	0.16	0.12	0.12	0.11	0.10	0.10	0.13	0.08	0.117	0.216
(6)	0.16	0.15	0.14	0.14	0.105	0.103	0.10	0.045	0.083	0.076	0.069	0.05	–	–	–	0.083	109 ε.

1 - Oxides injected
2 - Individual values
3 - Mean value
4 - Males
5 - females
6 - control

Our findings on the accumulation of manganese in the adrenals is quite consistent with some published data on the increased manganese content of the adrenals in intact animals [4, 21] and in dogs and rabbits that inhaled manganese oxides over a long period of time or that were injected subcutaneously with an isotope of the metal [9, 20, 18].

The references in the literature to adrenocortical function in manganese poisoning are conflicting. Boyer [19], for example, found an increase in the excretion of neutral 17-ketosteroids with urine (i.e., an intensification of adrenocortical activity), while others detected a decrease therein [27]. It will be noted that the amount of neutral 17-ketosteroids in the urine does not accurately characterize adrenocortical function because of the considerable fluction in the content of 17-ketosteroids,

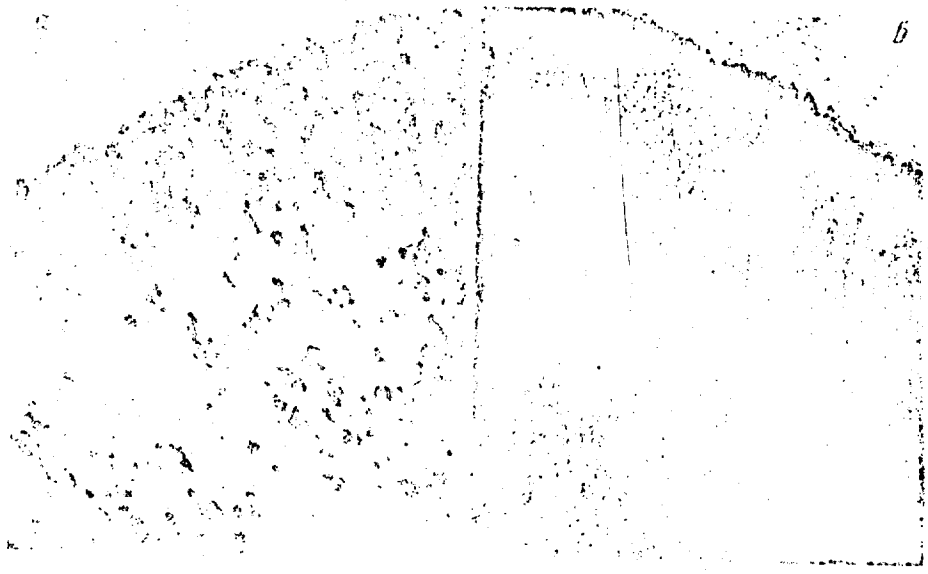

Fig. 1. Sudanophilic lipids in the adrenal cortex.

a - after injection of MnO; b - control. Magnification 100 X.

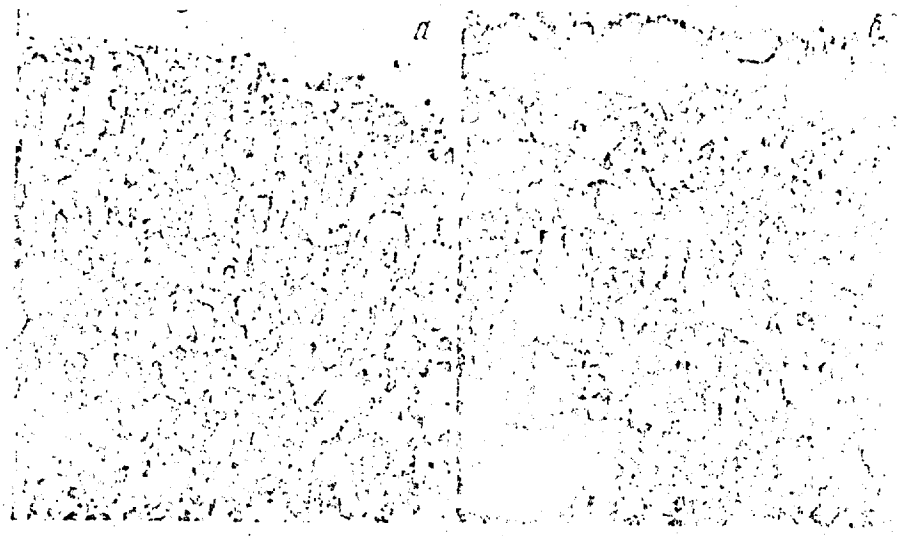

Fig. 2. Ketosteroids in the adrenal cortex.

a - after injection of MnO; b - control. Magnifioation 157 X.

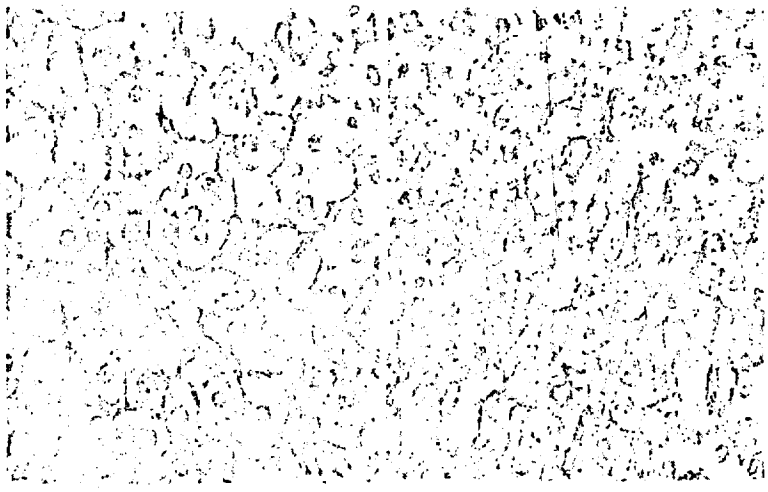

Fig. 3. Ascorbic acid in the adrenal cortex.

a - after injection of MnO; control. Magnification 314 X.

The nucleic acids were detected by Feulgen's method (DNA) and staining with pyronin-methyl green (RNA). Determination of the DNA content* of the nuclei in the adrenal cells revealed a decrease in the glomerular and reticular zones but about the same as in the control in the fascicular zone (Table 2).

Table 2

Average DNA Content of Nuclei in the Adrenal Cortex of the Experimental and Control Rats

[illegible] (1)	[illegible] (2)	[illegible] (3)	[illegible] (4)
MnO	2,4 ±0.036	2,18±0,037	2,3 ±0,036
MnO_2	2.57±0.023	2.2 ±0,037	2.34±0,032
[illegible] (5)	2.69±0.022	2.22±0.037	4,55±0,036

1 - Oxides injected
2 - Glomerular zone
3 - Fascicular zone
4 - Reticular zone
5 - Control

* A relative quantitative method of determining the intensity of staining of the nucleus was used to provide a more objective evaluation of the results. The intensity of staining was judged visually and measured in points, which were then analyzed mathematically.

The RNA content of the adrenal cortex likewise decreased slightly, particularly in the fascicular and reticular zones. RNA in the form of reddish-violet granules was found in both the pytoplasm and the nucleoli. The nucleoli of most cells in the glomerular zone did not have their characteristic reddish color, an indication of the presence of RNA. The nucleoli in the fascicular and reticular zones were reddish. However, the cytoplasm of many cells was lacking in reddish granules and where some were present, the intensity of color was less than in the control.

Thus, poisoning rats with manganese oxides produced both morphological changes and chemical changes in the adrenal cortex. These changes were more pronounced when the animals were poisoned with manganese dioxide, although the amount of manganese in the two oxides was identical.

The shifts observed - increased size and weight of the glands, some hyperplasia of the cortex, and presence of lipids, corticosteroids, ascorbic and nucleic acids - made up a pattern ordinarily characterized as increased adrenocortical function. The same phenomena occur when the adrenals are stimulated by the injection of ACTH. They can be interpreted as a nonspecific reaction of the body to poisoning. Manganese is known to produce lesions in the central nervous system, especially in some divisions of the brain (subcortical region). Hence the changes found in the adrenal cortex can be regarded as manifestations of impairment of neurohumoral regulation, particularly in the light of the thyroid changes occurring in animals poisoned with manganese oxides. These changes are manifested chiefly by functional inhibition.

Although our experiments revealed a morphological and histochemical picture of adrenocortical stimulation, the latter might well give way to inhibition after prolonged exposure to the toxic agent. There are references in the literature to the phase character of functional changes in the adrenal cortex under the influence of poisoning. For example, rats

poisoned with carbon tetrachloride exhibited initially an increase in the amount of corticosteroids excreted with urine and then a decrease [16a].

Thus, our experiments showed that poisoning with manganese oxide causes changes in the adrenals. They point up the need for dynamic observation of these glands in persons who come in contact with manganese oxides or who present symptoms of occupational poisoning.

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ИЗМЕНЕНИЯ В КОРЕ НАДПОЧЕЧНИКОВ БЕЛЫХ КРЫС ПРИ ОТРАВЛЕНИИ ОКИСЛАМИ МАРГАНЦА

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Вопрос о значении состояния эндокринной системы или отдельных ее звеньев для чувствительности организма к различным ядам, а также изменения эндокринных желез при различных интоксикациях, в том числе и промышленных, изучен далеко не достаточно как в клинике, так и в эксперименте.

Из клинических наблюдений, а также из отдельных экспериментов известно о наличии изменений в функции щитовидной железы при воздействии таких промышленных ядов, как окись углерода, свинец, ртуть, бензин, кобальт. Например, описаны явления гипертиреоза как результат острого отравления окисью углерода и увеличение щитовидной железы без нарушения ее функции в результате хронического воздействия окиси углерода (12, 24, 30). Явления гипертиреоза отмечали при хронической ртутной интоксикации (3, 6, 17). Имеются указания на развитие базедовизма при хроническом свинцовом отравлении. Явления гипертиреоза описаны и при отравлении свинцом животных (7, 28). Известно, что такие профессиональные яды, как фтор и кобальт, угнетают гормонообразование в щитовидной железе. Введение кобальта вызывает, кроме того, увеличение щитовидной железы (10, 16, 22 и др.).

Менее определенны данные, касающиеся изменений со стороны надпочечников в результате воздействия промышленных ядов. Самые общие и довольно противоречивые указания имеются об изменениях как самих желез, так и их функций, вызываемых такими ядами, как окись углерода, свинец, кадмий, тетраэтилсвинец, кремний и др. Так, имеются данные о повышении функции надпочечников в результате острого и хронического отравления окисью углерода (15, 30, 24). Увеличение размера и изменения со стороны функциональной активности коры надпочечников, судя по выделению 17-кетостероидов с мочой, отмечались также при воздействии двуокиси кремния и тетраэтилсвинца (26, 23). Увеличение надпочечников и снижение содержания аскорбиновой кислоты в них наблюдали при отравлении животных четыреххлористым углеродом (1). Одним из ранних проявлений бензольной интоксикации некоторые авторы считают расстройство функций надпочечных желез (7).

В результате исследования распределения ядов выявлено значительное накопление их в ряде эндокринных желез, в том числе и в надпочечниках. Это известно для таких металлов, как ртуть, свинец, кадмий, олово, хром и др., а также для ряда органических соединений (14, 19, 29).

Значение такого распределения еще далеко не выяснено. Отчасти оно может быть объяснено богатым кровоснабжением эндокринных желез (20). Есть указания и о каком-то специфическом влиянии яда на функцию некоторых эндокринных желез. Например, такие данные в отношении щитовидной железы имеются для фтора, кобальта, марганца (16, 8, 10, 25 и др.).

В предыдущих исследованиях (8) были выявлены уменьшение щитовидной железы, угнетение ее функции и снижение газообмена в результате отравления крыс окислами марганца. Эти данные вместе с общеизвестным значением коры надпочечников в общих адаптационных и обменных реакциях организма на различные внешние, в том числе и токсические, воздействия указывают на необходимость выяснения состояния коры надпочечников при отравлении животных соединениями марганца.

Внешние функциональные состояния надпочечников могут иметь значение и для расшифровки действия стероидных гормонов коры надпочечников в связи с эффективным их применением в терапии профессиональных отравлений. Известно, что полнокровие, кровоизлияния, дегенеративно-некротические изменения в надпочечниках, богатство их коркового слоя липоидами являются следствием скармливания или подкожного введения кроликам хлористого марганца или длительного

вдыхания собаками двуокиси марганца (5, 9, 17). У некоторых людей с хроническим отравлением марганцем обнаруживали снижение выделения 17-кетостероидов с мочой (27).

В нашем исследовании при помощи некоторых гистологических и гистохимических методов выявляли морфологические и функциональные изменения в коре надпочечников крыс при длительном отравлении окислами марганца.

В специальной серии опытов два окисла марганца (MnO_2 и MnO) вводили под кожу белым крысам в дозе 100 мг на 1 кг веса. Подобранные по исходному весу и одного пола, животные были разбиты на 3 группы по 15 крыс в каждой. Одной из групп вводили двуокись, второй — закись марганца, третья служила контролем. Инъекции взвеси окислов марганца в физиологическом растворе, а также только физиологический раствор контрольным животным повторяли через день. Всего крысы получили по 18 инъекций. Опыты проводились в течение марта — апреля.

Прижизненные проявления токсического действия окислов марганца выражались в падении веса крыс, адинамии и развитии у части животных центральных парезов задних конечностей. Одна из 15 крыс пала после 13 инъекций двуокиси марганца. Оставшиеся в живых как подопытные, так и контрольные в течение 7—8 дней после прекращения введения окислов были забиты. Уже при вскрытии животных обратил на себя внимание увеличенный размер надпочечников подопытных крыс по сравнению с контролем. Взвешивание этой железы и вычисление весового коэффициента подтвердили ее заметное увеличение по сравнению с контролем, как это показано в табл. 1.

В другой серии опытов с вдыханием аэрозоля двуокиси марганца увеличение размера надпочечников было обнаружено как у крыс, так и у кроликов. Средний весовой коэффициент у подопытных кроликов составлял 0,156, у контрольных — 0,094. В тех же опытах с вдыханием аэрозоля двуокиси марганца было установлено заметное накапливание марганца в надпочечниках кроликов. После $3^1/_2$ месяцев вдыхания пыли MnO_2 концентрация марганца в надпочечниках составляла 1,4 мг% на сырой вес и была выше, чем в печени, почках, головном мозгу и других внутренних органах тех же животных, но ниже, чем в щитовидной железе.

Наши сведения о накапливании марганца в надпочечниках довольно хорошо согласуются с некоторыми литературными данными о повышенном содержании марганца в надпочечниках в норме (4,21), а также при длительном вдыхании окислов марганца собаками и кроликами или при введении им под кожу изотопа этого металла (9, 20, 18).

О функциональном состоянии коры надпочечников при интоксикации марганцем в литературе имеются противоречивые указания. Так, Boyer (19) находил повышение выделения нейтральных 17-кетостероидов с мочой (т. е. усиление деятельности коры надпочечников), другие, наоборот, наблюдали уменьшение их выделения (27). Можно отметить, что определение содержания нейтральных 17-кетостероидов в моче недостаточно точно характеризует функциональное состояние надпочечников ввиду значительного колебания содержания 17-кетостероидов, в частности в зависимости от состояния печени, которая поражается при отравлении марганцем.

Для выяснения того, в какой степени выявленная в наших опытах гипертрофия коркового слоя надпочечников связана с изменением их функциональной активности, были применены гистохимические методы исследования: выявление суданофильных липоидов, кетостероидов и аскорбиновой кислоты, а также нуклеиновых кислот — дезоксирибонуклеиновой (ДНК) и рибонуклеиновой (РНК). Оказалось, что количество липоидов было значительно уменьшено. Насыщенная липоидами часть клубочковой зоны, в норме весьма богатая жирами, была сужена,

а промежуточная суданофобная зона расширена в основном за счет сдвига суданофильной зоны к периферии. Пучковая зона, как наружная, так и внутренняя ее части имели значительно меньше липоидных веществ, чем в контроле (рис. 1). Содержание кетостероидов[1] в коре надпочечников также изменилось. В норме они встречаются в основном в клубочковой и пучковой зонах. У крыс, которым вводились окислы марганца, количество синих гранул кетостероидов в цитоплазме клеток клубочковой зоны резко уменьшилось; в пучковой зоне кетостероиды встречались лишь в виде отдельных зерен, а многие клетки были лишены их. Зато синие гранулы заполняли сосудистые синусы, подчеркивая рисунок капилляров. В общем при отравлении крыс как закисью, так и двуокисью марганца кора надпочечников представляла картину довольно резкого обеднения кетостероидами (рис. 2).

Количество аскербиновой кислоты[2] в надпочечниках у подопытных крыс значительно снижалось, что было особенно заметно в пучковой и в меньшей степени в сетчатой зонах (рис. 3). Содержание аскорбиновой кислоты в клубочковой зоне ничтожно и в норме не изменялось даже под влиянием введения различных окислов марганца.

Нуклеиновые кислоты выявляли по методу Фёльгена (ДНК) и окраской пиронином — метиловым зеленым (РНК). Определение содержания ДНК[3] в ядрах клеток надпочечника показало уменьшение ее в клубочковой и сетчатой зонах, тогда как в пучковой оно было примерно таким же, как и в контроле (табл. 2).

Содержание РНК в корковом слое надпочечника также несколько уменьшилось, причем наиболее ярко это было выражено в пучковой и сетчатой зонах. РНК в виде красновато-фиолетовой зернистости встречалась как в цитоплазме, так и в ядрышках. В клубочковой зоне ядрышки большинства клеток не имели характерной для них красноватой окраски, указываю-

Таблица 1

Весовые коэффициенты правого надпочечника подопытных и контрольных крыс

Вводимые окислы	Индивидуальные значения																		Среднее значение	
																				самка
MnO_2	0,43	0,32	0,30	0,28	0,26	0,23	0,22	0,22	0,22	0,193	0,17	0,16	0,14	0,14	0,13	0,13	0,18	0,281
MnO	0,33	0,25	0,22	0,22	0,19	0,19	0,17	0,16	0,12	0,12	0,11	0,10	0,10	0,10	0,08	—	0,117	0,216
Контроль	0,16	0,15	0,14	0,14	0,165	0,103	0,10	0,045	0,083	0,076	0,069	0,45	—	—	—	—	0,083	0,09

[1] Кетостероиды определяли по методу Ашбель и Зеликмана в модификации В. П. Введенского, И. Б. Симон и Э. З. Юсфиной (2).

[2] Аскорбиновая кислота определялась по Бурну.

[3] Для более объективной оценки полученных результатов был введен относительный количественный метод определения интенсивности окраски ядра. Степень окраски оценивалась визуально в условных балловых величинах, которые далее обрабатывались математически.

щей на наличие РНК. В пучковой и сетчатой зонах ядрышки были красного цвета, зато цитоплазма многих клеток была лишена красноватой зернистости, там же, где она находилась, интенсивность ее окраски была меньше, чем в контроле.

Таким образом, в результате отравления крыс окислами марганца выявлены как морфологические изменения, так и изменения со стороны химического состава коры надпочечников. Эти изменения проявлялись более заметно при отравлении животных двуокисью марганца, чем его закисью, хотя количество марганца, вводимого в виде разных окислов, было одинаковое.

Обнаруженные сдвиги — увеличение размера и веса желез, некоторая гиперплазия коркового слоя, наличие липоидов, кортикостероидов, аскорбиновой и нуклеиновой кислот — укладывались в картину, обычно характеризуемую как повышение функциональной активности коры надпочечников. Такие сдвиги известны при стимуляции надпочечников посредством введения адренокортикотропного гормона.

Таблица 2

Среднее содержание ДНК в ядрах коркового слоя надпочечников у подопытных и контрольных крыс

Вводимые окислы	Клубочковая зона	Пучковая зона	Сетчатая зона
MnO . .	2,4 ± 0,036	2,18 ± 0,037	2,3 ± 0,036
MnO_2 . .	2,57 ± 0,023	2,2 ± 0,037	2,34 ± 0,032
Контроль	2,69 ± 0,022	2,22 ± 0,037	4,55 ± 0,036

Обнаруженные изменения можно трактовать как неспецифическую реакцию организма на токсическое воздействие. Известно, что марганец вызывает поражения в центральной нервной системе, в особенности в некоторых

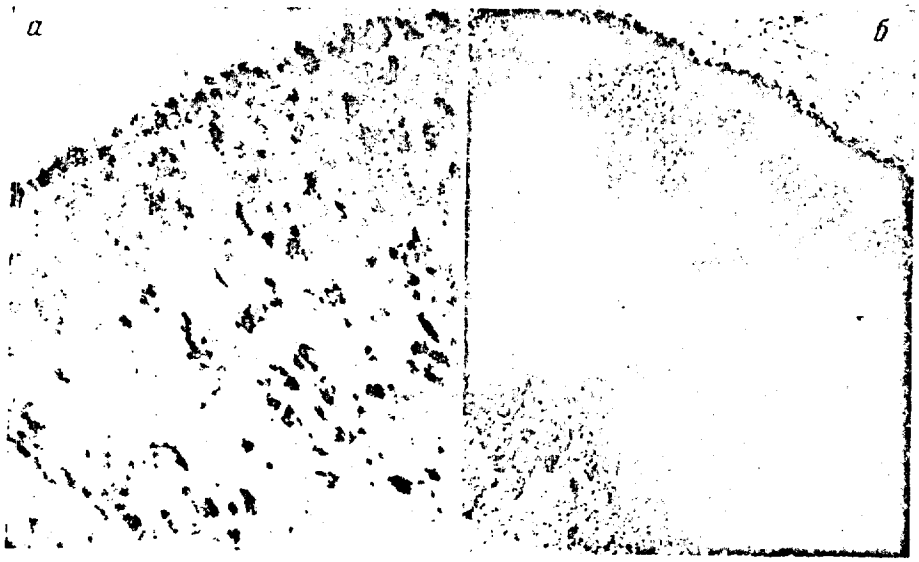

Рис. 1. Суданофильные липоиды в корковом слое надпочечников.
после введения MnO, б — контроль. Увеличение 100×.

отделах головного мозга (подкорковой области). Поэтому сдвиги, обнаруженные в коре надпочечников, могут рассматриваться как проявления нарушения нейро-гуморальной регуляции, тем более что при экспериментальном отравлении окислами марганца выявлены изменения и со стороны щитовидной железы. В последней они выражались преимущественно в ее гипертрофии.

Хотя в наших опытах мы выявили морфологическую и гистохимическую картины стимуляции коры надпочечников, возможно, что при длительном действии вызвавшего ее агента, эта стимуляция может смениться ее угнетением.

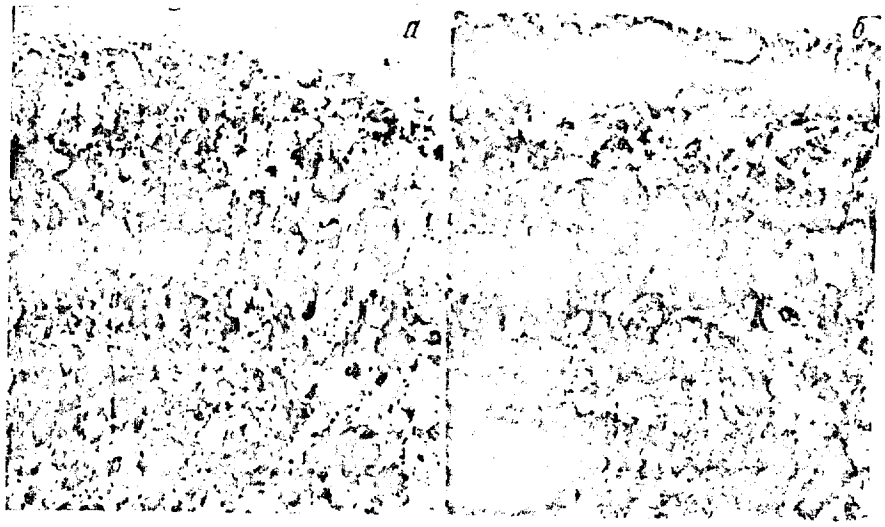

Рис. 2. Кетостероиды в корковом слое надпочечников.
а — после введения MnO; *б* — контроль. Увеличение 157 ×.

Рис. 3. Аскорбиновая кислота в корковом слое надпочечников
а — после введения MnO; *б* — контроль. Увеличение 311 ×.

Указания на такую фазовость изменений функции коры надпочечников под влиянием интоксикации имеются в литературе. Так, при экспериментальном отравлении крыс четыреххлористым углеродом обнаружено увеличение количества выводимых с мочой кортикостероидов в первый период и снижение уровня их в дальнейшем (16а).

Таким образом, проведенными опытами выявлены изменения в надпочечниках, возникшие под влиянием отравления окислами марганца.

Эти данные указывают на необходимость динамического наблюдения за состоянием этой системы при обследовании лиц, контактирующих с окислами марганца или у лиц с признаками профессионального отравления им.

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Поступила 13/V 1957 г.

CHANGES IN THE ADRENAL CORTEX OF WHITE RATS IN INTOXICATION BY THE OXIDES OF MANGANESE

E. N. Levina and *N. A. Minkina*

Summary

Manganese dioxide and oxide were injected subcutaneously to white rats (18 doses of 100 mg. per kg of body weight). The aerosol of manganese dioxide was inhaled by rabbits and rats for 3.5 months. In both experiments the weight of adrenal glands and the content of manganese in them showed a considerable increase. In hishological examination the cortical layer of rats suprarenals was hypertrophied, while the content of sudanophilic lipoids, as well as the amount of ketosteroids and ascorbic acid was considerably reduced. The content of nucleic acids (DNA and RNA) was also diminished. These changes were due to the increased functional activity of the cortex, a non-specific reaction of the body to the toxic influence.

ARCHIVUM IMMUNOLOGIAE ET THERAPIAE EXPERIMENTALIS
1965, 13, 50

EFFECT OF MANGANESE IN EXPERIMENTAL ANEMIA IN RATS

FIRST REPORT

by

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In 65 young rats nutritional anemia was induced and the animals were treated with manganous chloride. An improvement, as judged by the results of peripheral blood analysis and bone marrow examination, was not achieved.

The role of manganese in the body and its influence on the basic vital functions continues to be the subject of interest of many authors [3—5, 7, 8, 12, 32].

WOLF [37] in 1898 and LOEWI [25] in 1907 called attention to the beneficial action of trace elements, including manganese, in so-called secondary anemia. The effect of trace elements on the blood picture has been clarified since then by experiments with animals.

In 1925 HART et al [17], and in 1926 MITCHEL and SCHMIDT [27] induced anemia in rabbits and rats by means of milk diet. Named by the authors nutritional anemia, this condition has become an experimental model employed by all other investigators. Subsequent studies revealed that administration of milk diet leads to impoverishment of the body in iron, copper, manganese and other trace elements [10, 20].

Since that time, differences of opinion have arisen concerning the role of iron and trace elements in the regeneration of blood and induction of remissions in the course of anemia. Some investigators have concluded that restoration of blood constituents to normal values can be achieved by adding only pure inorganic iron to the milk diet [1, 27, 28]. Others claim that pure inorganic iron uncontaminated by trace elements does not improve the anemia [16, 36].

The greatest discrepancies are concerned with the problem whether manganese and other trace elements exert a direct action on anemia, or whether they act as catalysts through iron [14, 29, 34].

KRAUSS [24] found that manganese alone has no effect on hemoglobin levels, and that addition of manganese and copper to milk diet has the same effect as addition of copper alone. KEIL et al [19] also failed to observe any effect of

manganese on hemoglobin levels in milk-induced anemia. PETRANYI [31] showed that in nonanemic animals small doses of manganese increase the numbers of red blood cells and volume of circulating blood, and according to WACHTEL et al [35] also the hemoglobin levels. BRUGSCH [2] and FALUDI [11] reported that manganese salts give beneficial effects in anemic patients, especially in the secondary type of anemia.

Besides studies showing a beneficial action in anemia, others have denied any such effect of manganese. ORTEN et al [30] and KLEINBERG [23] noted that manganese diminishes the toxicity of cobalt, but does not itself exert any effect on hemoglobin or red blood cells.

Discrepant and often contradictory opinions concerning manganese have been voiced also by investigators of industrial intoxications. SCHWARZ and PAGEELS [33], FOUGHT [13] and KESIC and HAUSLER [21] in cases of manganese poisoning observed excessive increase in the number of red blood cells, as well as the symptoms of anemia.

The contradictory opinions encountered in the literature prompted a renewed study of this problem based on a large animal material in which anemia was induced by three different methods, especially since no studies on this subject have been published in recent years.

MATERIAL AND METHODS

1. *Preparation of the experimental animals*

In order to study the action of manganese, anemia was induced in 65 young rats by three different methods.

a) *Method of Elvehjem and Kemmerer*

Pregnant females one week before delivery were placed in special cages with glass plates covering the interior walls of the wooden frames. The floors were made of perforated zinc sheeting and were lined with cellulose. The young rats left with their mothers for about 25 days (period of suckling). Beginning on the tenth day after delivery, the mothers were removed from the cages for each feeding with the aim of preventing the young rats from having any access to normal food. Only cow milk in glass containers in unrestricted amounts was supplied in the common cages. After 25 days the mothers were removed definitely, and the young rats were fed only milk with added vitamins A and D. After three weeks on this diet the animals showed marked drop in hemoglobin and red blood cells.

b) *Modified Elvehjem-Kemmerer method*

The procedure was similar to that in the previous method. The modification consisted in giving the young rats normal food, like that which the mother received (with the exception of green fodder) between the 10th and 19th days after birth, enabling them to store a certain amount of iron and trace elements in their bodies. This modification was introduced on the basis of the work of ELVEHJEM and SHERMAN [10] and KALDOR and POWELL [18], who found that young animals very actively assimilate iron and trace elements and can later make use of their stores in the body. In this modification the period of induction of anemia was prolonged to 6—7 weeks from the moment of weaning of the young rats.

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c) *Bleeding*

Two-month rats, previously maintained on normal diet, were bled from the tail vein. During 14 days about 0·5 ml of blood was withdrawn daily. After each bleeding 0·5 ml of 0·15 M solution of sodium chloride was injected subcutaneously. Access to iron or trace elements during the experiments was prevented by maintaining the rats on milk diet exclusively during the period of experiments.

In the animals with anemia induced by three different methods preliminary determinations of hemoglobin levels, red blood cell and reticulocyte counts were made, and the behavior of the erythroblastic system was observed in the bone marrow. Blood was secured for analysis by incision of the tail vein.

2. *Laboratory methods*

a) Hemoglobin was determined as cyanhematin according to the method of KING and GILCHRIST [7]. The standard solution was prepared from crystalline hemin obtained by the method of DELORY [8] from rat blood. The determinations were made with a Pulfrich photocolorimeter in glass cuvets, employing the No. 6 green filter.

b) Red blood cells were counted in a Bürker hemocytometer. The mean of two countings was always recorded.

c) Reticulocytes were stained intravitally with brilliant cresyl blue; the smear was then stained additionally with the method of Pappenheim. One thousand red blood cells were counted in each smear.

d) Bone marrow was obtained from young rats by the following method: under ether anesthesia the skin and muscles were incised, exposing the femur laterally near the epiphysis. Through a small opening made in the bone a fragment of bone marrow was withdrawn and was smeared on a glass slide. The skin and muscles were then sutured in layers. The animals tolerated the procedure well, and no complications were encountered. The smears were stained according to Pappenheim. The ratio of nucleated red cells (erythroblasts) to nucleated white blood cells was determined by counting 500 nucleated cells.

3. *Determination of normal values of the studied blood and bone marrow components*

Determination of the values in 30 young rats showed that the normal levels of hemoglobin lie in the range 13·8—16·0 g/100 ml blood, mean 14·7 g/100 ml. The red blood cell counts varied in the range 5·7—7·5 million/mm³, mean 6·4 million/mm³. Reticulocyte counts ranged from 1·5 to 5·5%, mean 3·2%. In the bone marrow 25—30 nucleated red blood cells were found per 100 nucleated white blood cells (mean 28/100).

4. *Preparation of solutions of manganese salts*

Tetrahydrated manganous chloride (pro analysi, J. D. Riedel-E. de Haën Co.) was used in the experiments in 0·25% solution in redistilled water.

5. *Method of administration and dosage of manganese*

Manganous chloride was injected subcutaneously in 0·25% solution in doses of 5 mg/kg body weight, equivalent to 1·3 mg of metallic manganese per kg body weight daily. Manganese was given for four weeks, beginning from the appearance of the anemia. Hemoglobin and red blood cell determinations were made at intervals of 7 days. Reticulocyte counts were made after 2, 5, 7, 14, 21 and 28 days, and the bone marrow was examined after four weeks of administration of manganese. The control animals received injections of 0·1 ml of redistilled water throughout the same period of time.

6. *Determination of the lethal dose of manganese*

In order to determine the lethal dose of manganese in chronic poisoning, two-month rats were injected with manganous chloride during four weeks. With this purpose, 36 rats were divided into 6 groups of 6 animals each, which were injected with the following doses of manganous chloride: 10, 20, 30, 40, 50 and 60 mg per kg body weight daily. Doses of 10 and 20 mg failed to produce any symptoms of intoxication, and doses of 30 mg caused diminished gain in body weight. At the dosage level of 40 mg all the rats showed diminished gain in weight, and one out of six rats died. The dose of 50 mg of manganous chloride was lethal to three rats, and 60 mg was lethal to all the animals.

RESULTS

a) *Rats with anemia induced by the method of Elvehjem-Kemmerer.* This group consisted of 23 animals, of which 15 received manganous chloride injections, and 8 served as controls. Preliminary analyses after induction of anemia in the group of 15 rats gave the following mean values: hemoglobin 4·9 g/100 ml blood, red blood cells 2·51 mill./mm³, reticulocytes in peripheral blood 15·1%, and erythroblasts in the bone marrow 80% *.

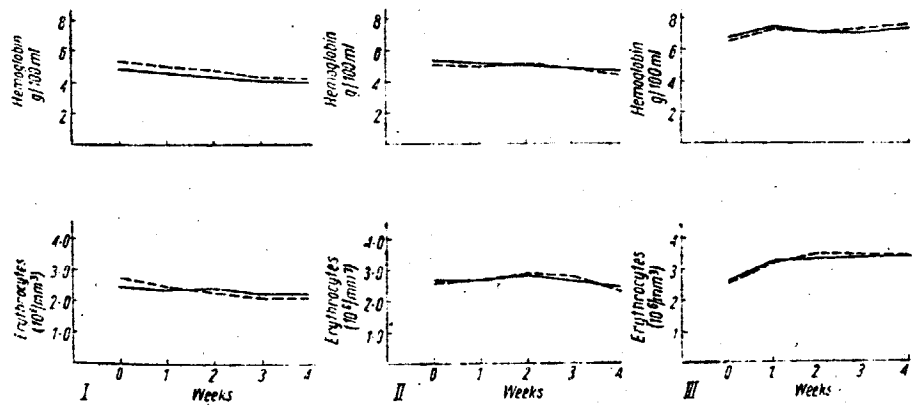

Fig. 1. Hemoglobin and erythrocytes after manganese administration. I = Elvehjem-Kemmerer method; II = modified Elvehjem-Kemmerer method; III = bleeding. ——— + Mn; ---- control.

In the control group of 8 rats the following mean values were obtained: hemoglobin 5·4 g/100 ml blood, red blood cells 2·82 mill./mm³, reticulocytes 14·5%, erythroblasts in the bone marrow 80% (Figs. 1, 2, 3).

* The numbers of erythroblasts in the bone marrow were calculated as percentages of nucleated red blood cells in relation to 100 nucleated cells of all types.

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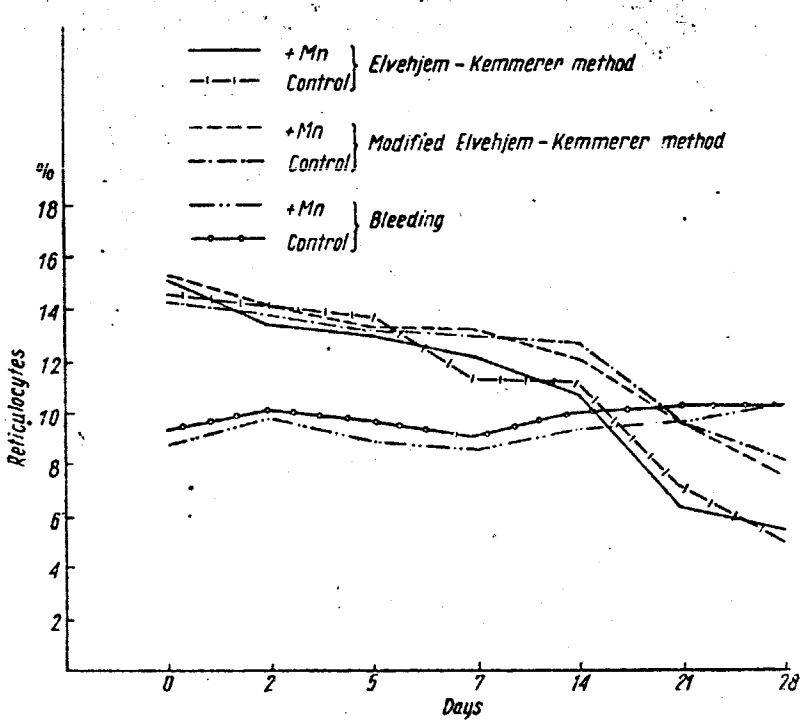

Fig. 2. Reticulocytes after manganese administration.

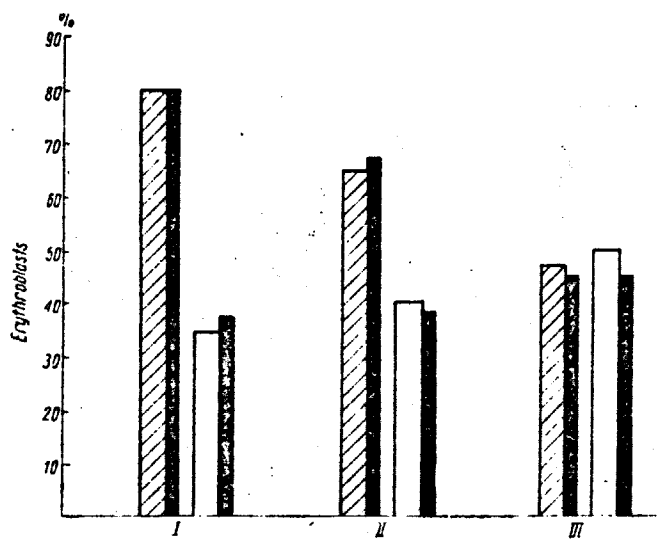

Fig. 3. Erythroblasts in bone marrow before and after manganese administration. I = Elvehjem-Kemmerer method; II = modified Elvehjem-Kemmerer method; III = bleeding. ▨ Before Mn; □ after Mn; ■ control animals.

In the course of four weeks during which manganese was administered the general condition of all the animals deteriorated; the animals lost appetite, were apathetic, showed pallor of the ear lobes and failed to gain weight. Out of the total of 23 rats, 5 died (2 in the group receiving manganese, and 3 in the control group).

Hemoglobin diminished progressively, both in the controls and in the rats receiving manganese. Toward the end of the observation period the mean hemoglobin level in both groups was 4·2 g/100 ml blood (Fig. 1).

The red blood cells behaved similarly. Their numbers gradually decreased, and after four weeks in the group of animals receiving manganese the mean was 2·28 mill./mm³, and in the control group 2·13 mill./mm³ (Fig. 1).

The reticulocytes in both groups showed a steady decline. In the group of 15 animals receiving manganese the mean value was 5·3%, and in the control group 4·8% (Fig. 2).

Erythroblasts in the bone marrow after four weeks showed a similar decline in both groups — mean 35% in the group receiving manganese, and 37% in the control group (Fig. 3).

b) *Rats with anemia induced by the modified Elvehjem-Kemmerer method.* In this group 12 rats were given manganese, and 6 served as controls. Preliminary examinations after induction of anemia in 12 rats gave the following mean values: hemoglobin 5·3 g/100 ml blood, red blood cells 2·7 mill./mm³, reticulocytes 15·2%, erythroblasts in bone marrow 65%.

The mean values in the 6 rats of the control group were as follows: hemoglobin 5·2 g/100 ml blood, red blood cells 2·59 mill./mm³, reticulocytes 14·2%, erythroblasts in bone marrow 67% (Figs. 1—3).

During the four weeks of administration of manganese the general condition of all the animals was better than in the group with anemia induced by the method of Elvehjem-Kemmerer. All the animals survived the experiment.

The laboratory findings after administration of manganese were as follows.

Hemoglobin in both groups of animals dropped slightly. The mean value in rats receiving manganese was 4·7 g/100 ml blood, and in the control group 4·8 g/100 ml blood (Fig. 1).

The red blood cells in the animals receiving manganese gave mean counts of 2·49 mill./mm³, and in the control group 2·39 mill./mm³ (Fig. 1).

Reticulocytes behaved similarly in both groups, showing steady decline. In the group of animals receiving manganese the mean proportion of reticulocytes was 7·4%, and in the control group 8% (Fig. 2).

In the bone marrow the proportion of erythroblasts declined in both groups — to 40% in the group on manganese, and 38% in the controls (Fig. 3).

c) *Rats with anemia induced by bleeding.* Out of 24 rats in this group, 12 were given manganese and 12 constituted the control group. Five days after the last bleeding preliminary examinations gave the following mean values in the first

group of 12 rats: hemoglobin 6·7 g/100 ml blood, red blood cells 2·65 mill./mm³, reticulocytes 8·7%, erythroblasts in bone marrow 47%.

In the second, control group of 12 rats the mean values were: hemoglobin 6·6 g/100 ml blood, red blood cells 2·53 mill./mm³, reticulocytes 9·2%, erythroblasts in the bone marrow 45% (Figs. 1—3).

With this method of inducing anemia the hemoglobin levels after the last bleeding were higher than in both preceding groups, and the proportions of reticulocytes and marrow erythroblasts were lower. The general condition of all the animals during administration of manganese remained good.

Hemoglobin in both groups after 7 days showed a slight but distinct increase; in the group receiving manganese the mean value was 7·5 g/100 ml blood, and in the control group 7·4 g/100 ml. In the following three weeks the hemoglobin level changed little, being 7·3 g/100 ml blood in the first group, and 7·5 g/100 ml in the control group (Fig. 1).

The red blood cells behaved similarly. After one week the group of rats receiving manganese showed an increase to 3·25 mill./mm³, and in the control group 3·22 mill./mm³ was noted. The counts then remained at this level to the end of the observation period (Fig. 1).

The reticulocyte counts in both groups after two days showed slight elevation, and then remained steady near the initial level observed after termination of the bleedings. The group of animals receiving manganese showed mean level 10·1 %, and the controls 10% (Fig. 2).

In the marrow the proportion of erythroblasts in both groups was similar — mean 50% in animals receiving manganese, and 45% in the controls (Fig. 3).

DISCUSSION

The findings after administration of manganous chloride indicate that this element has no influence on the course of anemia, regardless of the method of its induction. The studied blood and bone marrow components behaved similarly in rats receiving manganese and in the controls. Certain differences that were observed were attributable to the condition of the animals caused by the method of inducing anemia.

Anemia induced by the method of Elvehjem-Kemmerer is accompanied by the greatest impoverishment of the body in iron and trace elements, leading to marked visceral changes and enzymatic disorders [15, 26]. The course of anemia was therefore most severe, and the highest mortality was observed in this group.

In the modified Elvehjem-Kemmerer method of inducing anemia the young animals, having access to their mother's food, were able to store a certain amount of iron and trace elements. The time required to induce anemia was therefore

somewhat longer, the course of anemia was less severe. In this experiment all the animals survived.

Anemia induced by bleeding after two months on normal diet produced the least change in the general condition of the animals.

On the basis of these findings it was concluded that:

1. Manganese employed as the only treatment of anemia induced in rats by the method of Elvehjem-Kemmerer, modified Elvehjem-Kemmerer method or by bleeding, has no effect on the peripheral blood picture or on regeneration in the erythroblastic system of the bone marrow.

2. Differences in the behavior of the peripheral blood and bone marrow components are caused by the general condition of the animals, related to the method of inducing anemia.

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Received in January 1964

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Problemy Endokrinologii
1968, No. 14(3): 59-64

CONCENTRATION OF 17-HYDROXYCORTICOSTEROIDS IN PLASMA AND ASCORBIC ACID CONTENT OF THE ADRENAL GLANDS IN GUINEA PIGS AFTER THE ADMINISTRATION OF MANGANOUS CHLORIDE

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The effect of trace elements on the endocrine glands is currently under extensive study. The action of manganese on adrenocortical function has not been fully elucidated as yet.

Some investigators state that the amount of manganese is higher in the adrenal glands of animals than in other organs both under normal conditions and after they are administered the substance [1[5]. Structural changes have been described in the adrenals that are indicative of hyperfunction in rats poisoned with manganese oxides [6, 7]. After injecting guinea pigs with various doses of manganous chloride, Romano et al. [8] found histologic changes in the adrenal cortex that they regarded as a manifestation of the stimulating effect of this trace element on adrenal function. These data do not adequately characterize the effect of manganese on the adrenal cortex because they do not relate directly to its hormonal activity.

The purpose of this work was to study the effect of various doses of manganous chloride on the concentration of 17-hydroxycorticosteroids in peripheral blood plasma and on the ascorbic acid content of adrenal tissue in guinea pigs.

MATERIAL AND METHODS

The experimental animals were 140 guinea pigs weighing 260 to 320 g. There were 4 series of experiments. Manganous chloride was injected intramuscularly. The control received physiologic saline at the same time. In the first 3 series (86 animals), we studied the concentration of 17-hydroxycorticosteroids in plasma after the administration of 3, 10, and 100 μg of manganous chloride per animal. These particular doses were chosen because

morphological changes in the adrenal cortex, according to the literature, follow their use.

Blood was taken by the vacuum method from a front paw [9]. The amount of 17-hydroxycorticosteroids in the plasma was determined by the method of Porter and Silber as modified by N. A. Yudayev and Yu. A. Pankov [10]. The norm for each guinea pig was based on 3 or 4 determinations.

In series 4 (54 animals) we studied the ascorbic acid content of adrenal tissue in guinea pigs given 100 μg of manganous chloride. The experimental and control animals were decapitated at the same time, after which the adrenals were removed and thoroughly ground in the cold. The vitamin C level was determined by the micromethod of Birch et al. [11]. The experimental results were statistically analyzed by the Student-Fisher method.

RESULTS AND DISCUSSION

The animals in series 1 were divided into 2 groups, one receiving 3 μg of manganese chloride for 10 days, the other for 15 days. The concentrations of the 17-hydroxycorticosteroids found in plasma are given in Table 1. Analysis of the data shows that the amount increased in the peripheral blood plasma of both groups ($P < 0.01$). However, within a week of cessation of administration of manganous chloride, the concentration decreased slightly in the first group but remained high in the second.

The animals in series 2 were divided into 3 groups, each receiving daily injections of 10 μg of manganous chloride for 10, 15, and 20 days, respectively. It is evident from Table 2 that this dose of the trace element had no effect when given 5 or 10 days. After 15 injections the concentration of the steroids in plasma tended to increase, the increase becoming significant a week after the administration of the trace element was halted ($P < 0.05$). Group 3, like group 2, exhibited a slight increase in the concentration, but it disappeared after 20 injections.

Table 1

Change in Concentration of 17-Hydroxycorticosteroids ($\bar{x} \pm S\bar{x}$) in Plasma After the Administration of 3 μg of Manganous Chloride

1 Животные 1 серии опытов	2 Число животных	3 Длительность введения хлористого марганца (в днях)	4 Количество 17-оксикортикостероидов (в мкг в 100 мл плазмы)						
			5 исходный показатель	6 после 10 инъекций	P	7 после 15 инъекций	P	8 через 7 дней после конца введения	P
9 Группа	9	10	59,70 ± 2,2	75,72 ± 3,4	<0,01			65,66 ± 2,35	<0,1 >0,05
10 Контроль	7		50.48 ± 1.86	54,71 ± 3,78	>0,3			46,44 ± 3,91	>0,05 >0,3
11 Группа	8	15	29,14 ± 6.27			53,52 ± 3,31	<0,01	74,34 ± 4,76	<0,001
10 Контроль	9		39,03 ± 2,72			40,73 ± 4.35	>0,7	39,89 ± 2,01	>0,8

1 - Animals in series 1
2 - Number of animals
3 - Duration of administration of manganous chloride (in days)
4 - Amount of 17-hydroxycorticosteroids (in μg/100 ml of plasma)
5 - original level
6 - after 10 injections
7 - after 15 injections
8 - 7 days after end of administration
9 - Group 1
10 - Group 2

All the animals in series 3 received 100 μg of manganous chloride daily, group 1 for 20 days and groups 2 and 3 for 10 days. The results of a simultaneous investigation showed that in groups 1 and 2 no marked changes occurred in the level of the 17-hydroxycorticosteroids after 10 injections (Table 3). Hence the administration of manganese to group 1 was continued up to 20 days. After 20 injections the steroid content of the guinea pig plasma decreased ($P < 0.05$), but 4 days after the injections were halted it returned to the normal level. Noting that a daily dose of 100 μg for 10 days had no effect but reduced the concentration of the 17-hydroxycorticosteroids after 20 days, we conjectured that the steroid level might rise before then and we therefore limited the third group of animals to 5 injections of the above dose. It is evident from Table 3 that the steroid concentration increased ($P < 0.02$)

in the experimental animals but remained unchanged in the control during the same period of time.

Table 2

Change in Concentration of 17-Hydroxycorticosteroids ($\bar{x} \pm S\bar{x}$) in Plasma After the Administration of 10 μg of Manganous Chloride

(1)	(2)	(3)	(4) Количество 17-оксикортикостероидов (в µg в 100 мл плазмы)										
			(5) исходный показатель	(6) после 5 инъекций	P	(7) после 10 инъекций	P	(8) после 15 инъекций	P	(9) после 20 инъекций	P	(10) через 7 дней после конца введения	
1-я серия	8	10	48,90±3,91			58,10±7,23	>0,2		<0,1			51,76±4,59	>0
2-я серия	8	15	38,51±2,36			40,28±4,50	>0,7	50,14±5,90	>0,05			51,24±4,68	<0
3-я серия	10	20	51,28±2,80	53,36±5,30	>0,7			65,33±6,07	0,05	50,79±6,09	>0,9		
Контроль	9		39,03±2,72	42,60±1,60	>0,2	46,91±3,25	>0,05	40,73±4,35	>0,7	41,59±2,38	>0,1	39,89±2,01	>0

1 - Animals in series 2
2 - Number of animals
3 - Duration of administration of manganous chloride (in days)
4 - Amount of 17-hydroxycorticosteroids (in μg/100 ml of plasma)
5 - original level
6 - after 5 injections
7 - after 10 injections
8 - after 15 injections
9 - after 20 injections
10 - 7 days after end of administrat
11 - Group 1
12 - Group 2
13 - Group 3
14 - Control

Since 100 μg of manganous chloride may have different effects on the glucocorticoid function of the adrenal cortex, depending on the duration of administration, we used this dose to study the effect of the trace element on the ascorbic acid content of the adrenals. The guinea pigs in series 4 were divided into 3 groups, the first receiving 100 μg of the salt for 5 days, the second for 10 days, and the third for 20 days.

Table 4 shows that the daily administration of 100 μg of manganous chloride to the guinea pigs in group 1 for 5 days lowered the ascorbic acid content of the adrenal below control values ($P < 0.02$). After 10 daily injections of 100 μg of the preparation, the amount of ascorbic acid in the adrenals of the animals in group 2 was virtually indistinguishable from the control, with just a slight tendency for it to decrease ($0.05 < P < 0.01$).

The use of this dose for 20 days produced the opposite effect, i.e., an elevation of the ascorbic acid level of the adrenals ($P < 0.05$). The relative weight of the adrenals in the experimental and control animals remained within normal limits of variations [12].

Table 3

Change in Concentration of 17-Hydroxycorticosteroids ($\bar{x} \pm S\bar{x}$) in Plasma After the Administration of 100 μg of Manganous Chloride

1 Животные III серии опытов	2 Число животных	3 Длительность введения хлористого марганца (в дни)	4 Количество 17-оксикортикостероидов (в мкг в 100 мл плазмы)								
			5 исходный показатель	6 после 5 инъекций	P	7 после 10 инъекций	P	8 после 20 инъекций	P	9 через 4 дня после конца введения	
10 1-я группа	10	20	42,63±3,25			45,19±5,27	>0,6	28,28±4,75	<0,05	41,85±3,37	>
11 2-я »	10	10	52,61±3,39			53,22±3,34	>0,9				
12 3-я »	7	10	55,52±8,67	94,41±9,82	<0,02	56,02±6,85	>0,9				
13 Контроль	9		39,03±2,72	42,60±1,60	>0,2	46,91±3,25	>0,05	41,59±2,38	>0,4		

1 - Animals in series 3
2 - Number of animals
3 - Duration of administration of manganous chloride (in days)
4 - Amount of 17-hydroxycorticosteroids (in μg/100 ml of plasma)
5 - original level
6 - after 5 injections
7 - after 10 injections
8 - after 20 injections
9 - 4 days after end of administration
10 - group 1
11 - group 2
12 - group 3
13 - control

A comparison of the effect of the trace element on the ascorbic acid content of adrenal tissue with that on the concentration of 17-hydroxocorticosteroids in plasma revealed a definite correlation between them. For example, after 5 injections of 100 μg of manganouse chloride, the amount of ascorbic acid in the adrenals fell below the control while the steroid level of the plasma rose. After 10 days' administration of this dose, the amount of ascorbic acid in the adrenals of the experimental animals was the same as the control. The same phenomenon was observed with respect to the concentration of 17-hydroxycorticosteroids in the plasma. Longer administration (20 days) of 100 μg of manganous chloride increased the amount of vitamin C in the adrenals while the steroid level of the plasma dropped. Consequently,

under the given experimental conditions the changes in content of ascorbic acid in adrenal tissue and in concentration of 17-hydroxycorticosteroids in plasma were in opposite directions. This relationship between vitamin C and the steroids is confirmed by numerous published data according to which the ascorbic acid level of the adrenals is linked to an intensification of their function.

Table 4

Effect of Administration of 100 μg of Manganous Chloride on the Ascorbic Acid Content of Guinea Pig Adrenals

1 Животные IV серии опытов	2 Число животных	3 Длительность введения хлористого марганца (в днях)	4 Содержание аскорбиновой кислоты (в мкг на вес органа)	P
5 1-я группа	9	5	21.02 ± 1,01	<0.02
6 Контроль	9		31.30 ± 3.84	
7 2-я группа	9	10	29.17 ± 2,06	<0,1
6 Контроль	9		36,68 ± 2,93	>0.05
8 3-я группа	11	20	34,69 ± 2,07	
6 Контроль	7		28,32 ± 1,88	<0.05

1 - Animals in series 4
2 - Number of animals
3 - Duration of administration of manganous chloride (in days)
4 - Ascorbic acid content (in μg per weight of organ)
5 - Group 1
6 - Control
7 - Group 2
8 - Group 3

Thus, the trace element at the doses that it affects the content of the 17-hydroxycorticosteroids in plasma also alters the amount of ascorbic acid in the adrenals. This finding shows that the changes in vitamin C level of the adrenals induced by manganous chloride reflect to some extent the changes in secretion of the corticosteroids.

Our data show that the administration of manganous chloride may alter the glucocorticoid function of the adrenal cortex in guinea pigs. It is still an open question whether the effect is specific or dependent on the overall effect of manganese on the course of several metabolic processes in the tissues, thereby affecting the activity of the endocrine glands.

Hence the effect of manganese on adrenocortical function requires further study in order to elucidate the mechanism of the biological action of the trace element and to work out ways in which it can be used for prophylactic and therapeutic purposes.

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УДК 615.739.14-092:612.124.018:612.453+612.45.015.642

КОНЦЕНТРАЦИЯ 17-ОКСИКОРТИКОСТЕРОИДОВ В ПЛАЗМЕ КРОВИ И СОДЕРЖАНИЕ АСКОРБИНОВОЙ КИСЛОТЫ В НАДПОЧЕЧНИКАХ МОРСКИХ СВИНОК ПОСЛЕ ВВЕДЕНИЯ ХЛОРИСТОГО МАРГАНЦА

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Поступила 5 X 1965 г.

В настоящее время широко изучается вопрос о влиянии микроэлементов на железы внутренней секреции. Действие марганца на функциональное состояние коры надпочечников выяснено еще недостаточно.

Некоторые авторы указывают на повышенное (по сравнению с другими органами) содержание марганца в надпочечниках у животных в норме или после его введения [1—5]. Описаны структурные изменения в надпочечниках, свидетельствующие об их гиперфункции и наступающие при отравлении крыс окислами марганца [6, 7]. При введении морским свинкам различных доз хлористого марганца Romano с соавторами [8] обнаружили гистологические изменения в зонах коркового вещества надпочечных желез, которые авторы рассматривали как проявление стимулирующего влияния этого микроэлемента на функциональную деятельность надпочечников. Приведенные данные недостаточно характеризуют действие марганца на кору надпочечников, так как не освещают непосредственно вопрос об их гормональной активности.

Целью настоящей работы являлось изучение влияния различных доз хлористого марганца на концентрацию 17-оксикортикостероидов в плазме периферической крови и содержание аскорбиновой кислоты в ткани надпочечников морских свинок.

Материалы и методы

Исследования проведены на 140 морских свинках-самцах весом 260—320 г. Всего было поставлено 4 серии опытов. Хлористый марганец вводили внутримышечно. Контрольные животные получали в те же сроки физиологический раствор.

В первых 3 сериях (86 животных) изучали концентрацию 17-оксикортикостероидов в плазме крови после введения хлористого марганца в дозах 3, 10 и 100 мкг на каждую свинку. Выбор этих доз объясняется тем, что морфологические сдвиги в коре надпочечников, по данным литературы, наблюдаются именно при их применении.

Кровь брали вакуумным способом из кисти передней лапки [9]. Количество 17-оксикортикостероидов в плазме определяли методом Портера и Сильбера в модификации Н. А. Юдаева и Ю. А. Панкова [10]. Норму для каждой морской свинки выводили на основании 3—4 определений.

В IV серии опытов (54 животных) изучали содержание аскорбиновой кислоты в ткани надпочечников морских свинок, получавших 100 мкг хлористого марганца. Подопытных и контрольных животных одновременно обезглавливали, извлекали надпочечники, тщательно измельчали на холоде и определяли в них уровень витамина С микрометодом Вінф с соавторами [11]. Результаты опытов обрабатывали статистическим методом Стьюдента — Фишера.

Результаты и их обсуждение

Животные I серии опытов были разделены на 2 группы. 1-я группа получала по 3 мкг хлористого марганца в течение 10 дней, 2-я — в течение 15 дней. Результаты определений концентрации 17-оксикортикостероидов в плазме крови представлены в табл. 1. Анализ полученных данных показывает, что в плазме периферической крови животных обеих групп наблюдалось увеличение содержания 17-оксикортикостероидов ($P<0{,}01$). Следует отметить, что через неделю после прекращения введения хлористого марганца концентрация 17-оксикортикостероидов в 1-й группе животных несколько снизилась, а во 2-й сохранилась на высоком уровне.

Таблица 1

Изменение концентрации 17-оксикортикостероидов ($\bar{x} \pm S\bar{x}$) в плазме крови под влиянием 3 *мкг* хлористого марганца

Животные I серии опытов	Число животных	Длительность введения хлористого марганца (в днях)	Количество 17-оксикортикостероидов (в *мкг* в 100 мл плазмы)						
			исходный показатель	после 10 инъекций	P	после 15 инъекций	P	через 7 дней после конца введения	P
1-я группа	9	10	59,70± 2,2	75,72±3,1	<0,01			65,66± 2,35	<0,1 >0,05
Контроль	7		50,48± 1,86	54,51± 3,78	>0,3			46,44± 3,91	>0,05 >0,3
2-я группа	8	15	39,14±6,27			53,52± 3,31	<0,01	74,34± 4,76	<0,001
Контроль	9		39,03±2,72			40,73±4,35	>0,7	39,89± 2,01	>0,8

Животные II серии опытов были разделены на 3 группы; каждая из них получала ежедневные инъекции по 10 мкг хлористого марганца в течение 10, 15 и 20 дней соответственно. Как видно из табл. 2, введение этой дозы микроэлемента в течение 5 и 10 дней не дало эффекта. После 15 инъекций обнаружилась тенденция к увеличению содержания 17-оксикортикостероидов в плазме крови, а через неделю после прекращения введения это увеличение стало значимым ($P<0,05$). В 3-й группе, как и во 2-й, наблюдалось незначительное повышение концентрации стероидов, исчезавшее после 20 инъекций.

Все животные III серии опытов ежедневно получали по 100 мкг хлористого марганца: 1-я группа — в течение 20 дней, 2-я и 3-я — в течение 10 дней. Результаты одновременного исследования показали, что в 1-й и 2-й группе после 10 инъекций не отмечалось сколько-нибудь заметных изменений уровня 17-оксикортикостероидов по сравнению с исходным (табл. 3). В связи с этим введение марганца 1-й группе было продолжено до 20 дней. После 20 инъекций содержание стероидов в плазме крови морских свинок уменьшилось ($P<0,05$), однако через 4 дня после прекращения введения оно возвратилось к норме. Убедившись, что ежедневная доза хлористого марганца 100 мкг, вводимая в течение 10 дней, не оказывает эффекта, а за 20 дней снижает концентрацию 17-оксикортикостероидов, мы предположили, что увеличение уровня стероидов может происходить в более ранние сроки, поэтому в 3-й группе ограничились 5 инъекциями указанной дозы. Как видно из табл. 3, концентрация 17-оксикортикостероидов в этом случае повысилась ($P<0,02$). Содержание стероидов у животных контрольных групп за указанные периоды исследования не изменялось.

Исходя из того, что 100 мкг хлористого марганца в зависимости от длительности введения могут оказывать различное действие на глюкокортикоидную функцию коры надпочечников, мы применили эту дозу

Таблица 2

Изменение концентрации 17-оксикортикостероидов ($\bar{x} \pm Sx$) в плазме крови под влиянием 10 мкг хлористого марганца

Животные	Количество 17-оксикортикостероидов (в мкг в 100 мл плазмы)												
	Количество инъекций	Число животных	Исходный уровень	после 5 инъекций	P	после 10 инъекций	P	после 15 инъекций	P	после 20 инъекций	P	через 7 дней после конца введения	P
1-я группа	10	15	48,90 ± 3,91			58,10 ± 7,23	>0,2	50,14 ± 5,90	<0,1			51,76 ± 4,59	>0,6
2-я	15	15	48,51 ± 2,36			40,28 ± 4,50	>0,7	65,33 ± 6,07	>0,05	50,79 ± 6,09	>0,9	51,24 ± 1,68	<0,05
3-я	20	20	51,28 ± 2,89	53,36 ± 5,30	>0,7			0,05					
Контроль	10	9	39,03 ± 2,72	42,60 ± 1,60	>0,2	46,91 ± 3,25	>0,05	40,73 ± 1,35	>0,7	43,59 ± 2,38	>0,1	39,89 ± 1,01	>0,8

Таблица 3

Изменение концентрации 17-оксикортикостероидов ($\bar{x} \pm S\bar{x}$) в плазме крови под влиянием 100 мкг хлористого марганца

Животные III серии опытов	Число животных	Длительность введения хлористого марганца (в днях)	Количество 17-оксикортикостероидов (в мкг в 100 мл плазмы)								
			исходный показатель	после 5 инъекций	P	после 10 инъекций	P	после 20 инъекций	P	через 4 дня после окончания введения	P
1-я группа	10	20	42,63±3,25	31,41±9,82	<0,02	45,19±5,27	>0,6	28,28±4,75	<0,05	41,85±3,37	>0,8
2-я »	10	10	52,61±3,39			53,52±3,34	>0,9				
3-я »	7	10	55,52±8,67			56,62±6,85	>0,9	41,53±2,38	>0,4		
Контроль	9		39,03±2,72	42,50±1,60	>0,2	46,91±3,25	>0,05				

для исследования влияния хлористого марганца на содержание аскорбиновой кислоты в надпочечниках. Морские свинки IV серии опытов были разделены на 3 группы: 1-я получала по 100 мкг соли в течение 5 дней, 2-я — 10 дней, 3-я — 20 дней.

Из табл. 4 видно, что ежедневное введение морским свинкам 1-й группы 100 мкг хлористого марганца в течение 5 дней снижало содержание аскорбиновой кислоты в надпочечниках по сравнению с контролем ($P<0{,}02$). После 10-дневных инъекций 100 мкг препарата 2-й группе животных количество витамина С в надпочечниках почти не отличалось от контроля, хотя можно отметить тенденцию к его уменьшению ($0{,}05<P<0{,}01$). Применение этой дозы в течение 20 дней приводит к противоположному эффекту — увеличению уровня аскорбиновой кислоты в надпочечниках ($P<0{,}05$). Относительный вес надпочечников подопытных и контрольных морских свинок не превышал допустимых для нормы пределов колебаний [12].

При сравнении влияния микроэлемента марганца на содержание аскорбиновой кислоты в ткани надпочечников и на концентрацию 17-оксикортикостероидов в плазме крови обнаружено определенное соответствие между этими показателями. Так, после 5 инъекций 100 мкг хлористого марганца количество аскорбиновой кислоты в надпочечниках уменьшалось по сравнению с контролем, уровень же стероидов в плазме крови повышался. После 10-дневного введения этой дозы содержание аскорбиновой кислоты в надпочечниках подопытных животных не отличалось от контроля; то же самое мы наблюдали при определении концентрации 17-оксикортикостероидов в плазме. Более длительное воздействие (20 дней) 100 мкг хлористого марганца увеличивало количество витамина С в надпочечниках; уровень стероидов в плазме крови при такой постановке опыта снижался. Следовательно, в данных условиях эксперимента направленность сдвигов содержания аскорбиновой кислоты в ткани надпочечных желез и концентрации 17-оксикортикостероидов в плазме крови была противоположной. Установленное нами соотношение между

витамином С и стероидами подтверждается многими данными литературы, согласно которым падение уровня аскорбиновой кислоты в надпочечниках связывается с усилением их функции.

Таким образом, в тех дозах, в которых микроэлемент влияет на содержание 17-оксикортикостероидов в плазме крови, он способствует и изменению количества аскорбиновой кислоты в ткани надпочечников. Эти наблюдения показывают, что под влиянием примененного воздействия изменения уровня витамина С в надпочечниках отражают в известной мере сдвиги секреции кортикостероидов.

Таблица 4

Влияние введения 100 мкг хлористого марганца на содержание аскорбиновой кислоты в надпочечниках морских свинок

Животные IV серии опытов	Число животных	Длительность введения хлористого марганца (в днях)	Содержание аскорбиновой кислоты (в мкг на все орган)	P
1-я группа	9	5	21,02±1,01	<0,02
Контроль	9		41,30±3,84	
2-я группа	9	10	29,17±2,06	<0,1
Контроль	9		36,68±2,93	
3-я группа	11	20	31,08±2,07	>0,05
Контроль	7		25,32±1,88	<0,05

Полученные данные свидетельствуют о том, что введение хлористого марганца может изменять глюкокортикоидную функцию коры надпочечников морских свинок. Вопрос о том, специфичен ли выявленный эффект или он зависит от общего влияния марганца на течение ряда метаболических процессов в тканях, что косвенно сказывается на деятельности желез внутренней секреции, пока остается открытым. Поэтому влияние марганца на функцию коры надпочечников требует дальнейшего изучения, так как при этом может быть выяснен механизм биологического действия микроэлемента, а также намечены пути применения его с профилактической и лечебной целью.

Выводы

1. Хлористый марганец вызывает изменения концентрации 17-оксикортикостероидов в плазме крови, а также уровня аскорбиновой кислоты в надпочечниках морских свинок. Направленность этих изменений зависит от дозы и длительности введения марганца.

2. Инъекции 3 мкг хлористого марганца в течение 10 и 15 дней и 10 мкг в течение 15 дней увеличивают количество 17-оксикортикостероидов в плазме крови.

3. Введение 100 мкг хлористого марганца в течение 5 дней повышает уровень стероидов в плазме крови и уменьшает содержание аскорбиновой кислоты в надпочечниках.

4. Длительное воздействие (20 дней) 100 мкг хлористого марганца снижает концентрацию 17-оксикортикостероидов в плазме крови и увеличивает количество витамина С в надпочечниках.

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CONCENTRATION OF 17-OXYCORTICOSTEROIDS IN THE BLOOD PLASMA AND ASCORBIC ACID CONTENT IN THE ADRENAL GLANDS OF GUINEA PIGS FOLLOWING MANGANESE TETRACHLORIDE ADMINISTRATION

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Summary

Administration of manganese tetrachloride to guinea pigs provoked changes in 17-oxycorticosteroid concentration in the blood plasma, as well as of ascorbic acid level in the adrenal glands. Direction of these changes depends on the dose and duration of manganese administration. Injections of 3 microgram of the salt for 10--15 days and of 10 microgram -- for 15 days increased the content of 17-oxycorticosteroids in the peripheral blood plasma. Administration of 100 microgram of manganese tetrachloride for 5 days along with increase of the first index, reduced ascorbic acid content in the adrenal glands. A more prolonged effect (20 days) of this dose diminished steroid concentration in the blood plasma and increased the vitamin C level in the adrenal glands.

J. Nutr. 67: 309-317, 1958

STUDIES OF A MANGANESE-IRON ANTAGONISM IN THE NUTRITION OF RABBITS AND BABY PIGS [1,2]

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(Received for publication July 25, 1958)

Previous work by Hartman et al. ('55) showed that 2000 p.p.m. of manganese in the diet interfered with hemoglobin regeneration of lambs. The present investigation was undertaken to study several questions raised by this finding. Specifically, the experiments reported were conducted to obtain information about (a) the effect of excessive manganese in the diet on hemoglobin regeneration of rabbits and baby pigs, (b) the possibility of overcoming the manganese effect by additional iron in the diet, and (c) the minimal level of manganese in the diet that would interfere with hemoglobin regeneration.

EXPERIMENTAL

Four experiments were conducted, one with rabbits and three with baby pigs. The first experiment was designed to determine the effect of excessive manganese on hemoglobin regeneration of rabbits; the second experiment, to determine the effect of excessive manganese and of additional iron in the diet on the hemoglobin regeneration of baby pigs, and the third and 4th, to characterize the minimal level of dietary manganese which would retard hemoglobin regeneration.

[1] Published with the approval of the Director of Research, North Carolina Agricultural Experiment Station, as Paper no. 946 of the Journal series.
[2] Supported in part with a grant from the Herman Frasch Foundation.
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Experiment 1

Procedure. Six Dutch rabbits approximately 6 months of age were fed a diet of dried whole milk for a preliminary period of two months. During the latter part of this period, blood was drawn from the median artery and from ear veins to hasten the onset of anemia. When a pair of rabbits became anemic (5 to 6 gm of hemoglobin per 100 ml of blood), they were assigned at random, one to the control diet and the other to the manganese-supplemented diet (table 1), and were maintained on these diets for 6 weeks. The diets were fed ad libitum. Blood samples (0.02 ml) for hemoglobin determination were obtained at weekly intervals from ear veins. Hemoglobin was determined by the method of Shenk et al. ('34).

TABLE 1

Diets fed to rabbits in experiment 1

INGREDIENTS	CONTROL	MANGANESE [1]
	gm	*gm*
Chopped soybean hay	1600	1600
Glucose [2]	203	203
Casein (crude)	80	80
Wesson oil	112	112
Manganese mixture [3]	—	5

[1] Supplemented with 2000 p.p.m. of manganese.
[2] "Cerelose," Corn Products Refining Co., New York, N. Y.
[3] Contained 12.3 gm of $MnSO_4 \cdot H_2O$, mixed into 87.7 gm of glucose.

Results. An analysis of the variance of the weekly hemoglobin values over the 6-week period indicated that the hemoglobin regeneration of the control rabbits was significantly greater ($P \leq 0.01$) than that of the rabbits fed 2000 p.p.m. of manganese in the diet. As shown in figure 1, the greatest depressing effect of manganese on hemoglobin regeneration was observed at about the second week; by the 6th week the hemoglobin values of the manganese rabbits approached those of the controls.

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particularly in relation to the state of the liver which is impaired by manganese

We used histochemical methods to determine the extent to which the hypertrophy of the adrenal cortex noted in our experiments was due to change in their functional activity: detection of sudanophilic lipids, ketosteroids, ascorbic acid, DNA, and RNA. We found the quantity of lipids to be substantially decreased. The lipid-saturated part of the glomerular zone which is normally very rich in lipids was narrow while the intermediate sudanophobic zone was wide, mainly because of the shift of the sudanophilic zone to the periphery. The fascicular zone, both the external and internal portions, had much fewer lipid substances than in the control (Fig. 1). The ketosteroid content of the adrenal cortex had also changed.* These hormones are ordinarily found mostly in the glomerular and fascicular zones. In rats injected with manganese oxides, the number of blue ketosteroid granules decreased sharply in the cytoplasm of the cells in the glomerular zone; in the fascicular zone, the ketosteroids were found only as separate granules and many cells did not have any. On the other hand, blue granules filled the vascular sinuses, emphasizing the markings of the capillaries. In general, the adrenal cortex in rats poisoned either with manganous oxide or with manganese dioxide revealed a picture of marked depletion of ketosteroids (Fig. 2).

The amount of ascorbic acid** in the adrenals of the experimental rats decreased substantially, especially in the fascicular and, to a lesser degree, reticular zones (Fig. 3). The amount in the glomerular zone was insignificant and it normally did not change even after injection of the manganese oxides.

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*The ketosteroids were determined by the method of Ashbel and Seligman as modified by V. P. Vvedenskiy, I. B. Simon, and E. Z. Yusfina [2].

**Ascorbic acid was determined by Burnou's (?) method.

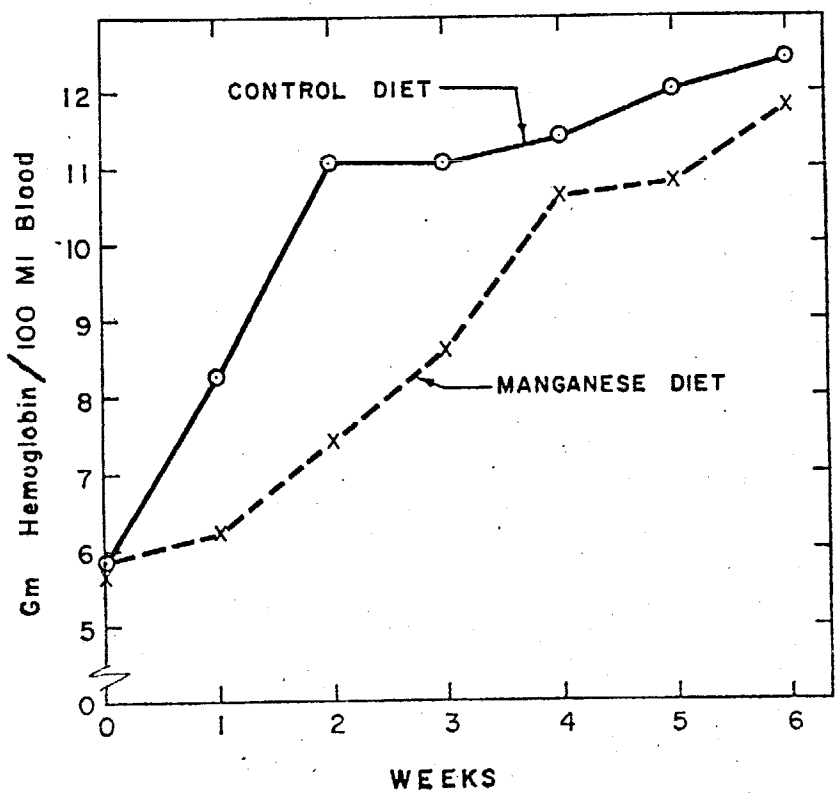

Fig. 1 Experiment 1, change in mean values of hemoglobin of rabbits fed the control and the manganese-supplemented diets during repletion.

Experiment 2

Procedure. Twelve newborn baby pigs deprived of colostrum were administered 10 ml of porcine gamma globulin,[4] 5 ml intraperitoneally and 5 ml subcutaneously, (Barrick et al., '54) and put into individual galvanized wire cages, which were covered with aluminum paint. The basal diet was fresh cow's milk fortified with vitamins A and D. Each pig was restricted to the amount that it would consume 5 times daily. When the pigs became anemic (approximately 5 gm hemoglobin per 100 ml blood), they were stratified into groups of

[4] Porcine gamma globulin was provided by the Armour Laboratories through the courtesy of Dr. M. A. Schooley.

4 according to weight and hemoglobin level and allotted to the following diets in a randomized block design: (1) basal + 25 p.p.m. Fe + 5 p.p.m. Cu; (2) basal + 25 p.p.m. Fe + 5 p.p.m. Cu + 2000 p.p.m. Mn; (3) basal + 400 p.p.m.

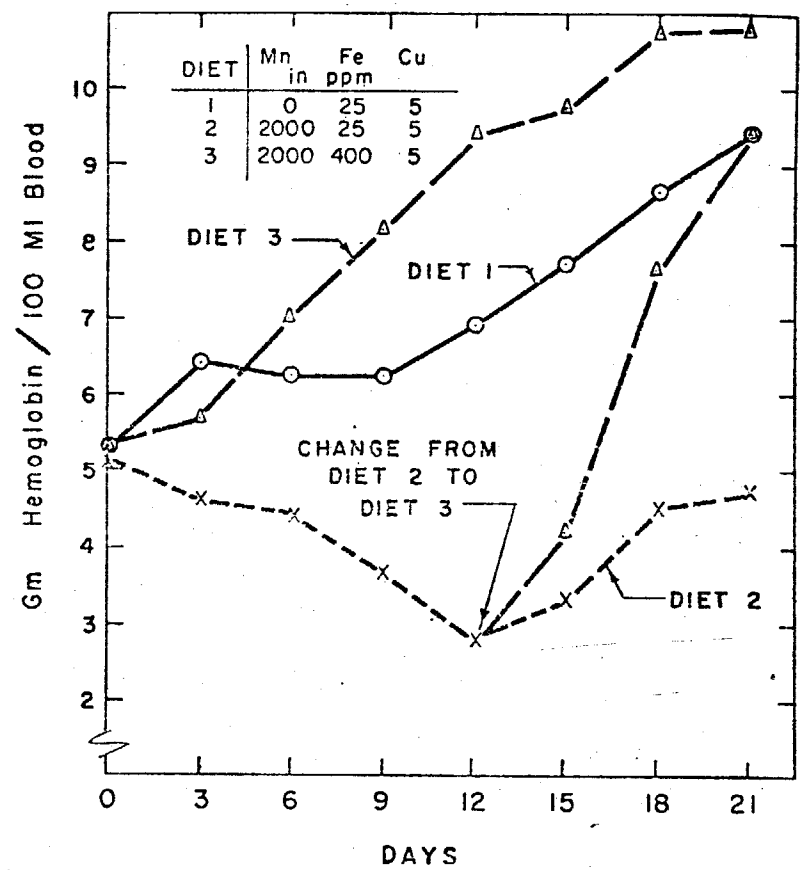

Fig. 2 Experiment 2, change in mean values of hemoglobin of baby pigs fed two levels of iron and two levels of manganese.

Fe + 5 p.p.m. Cu + 2000 p.p.m. Mn. After the pigs were fed these diets 12 days, two of the 4 pigs on diet 2 were changed to diet 3. Hemoglobin measurements were made at three-day intervals throughout the experimental period of 21 days.

Results. As shown in figure 2, hemoglobin regeneration in the baby pig was also depressed by 2000 p.p.m. of manganese

(diet 2). The manganese effect, moreover, was overcome by the addition of 400 p.p.m. of iron (diet 3). The two pigs changed over from diet 2 to diet 3 regenerated hemoglobin at such a rapid rate that by the end of the experiment their hemoglobin values were similar to those fed diets with either 400 p.p.m. of iron or with no supplemental manganese (fig. 2).

Experiment 3

Procedure. The basal diet and general procedure were similar to those of experiment 2. During the experimental period of 27 days, all diets were supplemented with 25 p.p.m. of Fe and 5 p.p.m. of Cu on the dry matter basis. Four levels of manganese supplementation were studied, zero, 125, 250 and 2000 p.p.m. Weights and hemoglobin measurements were taken at 4-day intervals.

TABLE 2

Changes in weight and hemoglobin of baby pigs fed various levels of manganese [1]

DIET NO.	LEVEL OF MN	AV. GAIN PER PIG	AV. HB INCREASE PER 100 ML BLOOD
	p.p.m.	*lbs.*	*gm*
	Experiment 3 [2]		
1	0	28.8	6.09
4	125	26.2	3.38
5	500	26.0	3.18
2	2000	22.7	1.40
L.S.D.[3]		6.04	1.52
	Experiment 4 [4,5]		
1	0	31.3	5.05
6	50	29.3	3.76
7	250	30.9	3.06
8	1250	27.8	3.24
L.S.D.		3.15	1.50

[1] Basal diet was made up of fresh cow's milk plus 25 p.p.m. of Fe and 5 p.p.m. of Cu and vitamins A and D.

[2] Three pigs per diet and an experimental period of 27 days.

[3] Least difference between any two means for significance at 5% level ($P \leq 0.05$).

[4] Six pigs per diet and an experimental period of 25 days for first three replications and 50 days for last three replications.

[5] After 25 days on experiment, iron of pigs on diets of last three replications in experiment 4 was increased from 25 to 50 p.p.m. (see text).

Results. The weight gains and the increase in hemoglobin values made during the experimental period are shown in table 2. The highest average gains were made by the pigs on the zero level of manganese and the lowest by the pigs on the 2000 p.p.m. level of manganese; the difference was significant ($P \leq 0.05$). The increases in hemoglobin levels of the pigs on the 125 and 500 p.p.m. were significantly lower ($P \leq 0.05$) than those on the zero level, and the pigs on the 2000 p.p.m. level made a significantly smaller ($P \leq 0.05$) increment in hemoglobin than did those on either the 125 or 500 p.p.m. levels. The over-all response to manganese appeared to follow a sigmoid curve; e.g., there was a sharp drop in the hemoglobin increment between the first and second levels of manganese, little change between the second and third levels, and a second sharp drop between the third and 4th levels of manganese (table 2).

Experiment 4

Procedure. The basal diet and preliminary period procedure were similar to those in experiments 2 and 3. The levels of manganese studied were zero, 50, 250 and 1250 p.p.m. The pigs were randomized to the diets as in experiment 3. Six replications of the 4 treatments were conducted, three at one time and three at a later time. All 4 diets in the first three replications were supplemented with 25 p.p.m. of Fe and 5 p.p.m. of Cu during an experimental period of 25 days. The last three replications of experiment 4 were carried out in new all-aluminum alloy cages instead of the galvanized wire cages. The 4 diets in the last three replications were supplemented with the usual levels of iron and copper, 25 and 5 p.p.m., respectively, but, since after 25 days the hemoglobin values of none of the pigs on experiment had risen, the iron supplement for all diets was increased from 25 to 50 p.p.m. and the experiment was conducted for an additional 25 days.

Results. The weight gains and the increments in hemoglobin values obtained are shown in table 2. As in experiment

three, the highest gains were made by the pigs on the zero level of manganese and the lowest by those on the highest level, 1250 p.p.m.; the difference was significant ($P \leq 0.05$). The hemoglobin increment made by the pigs receiving the 50 p.p.m. of manganese was not significantly lower than that of those on the zero level, whereas the hemoglobin increments made by the pigs on the 250 and 1250 p.p.m. were significantly lower ($P \leq 0.05$) than of those on the zero level. The shape of the over-all response curve to manganese was exponential rather than sigmoid as in experiment 3.

DISCUSSION

Excess manganese in the diet depressed hemoglobin formation in both mature rabbits and baby pigs. Although manganese retarded hemoglobin formation, regeneration was evident in both species. The hemoglobin regeneration of the rabbits on the high level of manganese was faster than that of the baby pigs, and eventually approached a normal value. This difference in response can be explained, in part at least, by the fact that the anemic baby pigs needed iron not only for hemoglobin repletion but also for hemoglobin increases associated with growth, whereas the mature anemic rabbits needed iron primarily for hemoglobin regeneration. Since the depressing effect of manganese on hemoglobin formation has now been shown to occur with rabbits, baby pigs and lambs (Hartman et al., '55), it appears probable that manganese may have a similar effect on other animal species. The data obtained from baby pigs suggest that the minimal level of manganese which interferes with hemoglobin formation under the experimental conditions prevailing in this investigation lies between 50 and 125 p.p.m. The depressing effect of manganese on growth of pigs was not observed with lambs (Hartman et al., '55). This growth depression was probably the result of the anemia rather than the direct effect of manganese on rate of gain.

The results in experiment 2, showing that additional iron in the diet could overcome the depressing effect of manganese, highlight a similarity between plants and animals in the effect of toxic levels of manganese on the metabolism of iron. The relationship showing that toxic levels of manganese brought about an iron chlorosis in plants has been dealt with by Somers and Shive ('42). They also found that the manganese toxicity of the plant could be overcome by additional iron. According to Somers and Shive ('42), manganese is antagonistic because its oxidizing potential is higher than that of iron. They reasoned that this property of manganese shifted the ionic state of iron to the ferric form which is biologically inactive for plants. This explanation has been challenged by Leeper ('44) and Hewitt ('50). Hartman et al. ('55) suggested that in animals excessive manganese antagonizes the enzyme systems that oxidize or reduce iron at the site of absorption.

The necessity for increasing the supplemental iron from 25 to 50 p.p.m. for the pigs in the last three replications of experiment 4, when the pigs were raised in aluminum cages, suggests that (a) the baby pigs in previous experiments were obtaining some iron from the wire cages, or (b) that 25 p.p.m. of Fe may be an inadequate dietary level for baby pigs. The possibility that the aluminum cages contained a substance which interfered with iron metabolism was deemed unlikely because of the fact that a radioiron (Fe^{59}) study, using two pigs in experiment 4 fed the unsupplemented manganese diet, indicated that 80% or more of the radioiron was absorbed and incorporated into hemoglobin.

SUMMARY

Four experiments, one with mature anemic rabbits and three with anemic baby pigs, were conducted. Excessive manganese in the diet, 2000 p.p.m., depressed hemoglobin formation in both rabbits and baby pigs. The minimal level of manganese in the diet that interfered with hemoglobin forma-

tion was estimated to be between 50 and 125 p.p.m. A supplement of 400 p.p.m. of the iron in the diet overcame the depressing effect of 2000 p.p.m. of manganese on the hemoglobin formation of baby pigs. A supplement of either 1250 or 2000 p.p.m. of manganese in the diet also depressed growth.

ACKNOWLEDGMENT

Acknowledgment is made to Clara R. Bunn and J. T. Judd for technical assistance, and to Frank A. Lane for the care and feeding of the baby pigs.

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ORAL MANGANESE CHLORIDE AS A HYPOGLYCAEMIC AGENT

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Since long, plant extracts have been used as home remedies for diabetes mellitus (Allen, 1927 ; McDonald and Wislicki, 1938, Nash *et al.*, 1950). Some of them have even been investigated for their hypoglycaemic effect (Collip, 1923, Large and Broeklesby 1938). However, there has been little experimental or clinical proof for their efficacy.

Recently, Rubenstein *et al.* (1962) published two case reports of young juvenile diabetics who showed marked hypoglycaemic response following ingestion of infusion of green leaves of an indigenous plant of America, called alfalfa. As the manganese contents of the leaves of alfalfa was found to be high (45·5 mg/kg.), it was considered to be responsible for this hypoglycaemic action. Accordingly 5-10 mg. of manganese chloride was given to those cases and it produced similar hypoglycaemic action as by leaves themselves.

These observations for the first time indicated that manganese chloride might have some hypoglycaemic action in cases of diabetes mellitus. To substantiate such an impression, a clinical trial of oral manganese chloride as a hypoglycaemic agent was carried out in 15 unselected cases of diabetes mellitus.

Material and Method

15 cases of diabetes mellitus of various age-groups and severity admitted to medical wards of Sarojini Naidu Medical College, Agra, constituted the material for study. A detailed history was taken and clinical examination along with routine investigations, were carried out to exclude the possibility of thyrotoxicosis, Cushing's disease and chronic pancreatitis.

The patients were kept on ordinary diet without any oral or parenteral hypoglycaemic drugs for 7 days. Following which, a standard glucose tolerance test was done after 50 g. of oral glucose in each case. The patients were allowed to take their normal diet again after this test. Two days later similar glucose tolerance test was repeated along with varying dose of manganese chloride given orally with 50 g. glucose. This procedure was in accordance with the method followed by Rubenstein *et al.* (*loc. cit.*). The results were analysed to judge the hypoglycaemic action of manganese chloride.

Observations

All these cases were elderly diabetics ranging between 30 to 62 years of age, except one who was a 14 year old juvenile diabetic. Six cases were of thin build, while 9 were of fatty build.

Table 1 shows values of initial glucose tolerance test in these 15 cases.

Table 1—Showing Initial Glucose Tolerance Test Values

Case no.	Fasting blood sugar (mg. %)	Rise after 50 g. glucose			Mean rise (mg. %)
		1st hr.	2nd hr.	3rd hr.	
1	333	500	400	375	125
2	95	143	167	100	42
3	180	286	222	180	50
4	188	280	250	200	55
5	210	334	286	224	71
6	285	500	400	400	148
7	286	550	500	400	164
8	167	250	400	334	161
9	333	500	445	375	107
10	222	400	334	286	118
11	186	250	334	250	92
12	286	500	350	300	97
13	200	334	286	250	90
14	185	286	334	250	105
15	190	284	350	250	105

Table 2 shows values of glucose tolerance test after varying doses of manganese chloride.

TABLE 2—SHOWING GLUCOSE TOLERANCE TEST VALUES AFTER VARYING DOSES OF MANGANESE CHLORIDE

Case no.	Fasting blood sugar (mg. %)	Dose of $MnCl_2$ (mg.)	Rise after 50 g. of glucose			Mean rise (mg. %)	Mean fall due to $MnCl_2$ (mg. %)
			1st hr.	2nd hr.	3rd hr.		
1	333	5	414	400	333	56	69
2	105	10	143	125	95	16	26
3	180	10	222	222	160	23	27
4	150	15	200	224	180	48	7
5	200	15	286	250	200	45	26
6	334	25	415	400	350	64	84
7	334	30	500	444	400	114	50
8	165	30	200	200	158	21	140
9	334	100	450	387	334	56	51
10	286	150	334	334	300	36	82
11	222	150	300	333	222	63	29
12	286	200	450	325	286	68	29
13	223	200	286	250	250	39	51
14	195	250	222	285	222	48	57
15	180	250	250	280	200	63	38

It is evident from Table 2 that there was always a noticeable reduction in blood sugar levels after manganese chloride. Further increasing doses of manganese chloride did not produce any appreciable difference in the fall of blood sugar levels.

Table 3 shows statistical comparison of mean blood sugar rise after 50 g. of glucose and after 50 g. glucose and manganese chloride.

TABLE 3—SHOWING STATISTICAL COMPARISON

Groups compared	Mean value	S.D.	Degree of freedom 'n'	Value of 't'	Probability 'p'	Significance
Blood sugar rise :						
Before $MnCl_2$	102	±37	28	24	<0·001	High
After $MnCl_2$	50	±24				

It is clear from Table 3 that manganese chloride produces a definite fall in the blood sugar levels of diabetic patients and the fall is statistically highly significant, but a single dose is not sufficient enough to control the hypoglycaemia.

In this series there were six cases (No. 1, 6, 7, 8, 12 and 14) of thin constitution and 9 cases of fatty constitution. A careful analysis of Table 2 indicates that fall of blood sugar due to manganese chloride was 71 mg. per cent on an average in these six cases of thin build type while it was only 37·5 mg. per cent on an average in cases of fatty build type.

Severity of diabetes does not appear to have any relation with the hypoglycaemic action of manganese chloride.

DISCUSSION

Manganese is a wellknown component and co-factor of many enzyme systems including some concerned in intermediary metabolism of carbohydrates (Dixon and Weble, 1958). But hypoglycaemic effect of manganese has not been reported so far.

The two cases reported by Rubenstein *et al.* (*loc. cit.*) were young juvenile diabetics and they were abnormal in many ways, e.g., one of them was totally insulin resistant, while the other required very high doses of insulin to control the diabetes. Both responded very well to alfalfa extract as well as to manganese chloride orally, and one of them after partial pancreatectomy, became insulin sensitive and resistant to action of manganese chloride and alfalfa extract.

Preliminary studies by these authors in several normal controls, obese and jevenile diabetics and diabetes due to chronic pancreatitis, did not show any significant fall in blood sugar levels after oral or intravenous administration of manganese chloride, even in very large doses.

However, in the present study it has been clearly shown that manganese chloride does produce fall in blood sugar levels on oral administration in diabetic patients and the fall is statistically significant but a single oral dose is not sufficient enough to control the hyperglycaemia.

The response was better in thin middle aged diabetics as compared to fatty diabetics. Further, increase in dose does not produce a corresponding decrease in blood sugar level. In one of our cases the response was judged by 25 mg. and 100 mg. of manganese chloride respectively, but a higher dose did not produce a significantly higher fall in blood sugar level.

Possibly manganese chloride acts by activation of enzymes concerned with the metabolism of carbohydrates as suggested by Dixon and Weble (*loc. cit.*). We are of the opinion that probably this activation is possible up to a certain limit only, beyond which any increase in dose, does not produce corresponding increase in hypoglycaemic action.

Two other possible mechanisms of action have been suggested by Rubestein *et al.* (*loc. cit.*). These are: (1) It may act peripherally by accelerating cellular uptake or utilisation of glucose either directly or by potentiating action of insulin or by interfering with the action of glucagon in forming glucose from stored glycogen of liver and (2) it may act on the pancreas, accelerating release of stored insulin into the blood stream or by inhibiting release of glucagon. Actually none of these hypotheses appear to be tangible as increase in the dose of manganese chloride does not produce an increase in the hypoglycaemic action.

Summary

Hypoglycaemic action of oral manganese chloride was evaluated on 15 cases of diabetes mellitus. It was found that—

Manganese chloride did produce a fall in blood sugar levels on oral administration and the fall was statistically significant; but a single oral dose was not adequate to control the hyperglycaemia.

Increasing doses of manganese chloride did not produce a corresponding fall in the blood sugar levels.

The response was better in thin middle aged diabetics as compared to fatty diabetics.

The drug in the doses administered did not produce any immediate side effect.

The mechanism of action of manganese chloride has been discussed.

Acknowledgment

The authors express their thanks to Sri T. C. Suchdav, Technician, Department of Biochemistry, S. N. Medical College, Agra, for technical assistance.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 241, No. 15, Issue of August 10, pp. 3488-3498, 1966
Printed in U.S.A.

Pyruvate Carboxylase

VII. A POSSIBLE ROLE FOR TIGHTLY BOUND MANGANESE*

(Received for publication, February 10, 1966)

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SUMMARY

Pyruvate carboxylase contains firmly bound manganese which affects the longitudinal relaxation rate of the protons of water as measured by pulsed nuclear magnetic resonance. The manganese shows an enhanced effect (ϵ_b = 4.2) on the relaxation rate when bound to the enzyme. Studies on the effect of added substrates and inhibitors on the proton relaxation rate have shown that components of the second partial reaction, *e.g.* the carboxylation of pyruvate by the enzyme-biotin ~ CO_2 complex, interact with the bound manganese of the enzyme and cause a reduction in the enhancement. In contrast, components of the first partial reaction, the formation of enzyme-biotin ~ CO_2 from adenosine triphosphate and HCO_3^-, do not significantly affect the enhancement. Examination of the enhancement of the bound manganese as a function of substrate or inhibitor concentration has permitted the determination of dissociation constants and enhancement values for the enzyme-substrate and enzyme-inhibitor complexes. The rate of inactivation of pyruvate carboxylase by avidin is increased by pyruvate and oxalacetate, and decreased by certain dissociable inhibitors, *e.g.* oxalate. Analysis of these data permits an independent determination of the dissociation constants of the enzyme-substrate and enzyme-inhibitor complexes. The dissociation constants derived from the proton relaxation rate measurements are in good agreement with those obtained from their effects on the rate of inactivation by avidin and also with inhibitor constants obtained from initial rate studies of the over-all reaction.

These data strongly support the thesis that the bound manganese plays a functional role in the transcarboxylation portion of the pyruvate carboxylase reaction, *i.e.* the transfer of a carboxyl group from the enzyme-biotin ~ CO_2 intermediate to pyruvate. This locus of action differs from that of the added divalent metal ion which is required for this reaction and which participates in the formation of the enzyme-biotin ~ CO_2 intermediate from HCO_3^- and ATP.

While dissociable inhibitors such as oxalate probably act by direct chelation of the bound manganese since the enhancement of the enzyme-inhibitor complex has a very low value (ϵ_c < 0.3), the corresponding values for the enzyme-substrate complexes are considerably higher (ϵ_c = 1.5 to 2.5), suggesting either that fewer metal-H_2O ligands are displaced by substrates or that conformational changes in the protein contribute to the situation in a different way with the substrates and the inhibitors. Several possible mechanisms for the involvement of the bound manganese in this reaction are discussed, and a mechanism is suggested which is compatible with the kinetic and magnetic resonance data.

Studies reported in the preceding paper (1) showed that pyruvate carboxylase (pyruvate-CO_2 ligase (ADP), EC 6.4.1.1) purified from chicken liver mitochondria contains tightly bound manganese in a stoichiometry approximating 1 mole of manganese per mole of biotin or 4 moles of manganese per mole of enzyme. Earlier studies on the exchange reactions catalyzed by highly purified pyruvate carboxylase and isolation of the enzyme-biotin ~ CO_2 intermediate have permitted the formulation of a minimal mechanism for this enzyme, involving two partial reactions (Reactions 1 and 2) (2, 3).

$$\text{Enzyme-biotin} + \text{ATP} + HCO_3^- \xrightleftharpoons{\text{acetyl-CoA, } Mg^{2+}} \text{enzyme-biotin} \sim CO_2 + \text{ADP} + P_i \quad (1)$$

$$\text{Enzyme-biotin} \sim CO_2 + \text{pyruvate} \rightleftharpoons \text{enzyme-biotin} + \text{oxalacetate} \quad (2)$$

$$\text{Sum: Pyruvate} + \text{ATP} + HCO_3^- \xrightleftharpoons{\text{acetyl-CoA, } Mg^{2+}} \text{oxalacetate} + \text{ADP} + P_i \quad (3)$$

Reactions 1 and 3 show a requirement for activation by a divalent cation which may be satisfied by the addition of either magnesium

* This work was supported in part by Contract AT (11-1) 1242 of the Atomic Energy Commission, by United States Public Health Service Grant GM 08320, and by National Science Foundation Grant G-23384.

‡ This work was done during the tenure of an advanced postdoctoral fellowship from the American Heart Association, at The Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania.

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or manganese (4).[1] However, the divalent cation required for activation may be removed by dialysis or chelating agents, and is therefore not related to the bound metal ion component of pyruvate carboxylase.

The bound manganese of pyruvate carboxylase has an enhanced effect ($\epsilon_b = 4.2$) on the proton relaxation rate of water (1). By qualitative and quantitative studies of the effects of added substrates and inhibitors on the enhancement, it has been possible to show a functional role for manganese in Reaction 2 and to obtain dissociation constants for enzyme-substrate and enzyme-inhibitor complexes which can be compared with Michaelis, inhibitor, and dissociation constants obtained by other methods.

These studies and possible mechanistic interpretations are presented below.

A preliminary account of some of these findings has already appeared (5).

METHODS

Pyruvate carboxylase was prepared as described previously (6) and was assayed spectrophotometrically in the direction of CO_2 fixation (2). Oxalacetate decarboxylation was measured spectrophotometrically by estimating ATP production with hexokinase and glucose 6-phosphate dehydrogenase. Specific activities are expressed as micromoles per min per mg of protein at 25°. Exchanges of pyruvate-^{14}C with oxalacetate and of $^{32}P_i$ with ATP were assayed as described previously (6). Protein was estimated spectrophotometrically (7).

Tritiated pyruvate was prepared and purified as described by Rose (8). The solutions were stored −20° and used for no more than 1 week after preparation. Tritium release was assayed in a system containing, in 0.5 ml, 100 μmoles of Tris-sulfate (pH 7.8), 3.5 μmoles of tritiated pyruvate (8.2×10^4 cpm per μmole), 1.2 μmoles of ATP, 2.5 μmoles of $MgSO_4$, 8 μmoles of $KHCO_3$, and 0.1 μmole of acetyl-CoA. Incubation was performed for 15 min at 23°, after which the reaction was stopped by addition of trichloracetic acid (final concentration, 3%), and denatured protein was removed by centrifugation. The supernatant fraction was neutralized to pH 7 with KOH and then placed on a Dowex 1-Cl^- column (8 × 1 cm) which had previously been washed with 10 ml of 1 N HCl followed by H_2O until the effluent pH was approximately 5 to 6. A 10-ml fraction, collected after adding the supernatant fraction and washings to the column followed by distilled water, was found to contain all the radioactivity released from tritiated pyruvate. One-milliliter aliquots from this fraction were added to 15 ml of dioxane-naphthalene scintillation mixture (9) and radioactivity was estimated in a Packard Tri-Carb scintillation spectrometer.

Analysis of Proton Relaxation Rate Data—The PRR[2] was measured and the observed enhancement (ϵ^*) was defined as described previously (1). Since in this system all the manganese is bound to protein, ϵ^* is equal to ϵ_b, the enhancement of the bound manganese. When substrates or inhibitors are added to pyruvate carboxylase, the observed enhancement (ϵ^*) decreases and approaches a limiting value (ϵ_c), the enhancement of the enzyme-substrate or enzyme-inhibitor complex.

The interaction of substrates (A) or inhibitors (A) with enzyme-bound manganese (E-Mn) may be described by

$$[E\text{-Mn-}A] \overset{K_d}{\rightleftharpoons} [E\text{-Mn}] + [A] \qquad (4)$$

and, hence,

$$K_d = \frac{[E\text{-Mn}][A]}{[E\text{-Mn-}A]} \qquad (5)$$

Also,

$$[E\text{-Mn}]_t = [E\text{-Mn}] + [E\text{-Mn-}A] \qquad (6)$$

The observed enhancement (ϵ^*) has been shown (10) to be a weighted average of the enhancements due to all forms of manganese. In the present case, we may write

$$\epsilon^* = \frac{[E\text{-Mn}]}{[E\text{-Mn}]_t}\cdot\epsilon_b + \frac{[E\text{-Mn-}A]}{[E\text{-Mn}]_t}\cdot\epsilon_c \qquad (7)$$

Solving Equation 7 for [E-Mn-A] gives

$$[E\text{-Mn-}A] = [E\text{-Mn}]_t\left(\frac{\epsilon_c - \epsilon^*}{\epsilon_b - \epsilon_c}\right) \qquad (8)$$

From the definition of the dissociation constant (K_d) of [E-Mn-A] (Equation 5), we may write

$$[E\text{-Mn-}A] = [E\text{-Mn}]_t\left(\frac{(A)}{(A) + K_d}\right) \qquad (9)$$

when (A) is the concentration of free substrate or inhibitor. Setting Equation 8 equal to Equation 9, taking reciprocals, and dividing by $(\epsilon_b - \epsilon_c)$ gives

$$\frac{1}{(\epsilon_b - \epsilon^*)} = \frac{1}{(\epsilon_b - \epsilon_c)} + \left(\frac{1}{(\epsilon_b - \epsilon_c)}\right)\frac{K_d}{(A)} \qquad (10)$$

If $(A)_t$ is greater than $[E\text{-Mn}]_t$, (A) approximates $(A)_t$, and a plot of $1/(\epsilon_b - \epsilon^*)$ against $1/(A)$ should be linear. Examination of the plot permits evaluation of ϵ_c from the ordinate intercept and K_d from the slope. Equation 10 is similar in form to an equation derived previously for the interaction of bovine serum albumin with manganese (10).

Analysis of Effect of Substrates and Inhibitors on Rate of Inactivation by Avidin—Dissociation constants (K_d) for substrates and inhibitors have also been obtained by measurement of the effects of these substances on the rate of inactivation of pyruvate carboxylase by avidin. In this method, which has been described in detail previously (11), pyruvate carboxylase and avidin are incubated in the presence of various concentrations of the substrate or inhibitor and the rate of irreversible inactivation of the enzyme by avidin is determined by removal of small aliquots for assay by the previously described spectrophotometric assay system for CO_2 fixation (2). The following relationship has previously been shown to apply to the rates of inactivation by avidin in the presence of a substrate (A) or inhibitor (A) (11).

$$\frac{v_a}{v_0} = \frac{k_2}{k_1} + \frac{K_d}{(A)}\cdot\left(1 - \frac{v_a}{v_0}\right) \qquad (11)$$

Here v_a and v_0 are the slopes of the pseudo-first order plots obtained for the inactivation of enzyme sites by excess avidin in the presence and absence of A, respectively; K_d is the dissociation constant of the E.A complex; and k_2/k_1 is the ratio of the fractional order rate constants for the inactivation of EA and E by avidin. The units of v_a and v_0 are min^{-1}, and A is expressed as molar. When v_a/v_0 is plotted against $(1 - v_a/v_0)/A$, the slope

[1] M. C. Scrutton and M. F. Utter, unpublished observations.
[2] The abbreviation used is: PRR, proton relaxation rate.

gives K_d and the ordinate intercept gives the k_2/k_1 ratio. Data of this type are shown for pyruvate (Fig. 2) and oxalate (Fig. 5) (see below). Other studies have shown that the reaction orders in enzyme sites and avidin were unaffected by the presence of either pyruvate or oxalate. Unchanged reaction orders in the presence of A are a necessary prerequisite for the derivation and application of Equation 11.

Methylmalonyl - CoA - oxalacetate transcarboxylase (EC 2.1.3.1) (specific activity, approximately 40) was the gift of Dr. H. G. Wood. Redistilled α-ketobutyric acid was the gift of Dr. D. S. Kerr. Sodium fluoropyruvate was obtained from K and K Laboratories; mesoxalic acid (A grade) from Calbiochem; oxamic acid and glyoxal from Matheson, Coleman, and Bell; and malonic acid from Eastman Organic Chemicals. All other reagents were reagent grade materials or were obtained as described previously (3, 6).

RESULTS

Effect of Reaction Components on Enhancement of Bound Manganese—Examination of the effect of reaction components on the PRR of a solution of pyruvate carboxylase (Table I) showed that only the components of Reaction 2 (pyruvate and oxalacetate) caused a marked decrease in the enhancement of the bound manganese from the initial value, $\epsilon_b = 4.2$. Addition of the components of Reaction 1 with the exception of $KHCO_3$ had no significant effect on this parameter. The decrease caused by $KHCO_3$ was significant, but occurred only in the presence of very high concentrations of this substrate. The concentration range (about 0.1 M) in which $KHCO_3$ caused 50% reduction in the enhancement of the bound manganese greatly exceeded the observed K_m (1×10^{-3} M) for this substrate in CO_2 fixation. Therefore, the binding of $KHCO_3$ observed by the reduction in the enhancement of the bound manganese probably represents the formation of an inactive complex between enzyme and HCO_3^- which is not involved in the main reaction pathway. Additionally, no reduction in enhancement of the bound manganese occurred on formation of the enzyme-biotin ~ CO_2 complex from ATP + HCO_3^- in the presence of Mg^{2+} and acetyl-CoA.

TABLE I

Effect of substrates and cofactors on enhancement of bound manganese

The system contained (in 0.1 ml), 5 μmoles of Tris-Cl (pH 7.8), 8 μmoles of Tris-Cl (pH 7.2), 32 μmoles of KCl, and approximately 1 mg of pyruvate carboxylase (specific activity, 10 to 13 (Experiment A) and 24 (Experiment B)). Additions were as shown. The final pH was 7.8. The observed enhancement values (ϵ^*) are calculated as described previously (1).

Reaction component added	ϵ^*
Experiment A	
None	4.2
1×10^{-3} M ATP	4.1
4.5×10^{-4} M acetyl-CoA	4.0
2×10^{-2} M $KHCO_3$	3.9
2×10^{-2} M $MgCl_2$	4.2
0.1 M potassium phosphate (pH 7.8)	4.1
3.7×10^{-3} M potassium pyruvate	3.0
1.7×10^{-2} M	1.8
2.0×10^{-3} M potassium oxalacetate	3.1
4.1×10^{-3} M	2.7
Experiment B	
None	4.3
1.2×10^{-3} M ATP, 1.8×10^{-2} M $KHCO_3$, 1.5×10^{-4} M acetyl-CoA	4.0
ATP, $KHCO_3$, acetyl-CoA + 1.7×10^{-3} M $MgCl_2$	4.1
ATP, $KHCO_3$, acetyl-CoA, $MgCl_2$ + 2.1×10^{-2} M potassium pyruvate	1.8

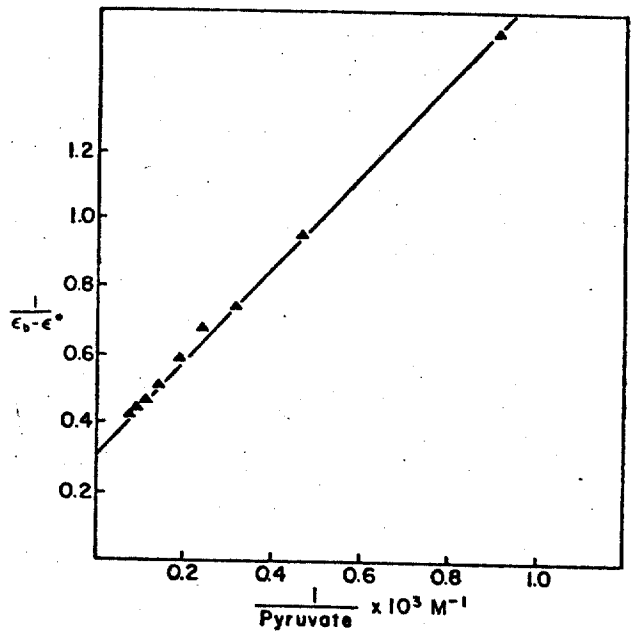


FIG. 1. Dissociation constant for pyruvate by PRR analysis. The titrating agent contained, in 0.1 ml, 5 μmoles of Tris-Cl (pH 7.8), 8 μmoles of Tris-Cl (pH 7.2), 32 μmoles of KCl, 0.725 mg of pyruvate carboxylase (specific activity, 10.6), and 5.65 μmoles of potassium pyruvate. Aliquots were titrated into 0.1 ml of a solution containing identical concentrations of enzyme, buffer, and KCl, and the PRR was measured after each addition. The final pH was 7.8. The ϵ^* values were calculated as described under "Methods."

Dissociation Constants for Enzyme-Substrate Complexes Obtained by PRR Titration and by Analysis of Rates of Inactivation by Avidin—By the use of the property of reduction in enhancement, the dissociation constant (K_d) of the enzyme-substrate complexes may be measured by titration of the bound manganese with pyruvate and oxalacetate as described by Cohn (12) and Mildvan and Cohn (13). The titration with pyruvate plotted according to Equation 10 is shown in Fig. 1. By analyses of the slope and ordinate intercept of this plot, K_d was obtained as 3.3×10^{-3} M and ϵ_c as 1.65.

Pyruvate and oxalacetate accelerated the rate of inactivation of pyruvate carboxylase by avidin, and analysis of the data according to Equation 11 permitted determination of K_d by this method. Experimental data showing the potentiation of the rate of avidin inactivation by pyruvate plotted according to Equation 11 are shown in Fig. 2. Analyses of the slope and ordinate intercept gave K_d as 4.8×10^{-3} M and the k_2/k_1 ratio as 1.32.

The K_d values obtained by these two methods for pyruvate and oxalacetate are compared with Michaelis (K_m) and inhibitor (K_i) constants for these substrates in Table II. Pyruvate carboxylase has also been shown to carboxylate α-ketobutyrate at approximately 3% of the rate of CO_2 fixation on pyruvate (4),[3] and

[3] D. S. Kerr and M. F. Utter, unpublished observations.

Michaelis, inhibitor, and dissociation constants for this substrate are also included in Table II. For pyruvate and oxalacetate, the K_d values obtained by the two methods agree closely, but differ from K_m and K_i determinations obtained in initial rate studies of the over-all reaction by one to two orders of magnitude. In contrast, all the constants obtained for α-ketobutyrate were in the same range. The latter substrate differed also in that the enhancement of the enzyme-substrate complex (ϵ_c) is less than 1 as compared with the ϵ_c for pyruvate and oxalacetate, which are greater than 1. Possible differences between the interaction of pyruvate carboxylase with α-ketobutyrate as compared with the interaction of this enzyme with pyruvate and oxalacetate are discussed in a later section.

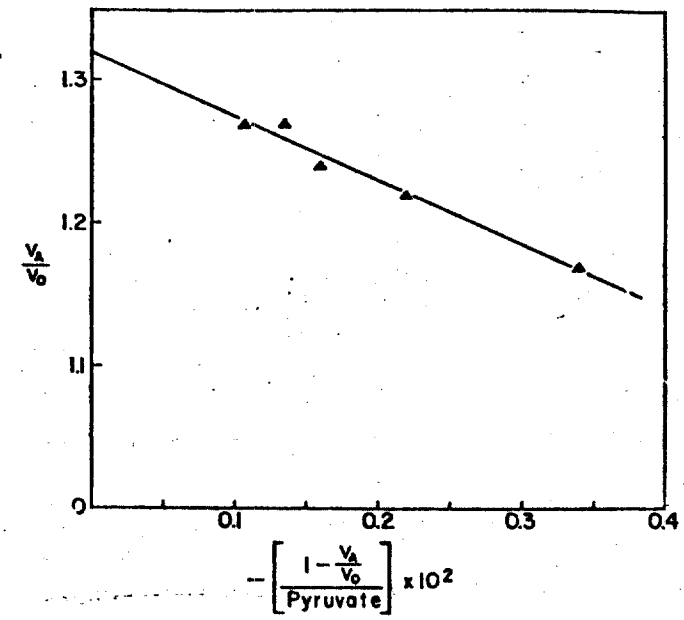


FIG. 2. Dissociation constant for pyruvate from its effect on the rate of avidin inactivation. The system contained, in 0.2 ml, 44 μmoles of Tris-sulfate (pH 7.8), 9 μmoles of $(NH_4)_2SO_4$, 200 μg of avidin (3.3 units per mg), 69 μg of pyruvate carboxylase (specific activity, 15.0), and various concentrations of pyruvate. The residual activity was measured spectrophotometrically after 2 and 4 min of incubation at 23°. The v_0 and v_a values were obtained from the slope of the pseudo-first order plots of the relationship between $\log_{10} E_t$ (residual enzymic activity) and t (minutes) at various pyruvate concentrations (*cf.* Reference 11).

The K_d values obtained for pyruvate and α-ketobutyrate represent true dissociation constants for the enzyme-substrate complexes, but the value found for oxalacetate is more complex since pyruvate carboxylase reacts with oxalacetate in the absence of other reaction components.

$$\text{Enzyme-biotin} + \text{oxalacetate} \rightleftharpoons \text{enzyme-biotin-oxalacetate} \rightleftharpoons \text{enzyme-biotin} \sim CO_2\text{-pyruvate} \rightleftharpoons \text{enzyme-biotin} \sim CO_2 + \text{pyruvate} \quad (12)$$

Furthermore, Reaction 12 does not come to a true equilibrium because of the nonenzymatic decomposition of enzyme-biotin ~ CO_2 to enzyme-biotin + CO_2. Therefore, the apparent K_d for oxalacetate determined by either the PRR or avidin inactivation methods is not a true dissociation constant for the enzyme-oxalacetate complex, but also contains a factor providing for the remaining reactions shown in Reaction 12 and terms due to the nonenzymatic breakdown of enzyme-biotin ~ CO_2. Similarly, the observed ϵ_c is not a true enhancement of the enzyme-oxalaceate complex. The relative contributions of these factors and terms to the observed K_d and ϵ_c cannot be assessed at present.

Properties of Certain Inhibitors of Pyruvate Carboxylase Which Interact with Bound Manganese—Further evidence relating to the role of the bound manganese in pyruvate carboxylase was obtained by examination of the properties of inhibitors of the over-all reaction, the action of which is localized in the second partial reaction, the carboxylation of pyruvate by enzyme-biotin ~ CO_2 (Reaction 2). These compounds have been found to reduce the enhancement of the bound manganese and also to protect pyruvate carboxylase against inactivation by avidin. The inhibitor and dissociation constants obtained by examination of the effects of these inhibitors on the initial rate of the over-all reaction, on the rate of inactivation by avidin, and on the enhancement of the PRR are summarized in Table III. Comparison of the inhibitor constants and dissociation constants obtained by the three inde-

TABLE II

Comparison of Michaelis constants, inhibitor constants, and dissociation constants for pyruvate, oxalacetate, and α-ketobutyrate

The K_m values for pyruvate and oxalacetate in the over-all reaction are taken from previous publications (4, 6). The K_m for α-ketobutyrate (see Footnote 3) was obtained by measurement of the rate of $^{14}CO_2$ fixation as a function of the concentration of this substrate. The K_i for α-ketobutyrate (see Footnote 3) was obtained by measurement of the effect of this compound on the rate of CO_2 fixation as a function of pyruvate concentration. CO_2 fixation was measured spectrophotometrically in this experiment. The K_i for pyruvate was obtained from the effect of this compound on the rate of oxalacetate decarboxylation measured as a function of oxalacetate concentration. Inhibition by both pyruvate and α-ketobutyrate was truly competitive and the K_i was obtained from a plot of $1/V_i$ against inhibitor concentration as described by Webb (14). The dissociation constants were obtained as described under "Methods." The values for ϵ_c are the enhancement of the enzyme-substrate complexes and are obtained from the ordinate intercept of the $1/(\epsilon_b - \epsilon^*)$ with respect to $1/(A)$ plot (see Fig. 1). The k_2/k_1 refers to the relative rates of reaction of enzyme-substrate ratio and enzyme with avidin (see Fig. 2).

Components	Over-all reaction		Avidin inactivation		PRR	
	K_m	K_i	k_2/k_1	K_d	ϵ_c	K_d
	M	*M*		*M*		*M*
Pyruvate	4.4×10^{-4}	5×10^{-4}	1.32	4.8×10^{-3}	1.65	4.5×10^{-3}
α-Ketobutyrate	4.9×10^{-3}	1.5×10^{-2}			0.4	3.5×10^{-3}
Oxalacetate	5.0×10^{-5}		1.78	2.38×10^{-3}	2.45	1.7×10^{-3}

TABLE III

Comparison of inhibitor constants and dissociation constants for some inhibitors of pyruvate carboxylase

The K_i values were obtained by measurement of inhibition of initial rate of CO_2 fixation as a function of pyruvate concentration, except for mesoxalate, when the inhibition of the initial rate of oxalacetate decarboxylation was measured as a function of oxalacetate concentration. For Type II and III inhibitors, K_i was estimated from a plot of $1/V_i$ against inhibitor concentration as described by Webb (14). For Type I inhibitors, K_i was obtained from secondary plots of the ordinate intercepts or slopes as shown for oxalate in Fig. 7. Other values were obtained as described in Table II.

Inhibitor[a]	Catalytic activity (K_i)	Avidin inactivation		PRR	
		k_2/k_1	K_d	ϵ_c	K_d
	M		M		M
Type I					
Oxalate	11.0×10^{-6}[b] 14.0×10^{-6}[c]	0.2	8.9×10^{-6}	<0.30	2.9×10^{-6}
Oxamate	1.6×10^{-3}	0.12	1.4×10^{-3}	<0.30	1.45×10^{-3}
Glyoxal	5.6×10^{-3}	0.12	6.0×10^{-3}	<0.38	7.8×10^{-3}
Fluoropyruvate	1.7×10^{-4}	0.05	1.3×10^{-4}	<0.30	2.3×10^{-4}
Type II					
Mesoxalate	2.1×10^{-3}	0.14	1.5×10^{-3}	<0.31	9.6×10^{-4}
Malonate	2.2×10^{-2}	0.2	8.2×10^{-3}	0.51	6.7×10^{-3}
Type III					
L-Malate	6.5×10^{-3}	0	2.3×10^{-3}	0.32	3.0×10^{-3}

[a] Other inhibitors, the properties of which have been examined, are: (*a*) Type I, bromopyruvate, glyoxalate, and oxanilate; (*b*) Type II, tartronate; and (*c*) Type III, α-ketoglutarate, isocitrate, maleate, and fumarate.

[b] Measured as a function of pyruvate concentration (see Fig. 3*A*).

[c] Measured as a function of oxalacetate concentration (see Fig. 3*B*).

TABLE IV

Effect of some carboxylic acids on rates of CO_2 fixation, exchange of pyruvate-^{14}C with oxalacetate, and exchange of $^{32}P_i$ with ATP by pyruvate carboxylase

CO_2 fixation was measured spectrophotometrically and ATP-P_i and pyruvate-oxalacetate exchange were measured as described under "Methods."

Inhibitor	Relative rates		
	CO_2 fixation	ATP-P_i exchange	Pyruvate-oxalacetate exchange
None	100	100	100
1.8×10^{-3} M L-malate	88.5	99.5	91.0
1.8×10^{-2} M malonate	70	94.8	71.5
3.0×10^{-4} M oxalate	8.7	98.0	14

pendent methods reveals good agreement. Calculation of the inhibitor constants in Table III required the assumption that these compounds were specific inhibitors of the carboxylation of pyruvate by enzyme-biotin ~ CO_2 (Reaction 2), and had no effect on the formation of enzyme-biotin ~ CO_2 from ATP + HCO_3^- (Reaction 1). This assumption was tested by examination of the effect of these inhibitors on the rates of the ATP-$^{32}P_i$ and ^{14}C-pyruvate-oxalacetate exchange reactions. In Table IV, oxalate (Type I) and malonate (Type II) are shown to be specific inhibitors of the exchange of ^{14}C-pyruvate with oxalacetate and to have no significant effect on the exchange of $^{32}P_i$ with ATP. The assumption involved in the calculation of the inhibitor constants appeared, therefore, to be valid. The data therefore strongly support the hypothesis that the bound manganese present in pyruvate carboxylase is involved in the carboxylation of pyruvate by the enzyme-biotin ~ CO_2 intermediate.

Although all the inhibitors described here affect the second partial reaction of pyruvate carboxylase, a closer examination reveals that the details of the mechanism of inhibition may differ. For convenience, these inhibitors have been subdivided into three types in Table III.

Type I Inhibitors, e.g. Oxalate—The inhibition of the initial rate of the over-all reaction by Type I inhibitors was uncompetitive with respect to pyruvate (Fig. 3*A*), but was competitive when measured as a function of the oxalacetate concentration (Fig. 3*B*). Furthermore, the relationship of $1/(\epsilon_b - \epsilon^*)$ with 1/(inhibitor) was nonlinear at high concentrations of inhibitor (Fig. 4) and the enzyme-inhibitor complex was only partially protected against inactivation by avidin ($0 < k_2/k_1 < 1$) (Fig. 5; Table III).

Type II Inhibitors, e.g. Mesoxalate—These compounds resembled the Type I inhibitors in showing nonlinearity of the relationship of $1/(\epsilon_b - \epsilon^*)$ and 1/(inhibitor) at high inhibitor concentrations and an enzyme-inhibitor complex which was partially protected against inactivation by avidin. However, the initial rate of the over-all reactions measured as a function of either pyruvate or oxalacetate concentration showed a mixed type of inhibition by these compounds (14), in contrast to the competitive or uncompetitive behavior observed with Type I compounds (Fig. 3). The mixed inhibitor behavior of mesoxalate on oxalacetate decarboxylation measured as a function of oxalacetate concentration is shown in Fig. 6.

Type III Inhibitors, e.g. L-Malate—These compounds showed a mixed type of inhibition of the initial rate of the over-all reaction when this parameter was measured as a function of pyruvate concentration. However, they resembled pyruvate (Fig. 1) in their effect on the enhancement of the bound manganese since the relationship of $1/(\epsilon_b - \epsilon^*)$ and 1/(inhibitor) was linear at all inhibitor concentrations. Additionally, the enzyme-inhibitor complex was completely protected against avidin inactivation

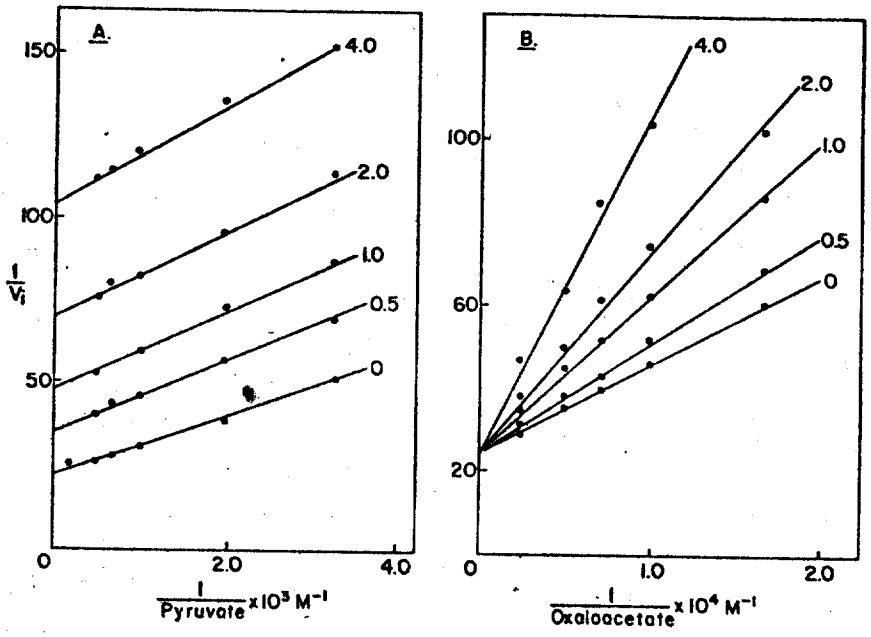


FIG. 3. Inhibition of pyruvate carboxylase by oxalate. The initial rates of CO_2 fixation and oxalacetate decarboxylation were measured spectrophotometrically as described in "Methods." Oxalate was present at the concentrations ($\times 10^{-5}$ M) indicated by the *figures*.

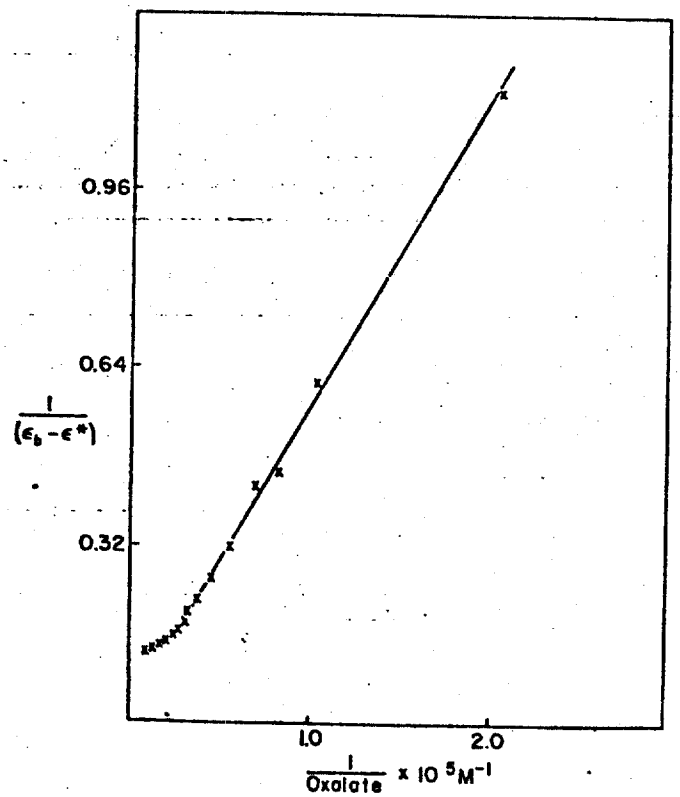


FIG. 4. Dissociation constant for oxalate by PRR analysis. The titrating agent contained, in 0.1 ml, 5 μmoles of Tris-Cl (pH 7.8), 8.5 μmoles of Tris-Cl (pH 7.2), 34 μmoles of KCl, 0.77 mg of pyruvate carboxylase (specific activity, 10.6), and 0.025 μmole of potassium oxalate. Titration was performed as described for Fig. 1. The final pH was 7.8.

(the k_2/k_1 ratio = 0). While L-malate was the most potent of the Type III inhibitors, many other compounds in this group, which comprised tricarboxylic acid cycle and related acids, have been examined (*cf.* legend, Table III). Inhibitor constants were not generally obtainable for the Type III inhibitors, since, at the concentrations required, anomalous effects were observed because of chelation of the divalent cation necessary for formation of enzyme-biotin ~ CO_2 from ATP + HCO_3^- (Reaction 1). However, dissociation constants obtained by either the PRR or avidin inactivation methods fell in the range of 0.01 to 0.05 M.

The mixed type of inhibition observed for Type II and Type III inhibitors would be predicted if these inhibitors can interact with both the free enzyme and the enzyme ~ CO_2 complex to form *EI* and *EI* ~ CO_2, respectively. However, it should be noted that the observed results could also result from difficulty in achieving concentrations of the other reactants sufficiently high that they have no effect on the initial rate measurements, although this explanation is less probable in view of the results obtained with Type I inhibitors using the same system.

Further analysis of the inhibition of the over-all reactions by oxalate was necessary to define the type of inhibition involved.

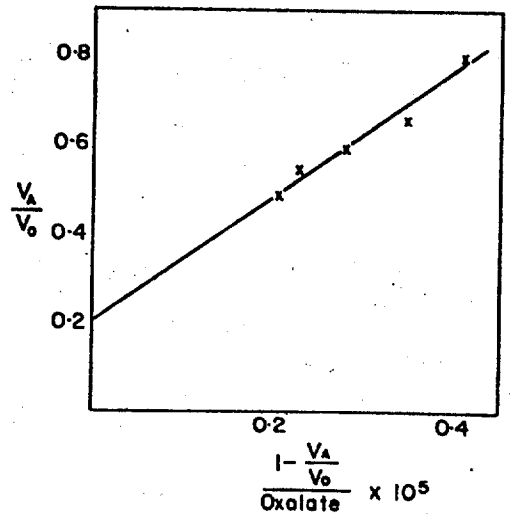


FIG. 5. Dissociation constant for oxalate by measurement of protection against avidin inactivation. The system was as described for Fig. 2 but with various oxalate concentrations. Analysis was performed as described for Fig. 2.

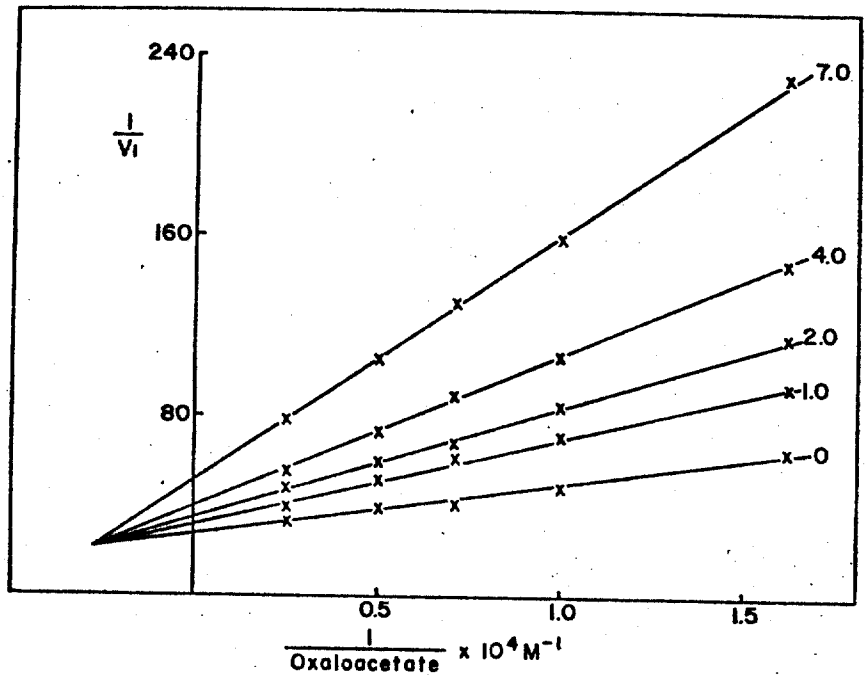


FIG. 6. Inhibition of pyruvate carboxylase by mesoxalate. The initial rate of oxalacetate decarboxylation was measured spectrophotometrically as described in "Methods." Mesoxalate was present at the concentrations ($\times 10^{-3}$ M) indicated by the *figures*.

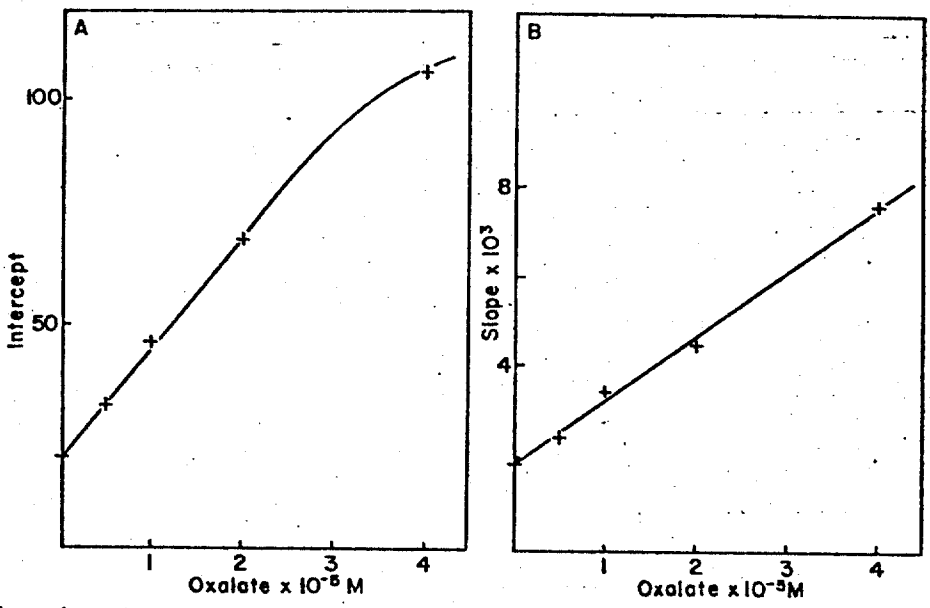


FIG. 7. Secondary plots of the ordinate intercepts from Fig. 3A and slopes of Fig. 3B against oxalate concentration

Secondary plots of the ordinate intercepts of Fig. 3A and the slopes of Fig. 3B against the oxalate concentration (Fig. 7) showed that a linear relationship existed in each case. This relationship is characteristic of simple uncompetitive (Fig. 7A) and simple competitive (Fig. 7B) inhibition. These results suggest that, in contrast to Type II and III inhibitors, the only significant interaction of Type I inhibitors is with the free enzyme to give *EI*, and that the formation of $EI \sim CO_2$ is not a kinetically significant pathway. The relationship of pyruvate with Type I inhibitors may be compared with the results obtained in initial rate studies of the relationship between pyruvate and the substrates of Reaction 1 (ATP and HCO_3^-). For these latter studies, an uncompetitive relationship was also observed,[4] and is explained if pyruvate interacts significantly only with $E \sim CO_2$. The binding of pyruvate to the free enzyme as shown by PRR (Fig. 1) and avidin inactivation (Fig. 2) studies, therefore, represents the formation of a complex which is not a part of the main reaction pathway. The observed difference between K_m and K_d for pyruvate (Table II) is therefore not unexpected.

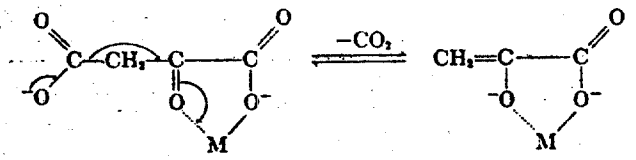
A possible deviation from the linear relationship shown in Fig. 7A may be found for the value of the ordinate intercept at the highest concentration of oxalate which is less than that pre-

[4] M. C. Scrutton and M. F. Utter, unpublished observations.

dicted. Similar deviations at the highest inhibitor concentration have been observed for all Type I inhibitors except bromopyruvate.

Enolization of Pyruvate—The results presented in the previous sections are entirely consistent with the proposal that the bound manganese of pyruvate carboxylase is involved in the carboxylation of pyruvate by the enzyme-biotin $\sim CO_2$ complex and in the formation of this complex from enzyme-biotin and oxalacetate (Reaction 2). Since these are transcarboxylation reactions, the role of the bound manganese may be analogous to that suggested by Steinberger and Westheimer (15) for the catalysis of the decarboxylation of dimethyl oxalacetate by metal ions. The intermediates proposed by Steinberger and Westheimer (15) (as written for pyruvate and oxalacetate) were those shown in Reaction 13. Spectral evidence for complexes of this type with divalent manganese had previously been presented by Kornberg, Ochoa, and Mehler (16). In the decarboxylation reaction (15), the most effective catalysts were Fe^{3+}, Al^{3+}, and Cu^{2+}, and, although the rate of decarboxylation in the presence of Mn^{2+} was only 4% of that found for Al^{3+}, the results suggest that Mn^{3+} might be a more effective catalyst. Reaction 13 predicts that pyruvate carboxylase should cause enolization of pyruvate in the absence of other reaction components, which might be detected by a release of tritium from the methyl group of tritiated pyruvate on incubation with the enzyme.

The assay for tritium release described under "Methods" has been studied as a function of time and enzyme concentration in a system which contained the components necessary for the overall carboxylation reaction (Fig. 8). The release of tritium was near with time to 20 min in the presence of 0.58 unit of enzyme



(13)

TABLE V

Requirements for tritium release from tritiated pyruvate

In Experiment A, the incubation system was as described in "Methods." Incubation was conducted for 15 min at 23° and H_2O was separated as described in "Methods." The values shown have been corrected for nonenzymic tritium release from tritiated pyruvate. For Experiment B, the incubation system contained, in 0.5 ml, 100 μmoles of Tris-sulfate (pH 7.8), 1.75 μmoles of tritiated pyruvate (8.2×10^4 cpm per μmole), and, where indicated, 0.5 μmole of potassium oxalacetate. The reaction was initiated by addition of 32 μg of pyruvate carboxylase (specific activity, 18.4) and incubation was conducted for 15 min at 23°. Analysis was made as described above.

System	Net activity in H_2O
	cpm
Experiment A	
Complete	15,800
Minus ATP	0
Minus Mg^{2+}	0
Minus HCO_3^-	3,415
Minus acetyl-CoA	0
Experiment B	
Enzyme + pyruvate	114
Enzyme + pyruvate + oxalacetate	5,080

(Fig. 8A) and with enzyme concentration to 1.1 units when a 15-min incubation period was used (Fig. 8*B*). Table V shows that release of tritium from tritiated pyruvate occurred only in the presence of the complete reaction mixture (Experiment A) or of oxalacetate (Table VB). Enolization of pyruvate in this system appeared, therefore, to require the presence of the enzyme-biotin $\sim CO_2$ complex. These results provide no support for the mechanism shown in Reaction 13, but are not necessarily inconsistent with such a mechanism. The equivocal nature of the results arises from the possibility that the identical proton,

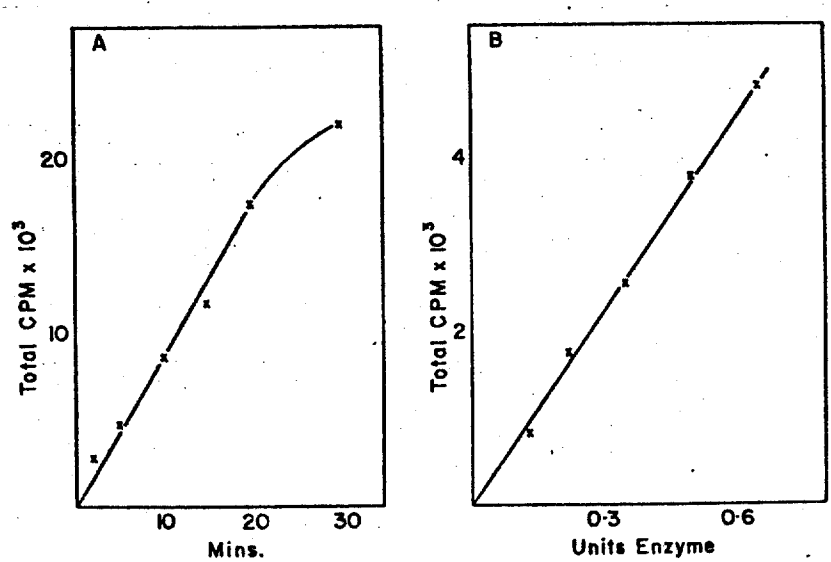


FIG. 8. Characteristics of tritium release from tritiated pyruvate by pyruvate carboxylase. The incubation system was as described in "Methods." For examination of the linearity with time (*A*), the reaction was initiated with 32 μg of pyruvate carboxylase (specific activity, 18.2) and incubation was conducted for the time indicated. The linearity of response to enzyme concentration was examined with an incubation time of 15 min and the reaction was initiated with various concentrations of pyruvate carboxylase (specific activity, 18.2) (*B*) as indicated. The total counts per min released have been corrected for nonenzymic release of $^3H^+$ from tritiated pyruvate.

which is removed from pyruvate on formation of the enzyme-pyruvate complex, may be replaced on this substrate when the complex dissociates. This phenomenon of proton retention has been shown for several enzymes (17, 18).

Preliminary results also suggested that pyruvate was not bound to the enzyme as a Schiff base intermediate, since the presence of pyruvate did not increase the extent of inactivation of pyruvate carboxylase by $NaBH_4$ at pH 7.8 as tested by the procedure of Rutter (19).

DISCUSSION

The data presented here strongly support the postulate that the bound manganese of pyruvate carboxylase is involved in the transcarboxylation portion of the reaction. Any detailed hypothesis describing the role of the metal ion in carboxyl group transfer from 1′-*N*-carboxybiotin-enzyme to pyruvate is necessarily speculative, but the experimental observations suggest certain guidelines for such hypotheses and for future experimentation. Any proposed mechanism must, as a minimum, be consistent with (*a*) the difference in the enhancements (ϵ_c) of the enzyme-substrate (Table II) and enzyme-inhibitor (Table III) complexes, (*b*) the failure to observe a reduction in the enhancement of the bound manganese when the enzyme-biotin $\sim CO_2$ complex is formed from ATP + HCO_3^- (Table I, Experiment B), and (*c*) the simple competitive relationship between oxalate and oxalacetate observed in initial rate studies of the over-all reaction (Figs. 3*B* and 7*B*).

Luz and Meiboom (20) have shown that the PRR of water in the presence of a complex of a paramagnetic ion, and hence the enhancement value of the complex, is directly proportional to the number of water molecules in the coordination sphere of the ion. Additionally, the PRR is inversely proportional to both the lifetime of the water molecules in the coordinated state and the rotation rate of the hydration sphere (13, 21). An increase in the rotation rate of the hydration sphere such as might result from a conformational change of the protein would reduce the enhancement to a value no less than 1, the enhancement of free manganese. For inhibitors such as oxalate, actual de-enhancement of the bound manganese is observed in the enzyme-inhibitor complex ($\epsilon_c < 0.3$), and this finding strongly suggests that the inhibitors coordinate directly to the bound manganese reducing the number of water ligands. The enhancement value of the complex is similar to that found for the complex of Mn(II) with EDTA (22), in which only 1 water molecule remains in the coordination sphere of the metal (23). Therefore, in the enzyme-inhibitor complexes, only 1 water molecule may remain in the manganese coordination sphere, and, since inhibitors such as oxalate can provide two ligands to the metal, the structure of the complex may be drawn as shown in Fig. 9, with the protein providing the remaining three ligands.

In contrast to the inhibitors, actual de-enhancement is not observed in the *E*-pyruvate and *E*-oxalacetate complexes ($\epsilon_c > 1.0$), and the possibility must be considered that these substrates do not interact with the bound manganese but bind at another site of the protein. In the latter case, the reduction in enhancement would be explained if binding of the substrate caused a conformational change which resulted in facilitation of the rotation of the manganese hydration shell or a decrease in the rate of exchange of the coordinated water (13, 20, 21). Unequivocal evidence for or against direct coordination of these substrates to the metal ion requires further experimentation, *e.g.* examination of the effect of the presence of the enzyme on the nuclear magnetic resonance spectrum of the methyl protons of pyruvate. Despite these reservations, the present data suggest that a direct interaction between the bound manganese and these substrates does occur since oxalate, which is believed to coordinate directly with the metal ion, shows a simple competitive relationship with oxalacetate (Figs. 3*B* and 7*B*). The enhancement of the enzyme-pyruvate complex ($\epsilon_c = 1.7$) then suggests that pyruvate provides only one ligand to the bound manganese (Fig. 9), leaving 2 water molecules in the manganese coordination sphere.

The proposed role of the metal ion in the formation of oxalacetate from $E \sim CO_2$ and pyruvate is shown in Fig. 10. The carboxylation of free enzyme (*A*) by ATP + HCO_3^- to give 1′-*N*-carboxybiotin-enzyme (*B*) does not result in interaction of the 1′-*N*-carboxyl group with the bound manganese, in accord with the finding that the enhancement was unaffected when the intermediate was formed in this way (Table I, Experiment B). The addition of pyruvate to *B* to give $E \sim CO_2$-pyruvate (*C*) causes a conformational change which brings the biotin ring into close proximity to the bound manganese. The conversion of $E \sim CO_2$-pyruvate (*C*) to *E*-oxalacetate (*D*) is then shown as a concerted process in which the proton leaves pyruvate synchronously with carboxyl group transfer to pyruvate from the 1′-*N*-carboxybiotin moiety. In this process, the electron-withdrawing effect of the metal would both facilitate the departure of a proton from the methyl group of pyruvate and would also assist the formation of a carbonium ion from 1′-*N*-carboxybiotin. Thus, in the mechanism shown in Fig. 10, the metal ion has a dual role (see below). Additionally, the nucleophilic sulfur atom might also aid the required polarization of the carbonyl group of biotin as shown in Fig. 10*C*.[5]

Recently, Caplow (24) has studied the rates of hydrolysis of esters of *N*-carboxy-2-imidazolidone, an analogue of 1′-*N*-carboxybiotin, by nucleophiles such as hydroxylamine. The rates of hydrolysis of these esters at an alkaline pH are accelerated up to 40-fold by 0.8 M $CaCl_2$, and barium and strontium salts also accelerate the process. These findings are clearly relevant to the proposed role of bound manganese in pyruvate carboxylase. Caplow (24) has proposed a mechanism for the transfer of the carboxyl group of 1′-*N*-carboxybiotin to pyruvate for pyruvate carboxylase which involves a chelate complex of the bound metal ion with the ureido carbonyl and the 1′-*N*-carboxyl groups of the 1′-*N*-carboxybiotin. Caplow's mechansim adequately explains the results obtained with the model reaction and cannot be excluded as an alternative for the mechanism proposed in Fig. 10. However, we prefer the latter hypothesis because it permits the metal ion to increase the nucleophilic character of the methyl group of pyruvate as well as to increase the susceptibility of the 1′-*N*-carboxybiotin to nucleophilic attack.

The proposed partial reaction mechanism shown in Fig. 10 implies that the sites on pyruvate carboxylase which are involved in formation of $E \sim CO_2$ from ATP and HCO_3^- (Reaction 1) and in carboxyl group transfer from $E \sim CO_2$ to pyruvate (Reaction 2) are not adjacent. Green (25) has previously suggested this possibility for biotin carboxylases and has proposed that the long flexible arm (14 A if extended) on which the biotin ring is mounted may permit movement of the ring between the sites.

[5] In a Dreiding molecular model of biotin, the sulfur atom was found to be 3.9 A from the carbonyl carbon atom. The 3*d* orbitals of sulfur, which contain the unbonded electrons, extend well beyond this distance.

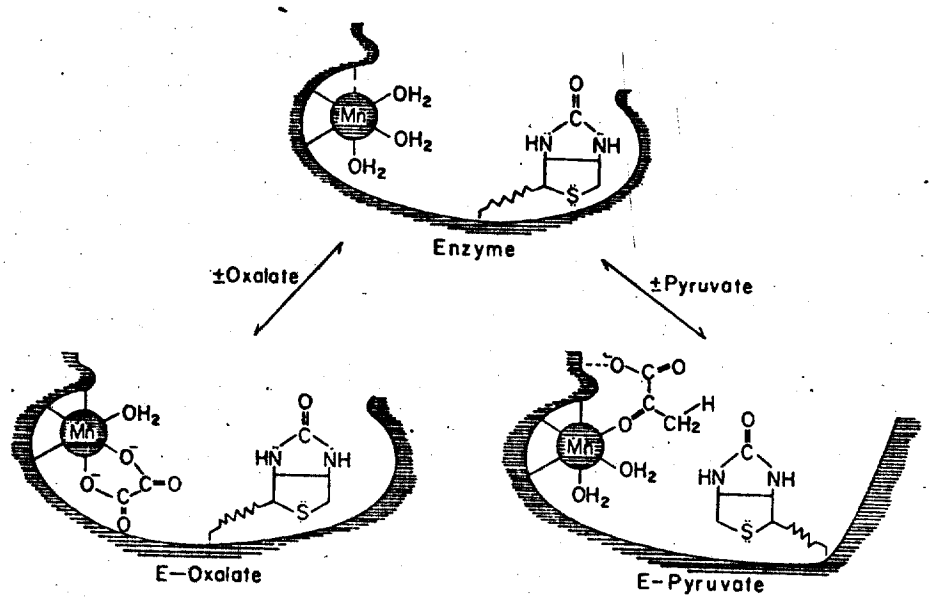


FIG. 9. Suggested nature of the complexes of pyruvate and oxalate with pyruvate carboxylase

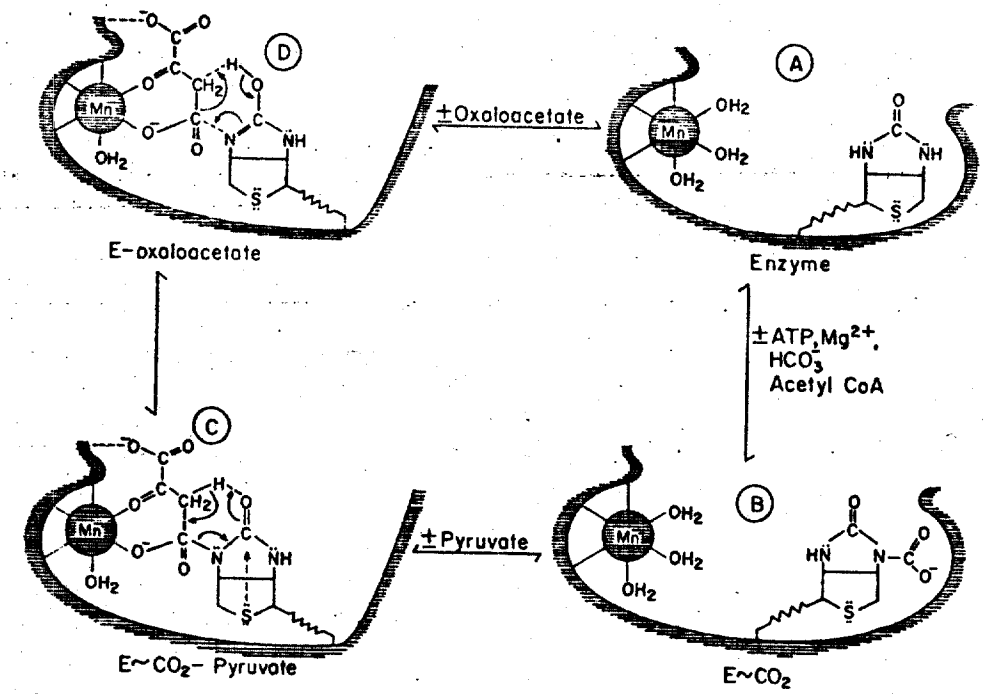


FIG. 10. Suggested role of tightly bound manganese in the pyruvate carboxylase reaction

A difference in the environment of the biotin ring in pyruvate carboxylase is further suggested by the contrasting effects on the rate of inactivation of the enzyme by avidin shown by pyruvate or oxalacetate (increased rate of inactivation) as compared with ATP (decreased rate of inactivation) (11).

Figs. 9 and 10 are entirely consistent with the observed relationships of the inhibitors with pyruvate and oxalacetate but the structure of the *E*-oxalacetate complex (Fig. 10*D*), in which this substrate provides two ligands to the bound manganese, suggests that the enhancement value of this complex should be similar to that observed for the *E*-oxalate complex ($\epsilon_c < 0.3$). The observed value for the *E*-oxalacetate ($\epsilon_c = 2.45$) is, however, much higher, and an apparent discrepancy exists, which may be rationalized in several ways. (*a*) As noted previously, it is not possible to obtain a true value for the enhancement of the *E*-oxalacetate complex since it is in equilibrium (Reaction 12) with several other structures (Fig. 10, *B* and *C*) and the observed value for ϵ_c is a weighted average of these various structures. (*b*) A change in the environment of the manganese may occur in the *E*-oxalacetate complex which hinders the rotational rate of the one remaining coordinated water molecule. (*c*) The rapid chemical exchange shown for the methylene protons of oxalacetate (Table V, Experiment B) may affect the longitudinal relaxation rate of the water protons in a manner analogous to an additional water molecule. (*d*) Disruption of one of the metal-protein ligands may occur on formation of the *E*-oxalacetate complex, permitting the coordination of an additional water molecule by the manganese and resulting, therefore, in the net

displacement of only 1 water molecule. (*e*) Manganese may coordinate an additional water molecule as a seventh ligand (22) on formation of the *E*-oxalacetate complex. While none of these alternatives can be excluded at present, either *a*, *b*, or *c* is considered to provide the more probable explanation.

The data obtained for α-ketobutyrate (Table II), as compared with pyruvate and oxalacetate, suggest that *E*-α-ketobutyrate complex resembles the *E*-oxalate complex more closely than the *E*-pyruvate complex. Thus, for α-ketobutyrate, $\epsilon_c = 0.4$, suggesting that this substrate is a bidentate ligand for manganese, as has been proposed for oxalate, and therefore forms a complex analogous to that depicted in Equation 13. A structural difference between the α-ketobutyrate complex and the pyruvate complex is compatible with the very different rates of CO_2 fixation by pyruvate carboxylase on these two substrates (4).

The results reported here, together with Caplow's studies (24), suggest that bound metal ions may be present in pyruvate carboxylases purified from other sources and in other biotin enzymes. Indirect evidence from inhibitor studies affords some support for this postulate since Losada, Canovas, and Ruiz-Amil (26) have shown that pyruvate carboxylase in bakers' yeast is inhibited by oxalate; Seubert and Huth (27) have found inhibition of pyruvate carboxylase in rat liver by oxalate, oxamide, and L-malate with inhibitor constants similar to those reported here for the chicken liver enzyme; and methylmalonyl-CoA-oxalacetate transcarboxylase from *Propionibacterium shermanii* is inhibited by oxalate.[6] Methylmalonyl-CoA-oxalacetate transcarboxylase has been examined for bound metal by the PRR method, and, while a small effect is found, the PRR after denaturation of an enzyme sample with perchloric acid gave an effect equivalent to only 0.1 μmole of manganese per μmole of biotin. Manganese cannot, therefore, be involved in this biotin enzyme, but the presence of some other metal with a much less significant effect on the PRR of water cannot be excluded.

No information is available which permits direct deductions concerning the environment of bound manganese in pyruvate carboxylase. The absence of de-enhancement by large chelating agents, *e.g.* EDTA (1), in contrast to the very marked effect of small bidentate ligands, *e.g.* oxalate, suggests that steric restrictions on access to the metal ion may exist. While the PRR data strongly suggest that oxalate combines directly with the bound manganese, experiments with ^{54}Mn-pyruvate carboxylase show unequivocally that the ^{54}Mn content is unaffected by incubation with oxalate. The unusually tight binding of manganese to this protein makes the investigation of the ligands involved a subject of considerable interest and studies on this aspect are now under way.

[6] P. Waegell and H. G. Wood, personal communication.

Acknowledgment—The authors are exceptionally indebted to Dr. M. Cohn for her advice and encouragement.

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Am. J. Physiol., 199: 77-83, 1960

Alimentary, blood and other changes due to feeding $MnSO_4$, $MgSO_4$ and Na_2SO_4[1]

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MOINUDDIN, JESSIE FISCHER AND HELEN WING-TSIT LEE. *Alimentary, blood and other changes due to feeding $MnSO_4$, $MgSO_4$ and Na_2SO_4.* Am. J. Physiol. 199(1): 77-83. 1960.—Rats were fed a basal diet or diets containing extra $MnSO_4$, $MgSO_4$ or Na_2SO_4 in three experiments. The major effects occurred in the experiment with the highest level of extra sulfates (138 mmoles/kg of feed) as follows. The $MgSO_4$-fed rats ate less feed than the Na_2SO_4-fed rats. Also, in the $MgSO_4$ group the activity of serum alkaline phosphatase was greater and the cleaned, dried cecum was considerably heavier than in the other groups, and the colon plus rectum was slightly longer than in the basal and $MnSO_4$ groups. The $MnSO_4$-fed rats, compared to rats of the other groups in this experiment, ate less feed, gained less body weight, required more feed per gram gain, drank more water, voided more urine, showed depigmentation of the labial surfaces of the incisor teeth, had less hemoglobin and serum inorganic phosphorus, and had cleaned, dried stomachs of lighter absolute weight. The erythrocyte count was higher in the $MnSO_4$ group than in the basal or Na_2SO_4 groups.

CERTAIN SUGARS IN THE DIET stimulate the growth of various regions of the gastrointestinal tract in rats. For example, diets containing 67% glucose or 67% sucrose, as compared to diets containing 67% cornstarch, increase the dry weight of the cleaned stomach (1). Diets containing 25% lactose (2) or 67% sucrose (1) increase the dry weight of the small intestine, and diets containing 15% cellobiose (1) or 10% arabinose (2) show some evidence of doing the same. Also, the 25% lactose (2), 10% arabinose (2) and 15% cellobiose (1) diets increase the dry weight of the cecum and of the colon plus rectum. These results, considered along with the number of sugar particles per unit weight of feed in each diet and with the digestibility or absorbability of each sugar, led to the idea that the increases produced in the growth of each organ by the designated sugars might be related to the osmotic effect of sugar particles in the lumen. However, results of feeding antibiotics indicated that microorganisms in the gut might play at least some part in some of the changes in gut weights. Thus, conceivably the sugars might be stimulating gastrointestinal growth in some way by serving as ready substrates for fermentation by the microflora of the lumen. Although we realized that there could be still other explanations of the stimulatory effects of dietary sugars, yet it seemed worthwhile to try to distinguish between osmotic effects of sugars and effects on microflora of the tract as possible causes of the stimulatory actions.

An indirect approach used in the present work was to see if gastrointestinal growth in the rat was increased by feeding substances (other than sugars) which have considerable osmotic effects in the lumen. For this purpose, we chose the hydragogue cathartics, magnesium sulfate and sodium sulfate. Manganese sulfate was tested also, since manganese has been found to stimulate the growth of the small intestine and of the ceca in chicks (3). It seemed unlikely that any of these three sulfates would affect the microflora of the lumen in the same way that the sugars might. However, some other effect of one or more of these salts on the microflora could not be ruled out. Also, there would be other uncertainties in the interpretation of any stimulation of growth by hydragogue cathartics. Thus, the present experiment was exploratory rather than a clear-cut test of alternatives. Other approaches to the problem are needed also.

METHODS

In three separate experiments, weanling male Sprague-Dawley rats were housed in individual cages and given tap water and their respective diets ad libitum for 4 weeks. In each experiment, there were six rats in each of the four dietary groups. In *experiments a* and *c*, feed intakes and feed:gain ratios were obtained for each week and for the total 4-week period. In all three experiments, records were kept of any diarrhea which occurred. The rats were weighed at the beginning of the 4-week period, at the end of each week, and at the end just before killing them. The rats were killed as described previously (2)

[1] Received for publication January 22, 1960.
Approved for publication as journal article no. 91-59 by the Associate Director of the Ohio Agricultural Experiment Station.

over a 3-day period. The gastrointestinal organs were freed from mesentery and excised. The small intestine and the colon plus rectum were hung full length, each with a 5-gram weight attached to the lower end. When these organs had straightened out completely, the lengths were measured. The organs were cleaned, examined, dried and weighed (2). In *experiment c*, a small snip of stomach was removed (for histological examination) before drying the organ, so that the dry weights of the stomach do not reflect the true weights as accurately as in *experiments a* and *b*.

In *experiment c*, additional observations were made as suggested by certain developments during the feeding period. These included visual observation of the teeth and of the volume of urine. Also, the amount of water drunk during the first 48 hours of the 3rd week was recorded. After 3½ weeks of the feeding period, blood was obtained from the tail for red and white blood cell counts and for determination of hemoglobin by the oxyhemoglobin method. At the time of killing the rats, blood was collected from the severed great vessels in the neck, and the serum from this blood was analyzed for alkaline phosphatase (4), inorganic phosphate (5), and protein (6). Many tissues were preserved for later analysis and examination, but it has not yet been possible to arrange for completion of this part of the study.

The basal diet, designated as the cornstarch diet, in all three experiments contained 67% cornstarch (Buffalo brand no. 3401, generously donated by the Corn Products Sales Co., New York City), 24% 'vitamin-free' casein, 5% Crisco, 4% salt mixture (U.S.P. XIV), and vitamins as given in detail elsewhere (7). The salt mixture was purchased from the Nutritional Biochemicals Corp., and it contained 3.8% magnesium sulfate (anhydrous) and 0.02% manganous sulfate monohydrate, but no sodium sulfate. For the diets high in sulfates of manganese, magnesium and sodium, part of the cornstarch of the basal diet was replaced, respectively, by extra manganous sulfate (monohydrate, Mallinckrodt, analytical reagent, A.C.S.), extra magnesium sulfate (anhydrous powder, Mallinckrodt, analytical reagent), or sodium sulfate (anhydrous powder, Merck, A.C.S.). In *experiment a*, the amount of the respective sulfate salt added, over and above that in the salt mixture, for the $MnSO_4$, $MgSO_4$ and Na_2SO_4 groups was 0.88 mmole/kg. This level was fed throughout the experiment. In *experiment b*, the respective sulfates were added initially at a level (again, over and above that in the basal diet salt mixture) of 8.64 mmole/kg of feed. This level was progressively increased at the same times for the $MnSO_4$, $MgSO_4$ and Na_2SO_4 groups in *experiment b* by doubling it on the 9th day of the feeding period to 17.28 mmole/kg, doubling this on the 17th day to 34.56, doubling the latter on the 25th day to 69.12 and there maintaining the level for the remaining 4–6 days. The final percentage by weight in the diet of the major sulfate constituent (now including the contributions of the basal salt mixture) was 1.2% $MnSO_4 \cdot H_2O$, 1.0% $MgSO_4$ and 1.0% Na_2SO_4 for the respective high sulfate diets in *experiment b*. In *experiment c*, the extra sulfates were added at the level of 138 mmole/kg of feed throughout the experiment.

In *experiments b* and *c*, the numerical results were analyzed statistically by analysis of variance, the hypothesis in every case being that the groups were equal and the alternative that they were not equal. In those cases where the F value indicated that there was a difference among the groups at the 5% level or less, a multiple range test (8) was applied to determine which among the groups differed from any other. The results of the latter test are indicated in the tables for the 5% and 1% levels of probability, and are referred to as significant in text statements if $P < 0.05$.

RESULTS

No data are presented for *experiment a*, since, at the low levels of sulfate salt additions in this experiment, all quantities examined were similar in all groups. Also, there were no significant differences among the groups in body weight gains within *experiment b*, so these gain data also are omitted.

Gains in body weight, intakes of feed and water, and feed : gain ratios (table 1). In *experiment c*, the rats in the $MnSO_4$ group, as compared to the rats in the other groups, gained less weight, ate less feed, required more feed per gram of gain and drank much more water. Although the $MgSO_4$-fed rats suffered chronic diarrhea (see later), they did not drink more water than the rats fed the cornstarch or Na_2SO_4 diets. The $MgSO_4$-fed rats ate significantly less feed than the Na_2SO_4-fed rats; they tended to eat less feed than the cornstarch diet rats also, but this latter tendency was significant only in the 1st week of the experiment. Thus $MgSO_4$ resembles another magnesium salt, $MgCl_2$, which also depresses the intake of feed when fed at excessive levels (9).

Volume of urine. In *experiment c*, rats in the $MnSO_4$ group voided more urine than rats in the other groups, as shown by much larger pools of urine on the waxed papers placed beneath the rats. The increase in volume

TABLE 1. *Gains in Body Weight, Intakes of Feed and Water, and Feed: Gain Ratios in Experiment c*

Dietary Group	Gain in 4 Wk.	Feed Intake in 4 Wk.	Over-All Feed: Gain Ratio for 4 Wk.	Water Intake, 1st 48 Hr. of 3rd Wk.
	gm	*gm*	*gm/gm*	*ml*
Cornstarch	186 (178–199)	386 (375–396)	2.08 (1.97–2.16)	48 (36–58)
$MnSO_4$	109* (101–124)	301* (280–319)	2.76* (2.52–2.89)	83* (53–99)
$MgSO_4$	180 (162–195)	356† (324–392)	1.98 (1.84–2.12)	48 (37–55)
Na_2SO_4	192 (180–209)	408 (371–453)	2.13 (2.03–2.19)	51 (40–60)

Figures given are averages; ranges are in parentheses. Average b. wt. at start was 55 gm in each of the groups. * Significantly different from the other 3 groups ($P < 0.01$). † Significantly different from the Na_2SO_4 group ($0.01 < P < 0.05$).

was estimated as at least twofold, but exact measurements were not made.

Teeth. During the 3rd week of *experiment c*, we noticed that the front surfaces of the incisor teeth of five out of six rats in the $MnSO_4$ group were white or very pale yellow in color. Only one rat in the $MnSO_4$ group had incisors of the normal orange-yellow color, whereas every rat in each of the other groups had normally colored incisors. These differences persisted until the end of the experiment.

Blood findings in experiment c (table 2). Hemoglobin in the $MnSO_4$ group was significantly lower than in the other three groups. Only one rat in the $MnSO_4$ group had a hemoglobin content falling within the range of the other three groups; this was the same rat which had normally colored incisor teeth. The numbers of red blood cells were significantly greater in the $MnSO_4$ group than in the cornstarch or Na_2SO_4 groups. The differences among the groups in numbers of white blood cells were not significant statistically. The differences in serum protein were not significant; however, three values in the $MnSO_4$ group were slightly below the normal range of 6.2–7.1 gm/100 ml (10). The $MgSO_4$ group exceeded all other groups in serum alkaline phosphatase activity. Serum inorganic phosphate was lower in the $MnSO_4$ group than in the other groups, in agreement with some other work in which fairly high levels of manganese were fed to growing rats (11, 12).

Diarrhea. In *experiment b*, there were no cases of diarrhea in the cornstarch group, one slight case in the $MnSO_4$ group and two slight cases in the Na_2SO_4 group lasting only 1 day each. In the $MgSO_4$ group, two of the six rats had no diarrhea, three had only slight diarrhea (one for 1 day, another on 2 nonsuccessive days, and the 3rd on 3 nonsuccessive days), while the remaining rat had slight diarrhea on 6 different days (including 4 in succession) during the middle periods of the experiment and moderate diarrhea for the 3 successive days following the last increase in $MgSO_4$ level. Also, on certain other days when diarrhea did not occur, the feces of this rat and of one other rat in the $MgSO_4$ group were soft and had a variety of unusual shapes: some pellets were extra long but of normal thickness, some were very thin but of normal length, and some showed the lines of 'working over' plainly. Also, several of the pellets had several small sideways projections at one end and thus resembled cloves. The pellets were black in all groups.

In *experiment c*, there was no diarrhea in the $MnSO_4$ group and only one instance of slight diarrhea lasting 1 day in the cornstarch group. Only one rat in the Na_2SO_4 group had any diarrhea at all. This occurred on 4 different days (including 3 in succession) in about the middle of the feeding period, and was slight in each instance. Diarrhea began in all the rats of the $MgSO_4$ group after 1–3 days of commencing the feeding, and continued until the end of the experiment. Once established, diarrhea in this group was severe on most days during the first 3 weeks, and slackened in severity slightly in some of the rats during the 4th week. However, there was no clear-cut indication of remission of the $MgSO_4$-produced diarrhea, such as occurs with the diarrhea produced by various sugar-containing diets (1, 2, 7). Haag and Palmer (13) also found that marked catharsis continued throughout many weeks of an experiment when rats were fed diets containing 0.8–0.9% magnesium as either the sulfate or the carbonate and amounts of calcium and phosphorus similar to those in the diets herein.

Appearance of organs. In *experiment b*, the cecal contents in the $MgSO_4$ group were quite runny (although the liquid contained some solid material) as compared to the more pasty or waxy cecal contents in the other groups. In the $MgSO_4$ group of *experiment c*, the contents of the cecum and colon were runny, and the cecum was noticeably enlarged compared to the cecum in the other groups. The tissues around the rectum in the $MgSO_4$ group seemed to be tougher and more difficult to separate from the rectum than is usual, perhaps indicating that some adaptation occurred external to the organ itself.

Weights and lengths of organs (table 3). In *experiment b*,

TABLE 2. *Blood and Serum in Experiment c*

Dietary Group	Tail Blood After 3½ Weeks of Feeding the Diets			Serum From Blood From Vessels in Neck, Obtained at Killing		
	Hemoglobin	Red blood cells	White blood cells	Protein	Alkaline phosphatase	Inorganic phosphate
	gm/100 ml	*millions/cu mm*	*thousands/cu mm*	*gm/100 ml*	*Bodansky U/100 ml*	*mg P/100 ml*
Cornstarch	14.4 (13.2–16.3)	6.91 (6.00–7.60)	9.19 (1.75–15.45)	6.7 (6.4–6.9)	84 (73–91)	5.3 (4.4–6.2)
$MnSO_4$	11.6* (10.0–14.0)	8.80† (7.40–11.00)	16.21 (11.95–21.20)	6.3 (5.7–7.0)	85 (81–89)	3.8§ (3.6–3.8)
$MgSO_4$	14.6 (14.1–15.4)	7.74 (6.90–8.75)	12.23 (6.70–16.60)	6.8 (6.6–7.0)	99‡ (90–106)	6.0 (4.8–7.0)
Na_2SO_4	14.6 (14.0–15.2)	6.94 (5.85–8.05)	11.72 (2.20–23.40)	6.5 (6.3–6.8)	78 (66–83)	5.3 (4.8–5.8)

Values given are averages; ranges are in parentheses. Six rats per group, except that the values and statistical analyses for phosphatase and phosphate are for 4 of the 6 rats in each group. The determinations of phosphatase in the sera of the other 2 rats of each group were ruined. The pooled sera of these remaining 2 rats of each group yielded values of 6.1, 3.7, 6.3 and 5.8 mg P/100 ml for the cornstarch, $MnSO_4$, $MgSO_4$, and Na_2SO_4 groups, respectively. * Significantly different from the other 3 groups ($P < 0.01$). † Significantly different from cornstarch and Na_2SO_4 ($0.01 < P < 0.05$). ‡ Significantly different from Na_2SO_4 ($P < 0.01$) and from cornstarch and $MnSO_4$ ($0.01 < P < 0.05$). § Significantly different from $MgSO_4$ ($P < 0.01$) and from cornstarch and Na_2SO_4 ($0.01 < P < 0.05$).

TABLE 3. *Body Weight, Weights of Cleaned, Dried Organs and Length of Small Intestine and Colon Plus Rectum in Experiments b and c*

Dietary Group	B. Wt.	Weights of Cleaned, Dried Organs				Lengths of Organs	
		Stomach	Small int.	Cecum	Colon+rectum	Small int.	Colon+rectum
	gm	*mg*	*mg*	*mg*	*mg*	*cm*	*cm*
				Experiment b			
Cornstarch	211 (193–235)	214 (196–235)	945 (877–996)	65 (52–74)	144 (130–164)	99 (96–105)	17.2 (16.5–18.5)
$MnSO_4$	210 (187–234)	231 (211–243)	904 (760–993)	73 (64–84)	150 (133–163)	100 (95–112)	17.6 (16.5–19.5)
$MgSO_4$	216 (171–237)	222 (184–244)	922 (844–1007)	80* (63–90)	145 (124–166)	98 (91–103)	17.8 (17.0–18.5)
Na_2SO_4	211 (172–246)	218 (189–247)	875 (798–1029)	63 (51–75)	142 (125–162)	96 (87–101)	17.1 (15.5–18.5)
				Experiment c			
Cornstarch	248 (232–260)	222 (202–238)	1011 (881–1174)	82 (70–97)	162 (145–184)	104 (99–107)	17.4 (16.5–19.0)
$MnSO_4$	171† (156–190)	179‡ (139–210)	915 (828–1025)	68 (60–75)	157 (141–180)	101 (94–105)	17.4 (15.5–19.0)
$MgSO_4$	243 (228–260)	217 (198–236)	1029 (961–1116)	161† (140–183)	165 (147–201)	105 (98–111)	19.7§ (18.5–21.0)
Na_2SO_4	256 (236–281)	229 (213–244)	1047 (1000–1134)	80 (71–90)	156 (144–167)	107 (104–112)	18.0 (16.0–19.5)

Values given are averages; ranges are in parentheses. Various groups are compared only within each experiment. In *experiment c*, weight of stomach is low in all groups due to removal of a small sample for histological examination. In *experiment c*, weights of small intestine were found to be different by analysis of variance ($0.01 < P < 0.05$), but no differences between single means were found using the multiple range test. * Significantly different from Na_2SO_4 and cornstarch ($0.01 < P < 0.05$). † Significantly different from the other 3 groups ($P < 0.01$). ‡ Significantly different from cornstarch and Na_2SO_4 ($P < 0.01$), and from $MgSO_4$ ($0.01 < P < 0.05$). § Significantly different from cornstarch and $MnSO_4$ ($0.01 < P < 0.05$).

the cecum was significantly heavier in the $MgSO_4$ group than in the cornstarch and Na_2SO_4 groups, but the differences were not very large.

In *experiment c*, the $MnSO_4$ group was exceeded by the other three groups in final body weight and in the absolute weight of the stomach. The absolute weight of the small intestine also tended to be least in the $MnSO_4$ group. However, the $MnSO_4$ group significantly exceeded the other three groups in the weight of the stomach and small intestine in relation to body weight (not given in table 3). The $MgSO_4$ group exceeded the other groups in the absolute and relative weights of the cecum. $MnSO_4$ exceeded Na_2SO_4 in relative cecal weight (not given in table 3). The absolute weights of the colon plus rectum did not differ significantly among the groups, but the relative weights of this organ (not given) were significantly greater in the $MnSO_4$ group than in the other groups. Most of the differences in weights of organs in *experiment c* appear to be related to the smaller size and body weight attained by the rats in the $MnSO_4$ group; there are difficulties in interpreting differences in weights of organs in such a situation. Thus, while the absolute weights of the small intestine, the cecum and the colon plus rectum in the $MnSO_4$ group were lowered less drastically than was the body weight, we don't know if the growth of these intestinal organs is stimulated by $MnSO_4$ or if it simply is not poisoned as greatly by $MnSO_4$ as is the growth of the body in general. The only clear-cut, interpretable effect of diet upon weights of organs in *experiment c* is the large increase in the weight of the cecum in the $MgSO_4$ group.

In *experiment c*, the colon plus rectum was longer in the $MgSO_4$ group than in the cornstarch or $MnSO_4$ groups. The ratio of weight to length of this organ (not given in table 3), like the absolute weight, did not differ significantly among the groups. Many other dietary substances (agar, for example) increase the colonic weight and weight:length ratio, as well as the length, in relation to the cornstarch group (14). Magnesium sulfate is the first substance we have found, among those which have any effect at all on the colon plus rectum, which appears to increase only the length.

DISCUSSION

Effects of $MnSO_4$. Most of the significant differences among the groups at the highest level of sulfates fed (that is, in *experiment c*) were due to the $MnSO_4$ group differing from the other three groups. These differences probably were due to manganese rather than to sulfate, since the $MgSO_4$ and Na_2SO_4 diets contained the same amounts of sulfate as did the $MnSO_4$ diet, and since more of the $MgSO_4$ and Na_2SO_4 diets were eaten. However, it remains possible that sulfate may affect the interaction of manganese and other elements.

We found that manganese, at the highest level we fed, caused decreases in growth, in intake of feed and in efficiency of conversion of feed to gain in body weight. Others have also reported that manganese in excess depresses the growth of young rats (11, 12, 15–17). Several changes possibly may be involved separately in causing this slow growth. First, an increased loss in the feces of phosphorus resulted from feeding excessive amounts of manganese (11), and low levels of inorganic phosphorus were found in the serum of manganese-fed young rats when the diets contained about 0.25% (12), 0.36% (11) or 0.47% (present paper) of phosphorus. Raising the dietary level of phosphorus to 0.80% raised the level of serum inorganic phosphorus and markedly improved growth (11). Second, the slowness of growth may have been related, in part, to the anemia caused by excessive manganese (17, present paper). Third, depletion of the rat's bodily thiamine by manganese and fourth, some alteration of energy metabolism mediated through the

thyroid gland are possible factors suggested by the work of others but regarding which we have no information from the present experiments.

Increases in the intake of water and in the volume of urine appear not to have been reported previously as symptoms of toxicity of manganese. In regard to possible causes of these increases, it may be pertinent that a somewhat prolonged decrease in the level of serum magnesium occurred in dairy calves fed supplemental manganese in experimental diets (18), and that a temporary decrease in serum magnesium occurred in cows grazing pastures of high manganese content and in rabbits, sheep or cows given manganese salts (19). Also, the volume of urine increased during magnesium deficiency in the rat (10). So it seems possible that excessive manganese increased the volume of urine by interfering with the absorption or metabolism of magnesium, thus inducing a deficiency of magnesium. Or possibly, since excessive manganese disturbs the iron picture (see later), the diuretic effect of $MnSO_4$ may involve interference with ferritin, the iron-storage protein which has been shown also to have an antidiuretic action (20). (This latter possibility is rendered somewhat less likely by the finding that apoferritin, the iron-free portion of ferritin, also is antidiuretic (20)). Other ways in which $MnSO_4$ might have produced the thirst and diuresis are conceivable and are not ruled out. Whether the increased intake of water may have caused the diuresis or vice versa is not known.

The depigmentation of the incisor teeth we observed in manganese-poisoned rats may have resulted directly from lowered iron, since rats given an excess of manganese have lower concentrations of plasma iron and lower stores of total body iron (17) and since it is known that simple iron deficiency causes loss of the yellow iron-containing pigment (21). Or, the manganese-produced depigmentation may have been secondary to a conditioned deficiency of phosphorus, since we found (herein) low values for serum phosphorus and since a diet low in phosphorus produced some bleaching of the yellow pigment (22). Hypoplasia of the enamel of the rat's upper incisor has been noted histologically after subcutaneous injection of manganese chloride, but it was not mentioned whether grossly apparent depigmentation, as seen in our manganese-fed rats, occurred also (23).

The present observations and some other work with several species indicate that an excess of manganese may notably affect the levels of hemoglobin and numbers of erythrocytes. Some of this work has been reviewed in various places (see, for example, 24). A decrease in hemoglobin and an increase in the red blood cell count, as reported in the present work, have been found in various species, although other effects or no changes also have been found. One per cent of manganese added to a stock diet for normal rats decreased hemoglobin but had no effect on the number of red cells (25). A combination of excessive oral and injected manganese in rats decreased hemoglobin (17) and, from the data presented, appeared to increase the numbers of red blood cells somewhat, although the increase in red cells is not indicated as being significant (17). Thus in the rat, lowering of hemoglobin by excessive manganese seems to be better established than is an increase in red blood cells. Also, excessive manganese promoted reticulocytosis in rats (17). Manganese in excess markedly affects both the iron picture (17, 26) and the copper picture (17, 26) in the body. However, whether manganese may produce or prolong anemia primarily by antagonism of iron or of something else (e.g. copper) is not known. Iron might appear to be involved mainly, since anemia was reversed by iron in manganese-fed pigs (27) but not by copper in rats given manganese (17).

Alkaline phosphatase of serum. Magnesium activates the alkaline phosphatase of rat serum (28, 29). The activity of this enzyme in rat serum is decreased when magnesium is deficient (30) and is either increased (31) or unchanged (32) when manganese is deficient in the diet. Thus it was of interest to learn the effects of dietary excesses of these metals on the activity, especially in view of the depression of serum magnesium by an excess of manganese in several mammalian species (18, 19), and also in view of the knowledge that magnesium and manganese may be at least partially interchangeable *in vitro* as activators of mammalian tissue alkaline phosphatases (33) and other enzymes. Our finding that the activity of serum alkaline phosphatase in the $MnSO_4$ group did not differ significantly from that in the cornstarch group may indicate that manganese was not specifically interfering with magnesium in our experiment, at least at the time we tested the enzymatic activity. However, it might also be that manganese did disturb the metabolism of magnesium in one or more other ways, but that manganese either did not displace magnesium from this particular enzyme or else was able to replace magnesium functionally as the activator of this enzyme *in vivo*. The slight but significant increase in the activity in the $MgSO_4$ group may indicate that the circulating enzyme(s) was not fully activated by magnesium under the conditions of the other diets. (Rosenthal *et al.* (29) found that plasmatic alkaline phosphatase of the rat was only about 75% as active in the absence as in the presence of added magnesium in the assay system, in a variety of nutritional states. We did not add any magnesium to our assay system.) It also seems possible that the extra magnesium of the $MgSO_4$ diet induced the formation and release (or simply the release) into the serum of more alkaline phosphatase, but we have no data on this point.

Gastrointestinal tract. An increase in the amount of manganese, added as manganese sulfate, from 2.6 ppm to about 50 ppm in the diet in *experiment a* had no effect on the weight of the cecum or small gut in our rats. This contrasts with the finding that the ceca and small gut of the chick grow heavier when the diet contains 50 ppm manganese as compared to 10 ppm manganese (3). The very much larger amounts of manganese in the high-manganese diet of our *experiment b* produced only a slight (statistically nonsignificant at the 5% level)

increase in cecal weight. Thus, it appears that the rat's cecum and small gut are either much less susceptible than the chick's to stimulation by dietary manganese, or else that other conditions in our experiments were too different from those in the experiment with chicks to be able to demonstrate a similar effect. The toxicity of manganese, particularly as reflected in poor growth, in *experiment c* prevents reaching any conclusion about effects on the rat's gastrointestinal tract of still higher levels of manganese.

Diarrhea and cecal distention, such as we noted herein with excessive $MgSO_4$ in *experiment c*, occur also with excessive dietary $MgCl_2$ or $MgCO_3$ (9). The weight of the cecum plus its contents was greatly increased by $MgCl_2$ or $MgCO_3$ (9); however, the weight of the cecal tissue alone was not given (9). Our findings with $MgSO_4$ show that the distention is accompanied by an actual increase in cecal tissue, not just by a stretching of the wall.

Many factors need to be considered before finally judging the possible usefulness of the hydragogue hypothesis to explain sulfate-produced increases in growth of any gastrointestinal region. Among these factors are the calculated numbers of extra particles per 100 gm feed which are supplied by the extra sulfate salts. These numbers for *experiment c*, assuming 100% ionization in the digestive tract, are 0.166×10^{23} for $MnSO_4$ and for $MgSO_4$, and 0.249×10^{23} for Na_2SO_4. (For comparison, the numbers of sucrose or glucose particles per 100 gm feed in previous experiments (1) were: 67% sucrose diet, 1.179×10^{23}; 25% sucrose diet, 0.440×10^{23}; 67% glucose (hydrate) diet, 2.037×10^{23}; and 25% glucose (hydrate) diet, 0.760×10^{23}. In Friedman's 50% talc diet (34), feeding of which stimulates growth of the small intestine in mice, perhaps by a hydragogue action, there are 0.794×10^{23} talc molecules per 100 gm of feed; the number of particles (molecules plus ions) from this talc probably would be somewhat greater than this, depending upon the degree of ionization in the lumen, which is unknown.) In addition to the number of particles per unit weight of feed, many other factors may affect the intensity and duration of a hydragogue action. Obviously, the total amount of feed eaten, and the amount eaten at any one meal, will influence the number of extra particles present in the tract. Other factors are extent of coprophagy, rate and completeness of digestion and absorption, rate of gastric emptying and intestinal transit, rate of excretion of absorbed substances in the bile or through the intestinal wall, and disposition by microbial action. If we consider only the number of small particles furnished by the diets, the present group of exploratory experiments does not adequately test the idea that a hydragogue action of certain substances is involved in their stimulation of organal growth. To cite just one example, in the stomach immediately after eating a similar weight of different diets (when very little absorption or emptying might have occurred), the number of extra small particles furnished by the diets high in sulfate salts is considerably less than the number of sugar particles furnished by the 67% glucose and 67% sucrose diets. The latter two diets are the only ones we have found which increase the gastric weight (1). Furthermore, the number of particles from the sulfate salts is smaller also than the number of sugar particles from 25% glucose and 25% sucrose diets, which diets fail to increase the gastric weight (1). So perhaps one might hardly have expected to have found any effect of our high sulfate diets on gastric weight, if a hydragogue effect is indeed involved in stimulation of gastric growth by the diets high in sugars, assuming that other factors are equal for the sulfate and sugar diets. However, as mentioned above, we cannot assume that these other factors are equal for all substances. Thus, differences in rate of gastric emptying could quickly change the concentration of different substances, and so alter the osmotic effect. Similar considerations apply to other factors and other regions of the tract. Until the many other factors are investigated thoroughly, the usefulness in gastrointestinal physiology of the present group of exploratory experiments is twofold. First, we have learned that feeding excessive amounts of $MgSO_4$ increases the weight of cecal tissue and appears to lengthen the colon plus rectum. Second, we have obtained some idea of the levels of sulfate salts which can be fed without untoward effects. Thus, in *experiment c*, the toxic effects of manganese sulfate and the digestive disturbances due to magnesium sulfate suggest that we could not sensibly raise the dietary levels of these two salts any higher, if it were necessary to do so to more adequately test the hydragogue theory of gastrointestinal growth stimulation. However, higher levels of sodium sulfate could be tested.

We are indebted to Mrs. Doris Elliot and Miss Margaret Thornbury for help with *experiments a* and *b*, respectively, to Mr. Robert Schlitz for the blood cell counts in *experiment c*, and to Dr. M. Moinuddin for help with the assays of *experiment c*.

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Voprosy Meditsinskoy Khimii
1968, No. 14(1): 27-31

EFFECT OF MANGANOUS CHLORIDE ON NITROGEN METABOLISM

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Poisoning caused by the entry of manganese compounds into the body of animals and man results in significant functional and morphological changes in the brain and viscera [1-4]. The metabolic disturbances underlying these changes have not been studied adequately.

Several investigators found that poisonings caused by various toxic agents (nitrochlorobenzene, aniline, ethylene dichloride, carbon tetrachloride, carbon monoxide), some insecticides, and bacterial toxins as well as many pathological states, especially traumas, exposure to heat, X irradiation, etc. markedly increase the ammonia content of the tissues. These facts led to the conclusion that the body, brain tissue in particular, react to injurious factors by disruption of nitrogen metabolism, which increases the ammonia content of the tissues, with the subsequent development of poisoning

Since the entry of manganese compounds into the body affects mainly the nervous system [1-3], we deemed it worthwhile to study the peculiarities of ammonia metabolism in the brain and other tissues.

The toxic action of ammonia is neutralized by the amination of α-ketoglutaric acid and amidation of glutamic acid, both free [10, 11] and bound to proteins [10, 12]. It was also of interest, therefore, to determine the amount of glutamine and amide nitrogen in proteins. This was the purpose of our study.

METHODS

Rats weighing 200 to 220 g were anesthetized with ether and injected intratracheally with 10% manganous chloride solution at the rate of 15 mg of manganese per rat. At higher concentrations of manganese the rats quickly died of pulmonary edema. The control animals received physiologic

saline under the same conditions. The rats were killed by decapitation 1, 6, and 30 days after poisoning. The control animals were sacrificed at the same time. These intervals were chosen because, according to the literature [13], after a single injection of manganese, increased concentrations of the substance can be detected within 24 hours in the blood and after 6 to 30 days in the brain and liver.

The tissues under study were quickly extracted, frozen in liquid air, and thoroughly ground. The ground frozen tissue was weighed and placed in 5% tetrachloroacetic acid.

The proteins were separated by centrifugation. Ammonia and amide nitrogen of glutamine were determined by the method of Silakova et al. [14]; amide nitrogen was determined in proteins by oxygen hydrolysis of the sediment obtained after separation of the trichloroacetic acid extract.

The results, expressed in milligram percentages of wet tissue weight, were statistically analyzed and the values were considered significant at $P < 0.05$

RESULTS

Intratracheal administration of physiologic saline under ether anesthesia produced a statistically significant decrease in the amount of ammonia, glutamine, and amide nitrogen in proteins in all the tissues under study in the control animals compared with the content of these substances in the tissues of normal animals (Figs. 1-4). These findings are consistent with the published data on the effect of anesthesia on ammonia metabolism [15, 16]. Hence the change in content of the nitrogenous substances observed in the tissues following the injection of manganous chloride is the sum of the changes caused by two factors - anesthesia and manganese salts. The true nature of the changes attributable solely to manganese is to be determined from the difference between the corresponding parameters in the experimental and control animals.

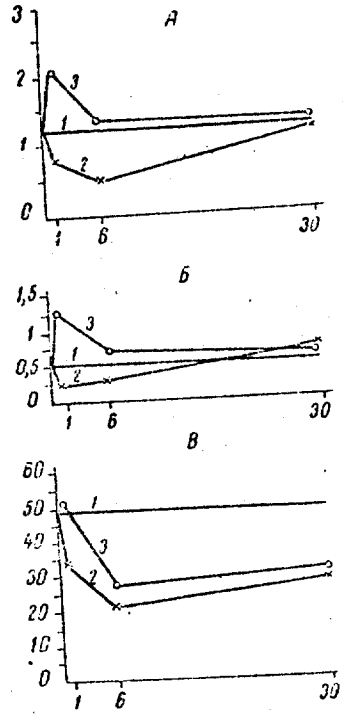

Fig. 1. Effect of manganous chloride on the amount of ammonia nitrogen (A), amide nitrogen in glutamine (B) and proteins (C) in the blood.

1 - normal animals; 2 - control animals; 3 - experimental animals. Abscissa: time (in days); ordinate: (A) - ammonia nitrogen in mg%); B - amount of amide nitrogen in glutamine (in mg%); C - amount of amide nitrogen in proteins (in mg%)

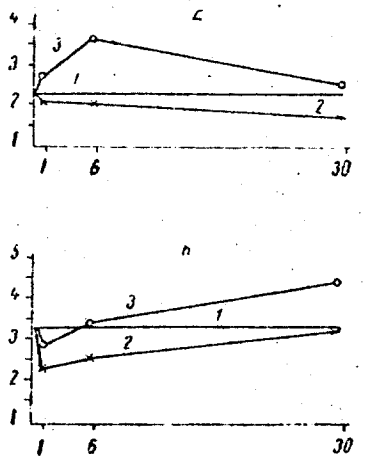

Fig. 2. Effect of manganous chloride on the amount of ammonia nitrogen (A) and amide nitrogen of glutamine (B) in brain tissue.

The symbols are the same as in Fig. 1

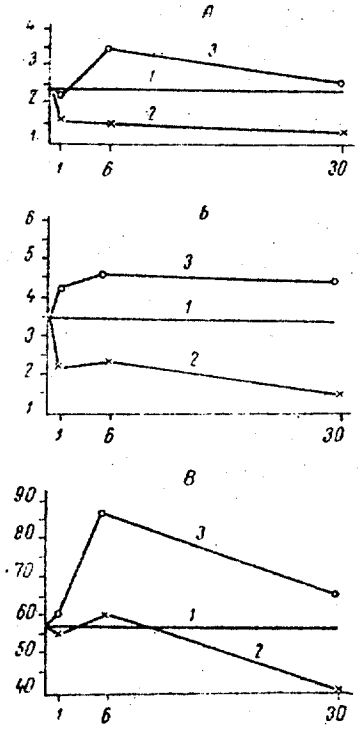

Fig. 3. Effect of manganous chloride on the amount of amide nitrogen (A), amide nitrogen in glutamine (B) and proteins (C) in liver tissue.

The symbols are the same as in Fig. 1.

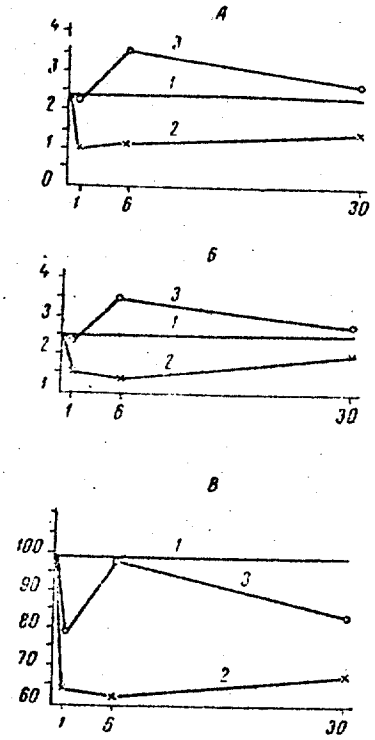

Fig. 4. Effect of manganous chloride on the amount of ammonia nitrogen (A), amide nitrogen in glutamine (B) and proteins (C) in skeletal muscle tissue

The symbols are the same as in Fig.1

It is evident from Fig. 1 that within 24 hours after intratracheal injection of manganous chloride, blood ammonia increased 2-fold, glutamine 3-fold, and amide nitrogen in proteins 1.3-fold compared to the control. On day 6 the content of these nitrogen fractions was still abnormal, exceeding the control 1.6-, 1.8, and 1.1-fold, respectively. By day 30 the levels were the same as the control.

The changes in the nitrogen fractions were somewhat different in the brain (Fig. 2). Within 24 hours after injection of manganese when, according to the literature [4, 13], its content in brain tissue has not yet increased, the amount of ammonia and glutamine in brain tissue was unchanged. As the manganous chloride passed through the blood-brain barrier, particularly on day 6, ammonia and glutamine increased on the average 1.6 and 1.3-fold, respectively, compared to the control.

By day 30 the amount of ammonia in the brain decreased while that of amide nitrogen in glutamine increased, exceeding the normal level by a factor of 1.4. Amide nitrogen in brain proteins remained unchanged throughout the period under study.

The changes in nitrogen metabolism following the injection of manganous chloride were greatest in the liver (Fig. 3). Within 24 hours ammonia increased 1.3-fold, glutamine 1.6-fold, and amide nitrogen in proteins 1.1-fold. By day 6 the changes were more intense: ammonia exceeded the control values 1.8-fold, amide nitrogen in glutamine 1.7-fold, and amide nitrogen in proteins 1.5-fold. By day 30 the levels of all the fractions were virtually the same as on day 6.

Fig. 4. presents data characterizing changes in the parameters under study in the skeletal muscles of the rats. These changes were also greatest on days 1 and 6 after injection of manganese.

On day 30 the ammonia content declined but remained 1.5 times higher. The level of the other nitrogen substances was normal.

The above data show that a single intratracheal injection of rats with 15 mg of manganous chloride sharply increases the amount of ammonia in all the tissues under study, a phenomenon that may be an important factor in the poisoning.

It is interesting to note that all the tissues do not respond with an increase to manganeous chloride poisoning with an identical increase in ammonia content. The latter, as pointed out, is highest in the liver. The differences may be due to differences in the permeability of the tissues to the manganous chloride or in their reactivity. This is undoubtedly significant because manganese compounds, according to the literature, are poisons affecting most clearly the nervous system. On the other hand, our findings show that the changes in ammonia content are most

prohounced in the liver rather than in brain tissue. Consequently, even with a lower ammonia content in brain tissue following manganese poisoning, nerve tissue function becomes more impaired. This is the basis for considering manganese compounds nerve tissue poisons.

Ammonia in the tissues becomes neutralized by the synthesis of nontoxic substances from it, glutamine in particular, through the amidation of both free and bound glutamic acid.

Our experiments showed that ammonia accumulates in the tissues under study while the processes aimed at neutralizing its toxicity intensify. As the ammonia concentration increases, so does the amount of both glutamine and amide nitrogen in proteins. The synthesis of glutamine was particularly pronounced in all the tissues, i.e., the amidation of free glutamic acid. Glutamic acid bound in protein molecules is likewise drawn into ammonia metabolism in the liver, skeletal muscles, and blood, but it participates in the detoxification of ammonia is somewhat less than does of free glutamic acid.

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УДК 612.015.33.014.46:546.711

ВЛИЯНИЕ ХЛОРИСТОГО МАРГАНЦА НА АЗОТИСТЫЙ ОБМЕН

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Интоксикация, обусловленная поступлением в организм животных и человека соединений марганца, сопровождается существенными функциональными и морфологическими изменениями в тканях мозга и внутренних органов [1—4]. Нарушения в обмене веществ, лежащие в основе этих изменений, изучены еще недостаточно.

Рядом исследований [5—9] установлено, что интоксикации, обусловленные различными ядами (нитрохлорбензол, анилин, дихлорэтан, четыреххлористый углерод, окись углерода), некоторыми инсектицидами, бактериальными токсинами, а также многие патологические состояния, в частности травмы, воздействие тепла и рентгеновых лучей и др., сопровождаются значительным повышением содержания аммиака в тканях. Эти факты послужили основанием для вывода, что общей реакцией организма и прежде всего ткани мозга на различные повреждающие факторы является нарушение процессов азотистого обмена, сопровождающееся увеличением содержания аммиака в тканях, вследствие чего развивается интоксикация, обусловленная аммиаком.

Поскольку при введении в организм соединений марганца наступающие поражения затрагивают прежде всего нервную систему [1—3], представляло интерес изучить особенности обмена аммиака в мозгу и других тканях организма.

Устранение токсического действия аммиака связано с аминированием α-кетоглютаровой кислоты и амидированием глютаминовой кисло-

ты, как свободной [10, 11], так и связанной в белках [10, 12]. В связи с этим представляло интерес изучить также содержание глютамина и амидного азота белков, что и явилось целью настоящих исследований.

Методы исследования

Крысам весом 200—220 г вводили под эфирным наркозом внутритрахеально 10% раствор хлористого марганца из расчета 15 мг марганца на крысу. При более высоких концентрациях вводимого марганца крысы быстро погибали от отека легких. Животным контрольной группы в тех же условиях вводили физиологический раствор. Животных забивали декапитацией на 1-е, 6-е и 30-е сутки после затравки. В эти же сроки забивали и животных контрольной группы.

Выбор указанных сроков был обусловлен тем, что, как известно из литературных данных [13], при однократном введении марганца повышенные концентрации его улавливаются в крови уже в 1-е сутки, а в мозгу и в печени на 6—30-е сутки.

Исследуемые ткани быстро извлекали, замораживали в жидком воздухе и тщательно размельчали. Размельченную замороженную ткань взвешивали и помещали в 5% ТХУ.

Белки отделяли центрифугированием. В безбелковых экстрактах определяли аммиак и амидный азот глютамина по методу Силаковой и сотрудников [14]; амидный азот белков определяли кислотным гидролизом осадка, полученного после отделения трихлоруксусного экстракта.

Полученные данные выражали в миллиграмм-процентах в расчете на сырой вес ткани.

Все полученные результаты подвергали статистической обработке и достоверными считали величины при $P<0,05$.

Результаты и их обсуждение

Внутритрахеальное введение физиологического раствора под эфирным наркозом приводило к статистически достоверному снижению содержания аммиака, глютамина и амидного азота белков во всех исследуемых тканях контрольной группы животных по сравнению с содержанием этих веществ в тканях нормальных животных (рис. 1—4), что соответствует имеющимся в литературе данным о влиянии наркоза на обмен аммиака [15, 16]. В связи с этим изменение содержания исследуемых азотистых веществ, наблюдаемое в тканях при введении в организм хлористого марганца, представляет собой сумму изменений, вызванных обоими факторами: наркозом и солями марганца. Истинная картина изменений, обусловленных только введением марганца, может быть определена как разница между соответствующими показателями в опытной и контрольной группе животных.

Как видно из данных, приведенных на рис. 1, уже через сутки после внутритрахеального введения хлористого марганца содержание аммиака в крови увеличивается в 2 раза, глютамина — в 3 раза, амидного азота белков — в 1,3 раза по сравнению с содержанием их в крови контрольных животных. На 6-е сутки интоксикации содержание изучаемых азотистых фракций все еще достаточно изменено: содержание аммиака превышает контроль в 1,6 раза, глютамина — в 1,8 раза, амидного азота белков — в 1,1 раза. К 30-м суткам содержание аммиака, амидного азота глютамина и белков в крови не отличается от содержания этих азотистых веществ у контрольных животных.

Несколько иным образом изменяется содержание аммиака, амидного азота глютамина и белков в мозгу (см. рис. 2). Через сутки после введения марганца, когда, согласно литературным данным [4, 13], его содержание в ткани мозга еще не повышено, содержание аммиака и глютамина в ткани мозга не изменено. По мере перехода хлористого марганца через гемато-энцефалический барьер в мозг, в частности на 6-е сутки, содержание аммиака увеличивается в среднем в 1,6 раза по сравнению с содержанием его в мозгу контрольных животных, а глютамина — в среднем в 1,3 раза. К 30-м суткам содержание аммиака в мозгу снижается; содержание амидного азота глютамина возрастает,

превышая в 1,4 раза его содержание в норме. Содержание амидного азота белков мозга в течение всего изучаемого нами периода не изменялось.

Наибольшие изменения изучаемых показателей азотистого обмена при введении хлористого марганца наблюдались в печени (см. рис. 3). Уже в 1-е сутки содержание аммиака увеличивалось в 1,3 раза по сравнению с содержанием его у контрольных животных; содержание глютамина возрастало в 1,6 раза, а амидного азота белков — в 1,1 раза. К 6-м суткам эти изменения нарастали: содержание аммиака в 1,8 раза, амидного азота глютамина в 1,7 раза, а амидного азота белков в 1,5 раза превышало содержание этих веществ в печени контрольных животных. К 30-м суткам содержание аммиака, амидного азота глютамина и амидного азота белка оставалось практически на том же уровне, что и на 6-е сутки.

На рис. 4 приведены данные, характеризующие изменения исследуемых показателей в скелетных мышцах крыс. Наибольшие изменения в этой ткани также наблюдались на 1-е и 6-е сутки после введения марганца.

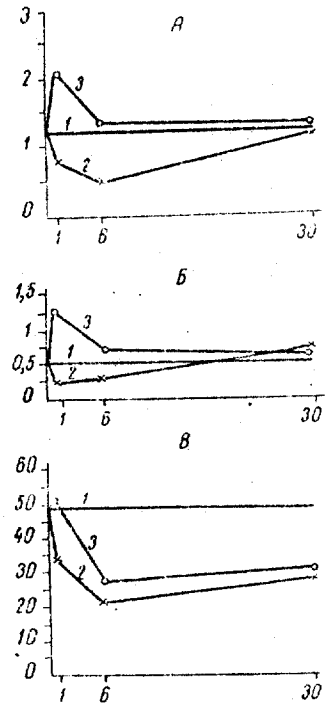

Рис. 1. Влияние введения хлористого марганца на содержание азота аммиака (*А*), амидного азота глютамина (*Б*) и белков (*В*) в крови.

1 — нормальные животные; *2* — животные контрольной группы; *3* — животные опытной группы. По оси абсцисс — время (в сутках); по оси ординат (*А*) — содержание азота аммиака (в *мг*%); *Б* — содержание амидного азота глютамина (в *мг*%); *В* — содержание амидного азота белков (в *мг*%).

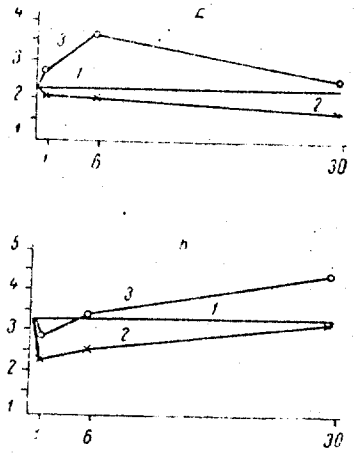

Рис. 2. Влияние введения хлористого марганца на содержание азота аммиака (*А*) и амидного азота глютамина (*Б*) в ткани мозга.

Обозначения те же, что на рис. 1.

На 30-е сутки содержание аммиака снижалось, но все еще было в 1,5 раза выше, чем в норме. Содержание остальных азотистых веществ не отличалось от нормального уровня.

Полученные данные свидетельствуют, что в результате одноразового внутритрахеального введения крысам 15 мг хлористого марганца резко увеличивается содержание аммиака во всех исследованных тканях, что, возможно, и является существенным фактором интоксикации.

Интересно отметить, что не все ткани отвечают равной степенью повышения содержания аммиака на интоксикацию хлористым марганцем. Наибольшее увеличение содержания аммиака, как установлено, происходит в печени. Эти отличия могут являться результатом различий проницаемости отдельных тканей по отношению к введенному хло-

ристому марганцу, а также различий реактивности тканей. Последнее обстоятельство, несомненно, имеет существенное значение, поскольку соединения марганца, согласно имеющимся в литературе данным, являются ядами, наиболее отчетливо поражающими нервную систему. Вместе с тем данные, полученные нами, свидетельствуют, что наиболее выраженные изменения в содержании аммиака наступают не в ткани мозга, а в печени. Следовательно, даже при более низком содержании аммиака в ткани мозга при интоксикации марганцем нервная ткань отве-

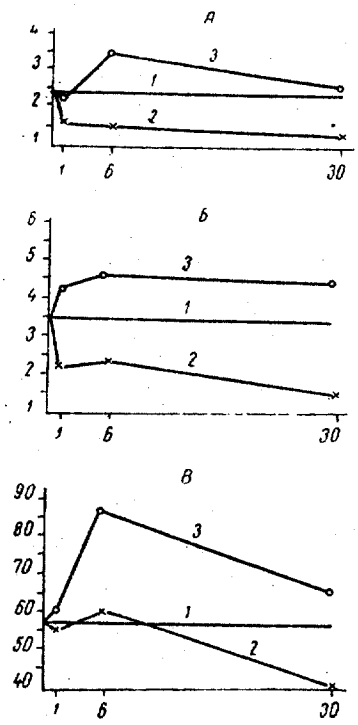

Рис. 3. Влияние введения хлористого марганца на содержание азота аммиака (*А*), амидного азота глютамина (*Б*) и белков (*В*) в ткани печени.

Обозначения те же, что на рис. 1.

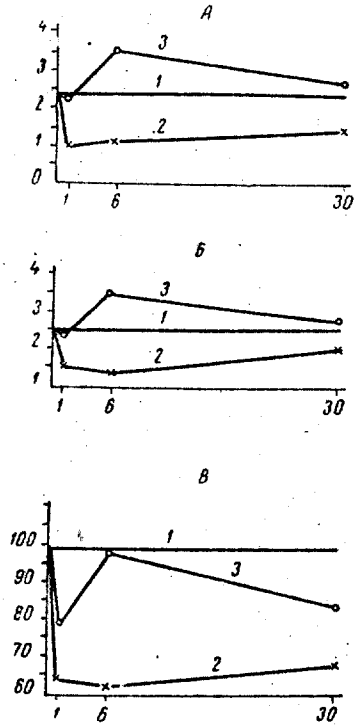

Рис. 4. Влияние введения хлористого марганца на содержание азота аммиака (*А*), амидного азота глютамина (*Б*) и белков (*В*) в ткани скелетных мышц.

Обозначения те же, что на рис. 1.

чает более резким нарушением своей функции, что и явилось основанием считать соединения марганца ядами нервной ткани.

Аммиак в тканях устраняется путем синтеза из него нетоксических веществ, в частности глютамина, путем амидирования как свободной, так и связанной глютаминовой кислоты.

Исследования показали, что в изученных нами тканях имеет место не только накопление аммиака, но и усиление процессов, направленных на устранение его токсического действия. С возрастанием концентрации аммиака в тканях возрастало содержание как глютамина, так и амидного азота белков. Во всех тканях более выражен был синтез глютамина, т. е. амидирование свободной глютаминовой кислоты. Глютаминовая кислота, связанная в молекулах белков, также вовлекалась в обмен аммиака в тканях печени, скелетных мышц и крови, но степень ее участия в детоксикации аммиака была несколько меньшей, чем свободной глютаминовой кислоты.

Выводы

1. Интоксикация, обусловленная одноразовым внутритрахеальным введением крысам 10% раствора хлористого марганца в дозе, соответствующей 15 мг марганца на крысу, сопровождается увеличением содержания аммиака в крови, мозгу, печени и скелетных мышцах на 1-е и особенно 6-е сутки после введения марганца. К 30-м суткам содержание аммиака в крови приближается к его содержанию у нормальных животных, а в тканях мозга, печени и скелетных мышц еще остается на высоком уровне.

2. Наряду с увеличением содержания аммиака в тканях усиливаются процессы, направленные на устранение его токсического действия: увеличивается содержание свободного глютамина и амидного азота белков.

3. Наиболее выраженные изменения в обмене аммиака имеют место в ткани печени, несколько менее выраженные — в ткани мозга.

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Поступила 21/V 1966 г.

«Вопросы медицинской химии», 1968, т. XIV, в. 1.

EFFECT OF MANGANOUS CHLORIDE IN THE NITROGEN METABOLISM

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Intoxication induced by intratracheal administration of 10% solution of $MnCl_2$ into rats at a dose 15 mg/rat was followed by the disorder in ammonia metabolism. Within 24 h after manganous administration the ammonia content in the blood, brain, liver and skeletal muscles was increased. At the 6th day after manganous administration the ammonia content in these organs is still greater. At the 30th day the ammonia content in the blood was near to that in the control animals. But in the brain, liver and skeletal muscles the ammonia content remaines at a high level. Apart from the increase in ammonia content in tissues the rate of process of its detoxication was accelerated. Free and protein bound glutamine content is increased. The increase in glutamine and protein nitrogen correlates with that in ammonia content. The most pronounced changes in ammonia content in tissues therate of process of its detoxication was accelerated.. brain.

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Perfusion of damaged liver and kidneys in progressive disease of these organs

by

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Director: Prof. G. von Bergmann, M.D.

Sent in: Oct. 16, 1945.

In the study of the development endogenous degenerative kidney diseases it was found that the kidney damage caused by endogenous fresh liver and kidney extracts in dogs and cats often persisted for several months. According to findings so far, in addition to the proteins of these organs it is mostly the enzyme systems that are most active. The fact that there is increased perfusion in the area of the liver, kidney and heart due to substances that play a role in the activity of the organ appears to counteract this process. If we study fresh endogenous organ extracts for perfusion of the organ from which they originate, no obstruction of the blood supply can be established with Rein's flowmeter, or at any rate it is very rare. An interesting feature is that in the animal that was not pretreated, the intravenous administration of 0.1 mg cantharidin, 0.1 mg aconitine, 0.5-2 mg sparteine and up to 1.0 mg veratrine per 10 kg increases the volume of perfusion of these vital organs with almost complete regularity. These stimulants administered as model substances are thus capable of setting in motion the receptors in the organs as mediators of increased perfusion; this mechanism is also believed to be capable

of counteracting the further development of disease in these organs, regardless of whether of an inflammatory or a degenerative process. All dosages generally relate to 10 kg.

According to the present status, all metabolic processes in the cell in organic processes of a pathologic nature are known to be intensified. Thus we may assume an excess supply of nutritive perfusion and the volume of perfusion may be regarded as an important factor for the destiny and the course of pathologic processes in the organs. However, it is essential for such considerations to study organ perfusion as far as possible in an intact organism, and for this reason the damage-free registration of the perfusion volume according to Rein is of great value for us and also for therapeutic studies. Furthermore it is essential to imitate pathologic processes as far as possible, so as to be able to administer drugs that are at least roughly effective in diseases that occur.

The studies were made on large dogs under Chloralose anesthesia. Natural respiration was stressed. In the present study we investiaged disturbances of the liver and kidney, in particular the problem of the progression of diseases of these organs. The condition of these organs is easier to check than that of other organ systems, at least in animal experiments, since it can be tested on secretions from bile and urine. Renal perfusion was investigated by registration of the perfusion volume in the renal vein, and liver perfusion by registration of the volume of perfusion in the hepatic artery and the portal vein with Rein's flow meter. In each case the hemodynamic conditions were determined by

continuous registration of the atrial pressure in the right atrium, the blood pressure (glass plate manometer) and measurement of perfusion of the inferior vena cava. Inflammatory and degenerative damage was studied in both organs.

A. Diseases of the liver

1. Inflammatory liver damage.

Following Findlay's procedure, who made similar experiments on rats and guinea pigs with manganese chloride, we used this poison which gives rise to liver sclerosis starting out from the connective tissue surrounding the bile ducts.

The animals received 20 mg per 10 kg manganese chloride i.m. every two days. Two animals survived 4 weeks, 2, 8 weeks and 2, 16 weeks. By 10 weeks of manganese chloride treatment a kind of cholangitis-like "cirrhosis" could be obtained with incipient congestion of the portal vein.

Earlier experiments on animals that were merely anesthetized had shown that veratrine above all, a substance with a particularly strong inotropic effect, can greatly intensify perfusion, which may be explained by the effect on the vasodilating nerves. Among a large number of substances studied, urea, as a product of liver activity, was also found to result in an increased liver perfusion. Liver concentrates such as Pernemyl, Campotone, Hepartrate and Hepatopsone had a similar effect.

The application of these substances, which were of particular interest to us as models of the effect of stimulants and of active principles, caused a considerably greater increase of the arterial and portal liver perfusion than was the case in undamaged

animals. With doses of 0.5 mg veratrine, 1.0 g urea in 20 % solution and 0.5 cc Pernemyl forte i.v. the perfusion-intensifying effect in 10 experiments each was up to 40% stronger compared to undamaged animals, the doses once again related to 10 kg. Such elevating effects on perfusion were strongest in animals that had received manganese chloride for 4 weeks. The doses of manganese chloride administered in geometrically increasing doses up to 16 weeks revealed a gradual drop in the elevating effect on curves of perfusion increase. This behavior may represent an interference between sclerosis and sensitization of the vascular connective tissue system in inflammatory processes of the liver for stimulants and active principles, compared to the possibility of almost active excess perfusion.

Further experiments on another 6 dogs, 2 of which received manganese chloride for 4 weeks, 2 for 8 weeks and 2 for 16 weeks, dealt with the administration of substances that reduced liver perfusion. 20 γ adrenalin and 20 mg veritol were administered i.v. The obstruction of perfusion in the hepatic artery and portal vein was 28% less than in healthy anesthetized animals. The maximum weakneing effect occurred in animals treated with manganese chloride for 4 weeks, while a reduction of the weakening effect could be observed up to 16 weeks of manganese chloride treatment.

It was found in numerous other experiments that the arterial and portal liver perfusion could be reduced by cardiac glycosides. It has not yet been determined whether this vascular area, which also plays a role in cardiac decompensation, is by-passed, which seems reasonable, since the degradation of cardiac glycosides

takes place in part in the liver.

In order to reproduce the effect of inflammatory stimuli with special sensitization of the vascular systems, in 10 experiments on 4 dogs 50 γ g-strophanthin was administered after the administration of 0.5 mg veratrine, whose acute effect had ended 20 min previously. With this procedure, up to 20% increase in portal perfusion could be observed compared to the initial value. Stimuli with veratrine and presumably also inflammatory stimuli in the liver cause sensitization leading to excess perfusion with each new stimulus.

2. Degenerative liver damage

In order to induce degenerative damage in the liver parenchyma, 2 cc chloroform per 20 kg per day was administered i.v. to 8 dogs for 8 days. The study of the volume of perfusion of the hepatic artery and portal vein with the substances of primary interest, namely veratrine, urea and also liver extracts with an anti-anemia effect in the dosages cited, showed an increase that remained within the range of the effect on healthy animals. In particular after 4 mg Metasympatol i.v. there was increased perfusion both in the hepatic artery and the portal vein when preceded by experimental damage to the liver parenchyma. An important observation was that 10 g pyruvic acid or 2 g sodium lactate only increased the perfusion of the liver in healthy animals, while if there was degenerative liver damage there was a considerable increase both in the hepatic artery and the portal vein.

B. Kidney diseases.

1. Inflammatory kidney diseases.

In general it is possible to produce a kidney disease that is not unlike human nephritis according to the method of Masugi, and like Fahr and Sarre we too have often observed simultaneous glomerulonephrosis. Renal perfusion, measured on the perfusion volume of the renal vein, could be increased particularly well in untreated animals with 1 cc Padutin, 1 g per 10 kg dextrose and 2 mg adenosine triphosphate. In 10 dogs treated for 12 days with anti-dog renal toxin with the same dosages, acute glomerulonephritis was histologically established after this period. The anti-dog renal toxin was obtained from cats.

If the renal perfusion was measured in proportion to the inflammatory stimuli administered with anti-dog renal toxin, it was found, in confirmation of the data of Sarre, that the perfusion volume of the kidneys remained unaltered. Other experiments were carried out to produce additional stimulation with 0.2 mg veratrine i.m. Thereupon a considerable increase was produced in kidney perfusion. Together with a rise in atrial pressure, diuresis generally started up again with this procedure. It is therefore assumed that it was not an inflammatory stimulus in the usual sense that was produced by veratrine, but that predominantly vasodilating nerves were stimulated. In the presence of inflammatory processes in the kidney there is apparently a particular responsiveness of the vasodilating processes. Veratrine itself cannot be used therapeutically, at any rate not in the dosage cited; yet it can be assumed that the process of sensitization of vasodilating nerves can be particularly well

utilized in inflammatory kidney diseases. We are in the process of subjecting endogenous substances to particularly thorough investigations from this angle.

In this connection other experiments on 8 cats are of importance, in which the cats were rendered nephritic in the same manner as the dogs according to Masugi's method. In this case the anti-renal toxin was obtained from dogs. In view of the observation of a particular sensitivity of renal vessels in experimental nephrotoxic nephritis, the administration of general vasodilating substances was also of importance. Particularly distinct effects could be observed in the form of increased renal perfusion after the administration of 5 mg Eupaverin per 5 kg cat and 0.2 cc Padutin per 5 kg cat i.v. Adenosine (5 mg/5 kg), sodium lactate (0.2 g/5 kg) and 0.5 mg per 5 kg adenosine triphosphate had a distinctly weaker effect than the former substances in renal perfusion tests on cats with Masugi nephritis.

In 4 other cats with Masugi nephritis the effect of 10 γ/5 kg i.v adrenalin was tested. A distinct sensitization was observed against the vasoconstricting stimuli in Masugi nephritis. Thus all agents with a vascular effect must always be used taking the condition of the organ into account. This applies particularly to inflammatory diseases of the kidney.

Finally studies were made on 4 dogs with fresh cantharidin damage to the kidneys. In these experiments Otrivin, which otherwise has a strong vasoconstricting effect in doses of 5 mg/10 kg dog, gave rise to dilation of the renal vessels in 8 out of 10

experiments. For the problem of the progression of diseases of these organs this means that inflammatory stimuli have different valences and probably give rise to different processes; in particular the vascular reactions in inflammatory diseases induced in different ways often differ from one another; allergic Masugi nephritis sensitizes against the effect of phenylalcanolamines by constriction, damage caused by cantharidin tends more to be followed by an increased perfusion after administration of these substances. Such considerations play an important role in the testing of drugs in various forms of nephritis. Even a kidney whose perfusion has been obstructed by drugs can reveal increased perfusion in the form of an inverse reaction; when spasms of the renal vessel were induced with Pitressin (4 cats) in doses of 2 units per kg, resulting in a considerable drop in renal perfusion and diuresis, the administration of 10 γ adrenalin per kg cat led to an increase in renal perfusion over the initial value in 4 out of 10 experiments, and diuresis was also started up, even though the posterior lobe hormone has a prerenal diuresis-inhibiting effect.

The situation and direction of the vascular effects in general and in the kidney in particular is dependent not only on the pathologic state of the organ, but upon the tonicity of the vessels caused by certain pathogenic substances. This factor will become apparent in the progression of diseases of these organs, since the effects of organ-specific substances come up against a completely diversely tuned orchestra of vascular reactivity.

Furthermore it is not always possible to anticipate therapeutic measures on the basis of this situation. Instead, it is necessary to chose the medication on the basis of the state of the organ or vessel, bearing in mind the intensity of the disease.

Renal perfusion, like the perfusion of the coronary vessels, the hepatic artery and the portal vein, is relatively insensitive. However, inflammatory renal diseases are capable of eliciting certain reactions to vasopressor substances, which is in agreement with the studies of Enger on vasopressor substances from the kidney itself.

2. Degenerative renal damage

In exhaustive investigations on 16 dogs, acute degenerative damage was induced by i.v. administration of 10 cc 20% endogenous fresh liver extract per 10 kg. The studies on 30 dogs published earlier did not reveal any different vascular reactions from those of healthy animals, save that the effects are sometimes somewhat more distinct than in the unpretreated animals.

In the present experiment on 16 dogs the liver extract was administered 4 days before the experiment. Perfusion voumes were studied together with the registration of blood pressure, pressure in the right atrium and perfusion in the inferior vena cava.

Calculated for 10 kg dog, among active substances 1 mg adenosine triphosphate and 50 γ acetylcholine, among additional substances 1-5 g urea (20%) and liver extract, and among other

endogenous substances 1-5 g dextrose (20%) were found to be effective. The intensity of action, according to our earlier investigations, was 30% higher than the values of unpretreated animals.

The dogs with hepatogenic disturbances in diuresis showed signs of degenerative damage in the epithelia of the urinary tubules. Therapeutically the administration of urea only had a slight effect on renal perfusion. The administration of dextrose had a somewhat stronger perfusion-increasing effect.

The hepatogenic inhibition of diuresis, accompanied by morphologic degenerative changes in the kidney, could be penetrated by means of dextrose and urea; the effect of urea was sometimes more distinct than that of dextrose with the same dosages. This leads to the following conclusions: there is inhibition of diuresis in tubular renal disease as well. An effect on these is not necessarily linked to perfusion. However, renal disease of a degenerative nature heals the sooner, the earlier diuresis is set in motion. This could be demonstrated on 10 guinea pigs in which the same measures were taken in identical manner, with hepatogenic kidney damage induced by 1 cc 20% endogenous liver extract. One group of 5 animals received 0.5 g urea twice per day in 10% solution through a stomach tube, while the other group remained untreated. Of the untreated animals two died. The animals that received urea recovered rapidly, while the surviving untreated animals became cachectic, as will be demonstrated in another work on progression of diseases of these organs in dogs that had been treated with liver or kidney extract. In the

autopsy 10 days after the operation the animals that received urea rarely, and at the most, showed a drop-like disintegration of the cells, while the untreated animals revealed extremely severe vacuolar and severe fatty degeneration. These observations suggest that in diseases of the parenchyma the removal of degradation products also proceeds by virtue of secretion.

The stimulation of secretion of glandular parenchymas by physiologic means causes a more rapid healing and wherever possible prevents the transition to stages of cellular necrosis. These routes are not dependent upon perfusion; there is, of course, a relationship with this physiologic mechanism of equilibrium, for glucose with its more positive effect on renal perfusion had a stronger effect than urea in degenerative kidney damage in 2 out of 20 experiments when the damage produced was very pronounced and led to cellular necrosis. Of substances that play a role in intermediate metabolism pyruvic acid and lactic acid were tested. Both are capable of stimulating urinary secretion to a slight degree. However, pyruvic acid, as was demonstrated on 4 dogs, had a much stronger effect in animals with degenerative kidney damage than in normal animals. The dogs were examined under chloralose anethesia in acute experiments after the administration of 0.1 cc di-toxin i.v. In normal animals the effect of pyruvic acid on renal perfusion was slight, while the degenerative damage apparently caused sensitization to its effect. It is also important that after prior administration of pyruvic acid the effect of lactic

acid on renal perfusion is extremely slight both in normal animals and in animals with degenerative renal damage.

It would appear that in degenerative diseases of the kidney and liver, pyruvic acid and lactic acid improve perfusion and thus counteract the progression of the disease physiologically. There is no doubt that upon intensifying the metabolism of these organs following degenerative disease many intermediate products are not oxidized. This deficiency, however, leads to added nutritive blood being conducted to the diseased cell, possibly leading to processes of equilibrium.

In order to obtain some indication of the degree of sensitivity of the vascular reactions of the liver, kidney and heart, 4 dogs received 8 γ acetylcholine after acute i.v. pretreatment with 0.1 cc/10 kg di-toxin. While this dose of acetylcholine otherwise has almost no effect on blood pressure, in this case there was a very distinct drop. Thus the fact that the coronary perfusion remains the same, and the slight increase in the perfusion of the hepatic artery can be taken as a relative excess perfusion of these vascular areas. Renal perfusion was even more distinctly increased in comparison with these.

Thus renal perfusion may be regarded as particularly distinctly sensitizeable by pathologic phenomena. The liver and heart also suffer degenerative damage from di-toxin, but their vascular system does not become sensitized to the same degree.

The fact that otherwise subliminal doses of acetylcholine are effective in the presence of damage to these organs suggests that the humoral transfer substance of the vagus acts more

strongly under pathologic conditions than in the presence of normal organs. Di-toxin also alters the vascular reaction of acetylcholine in general, as shown by the effect on blood pressure of otherwise subliminal doses.

Summary and conclusions

Study of the influence of drugs on the perfusion of the damaged liver and kidney reveals the possibility of separating pathologic reactions in the vascular connective tissue system and the parenchyma cells.

Inflammatory processes reveal particularly strong sensitization to influences that are capable of increasing or reducing perfusion. Thus both in the liver and kidney a particularly strong general increase is observed in perfusion after i.v. administration of veratrine in the presence of inflammatory diseases; in the case of livers damaged with manganese chloride and kidneys damaged with anti-renal toxin this appears chiefly to stimulate vasodilator nerves. Such an effect of veratrine is made particularly apparent by the reversal of the otherwise more reducing effect of cardiac glycosides on liver perfusion. In animals with Masugi nephritis veratrine gives rise to such a striking increase in perfusion of the kidney that diuresis is started up again.
In general in inflammatory diseases of the liver and kidney the sensitization of the vasodilating nerves provides a possibility to counteract the pathologic process. This tendency to react to vasodilating substances with increased perfusion also applies to products of the organs, such as urea and protein-free liver extracts in the case of the liver and pyruvic acid in the case

of the kidney. Adenosine and adenosine triphosphate are also more effective in the Masugi kidney than the healthy kidney. It is important that the direction of the vascular reaction in inflammatory diseases of the kidney diverges from the norm as established so far in that in the Masugi kidney adrenalin has a particularly strong effect, while for instance Otrivin after cantharidin-induced damage may elicit the reverse effect. In general, inflammatory diseases require a selection of drug and dose based on the state of the organ and vessels; the intensity of the disease modifies the dosage.

Degenerative diseases of the liver and kidney also reveal a greater readiness to react to drugs that increase perfusion. This applies to the stimulant veratrine in the case of the liver and kidney, more particularly to the active substances urea and dextrose, and for the kidney, to pyruvic acid. The effect of urea in endogenous degenerative renal damage after the administration of endogenous liver extract is associated with more rapid healing and has some connection with perfusion. In the liver and kidney the maintenance of secretion in diseases of the epithelial cells is decisive for recovery of cellular activity. The more intensive the degenerative damage, the more the intactness of secretion depends upon increased perfusion; only glucose, which has a stronger perfusion-increasing effect, sometimes still has some diuretic effect once epithelial necrosis of the kidneys has begun.

Pyruvic acid, lactic acid and acetylcholine have a particularly strong perfusion-increasing effect in diseases of the

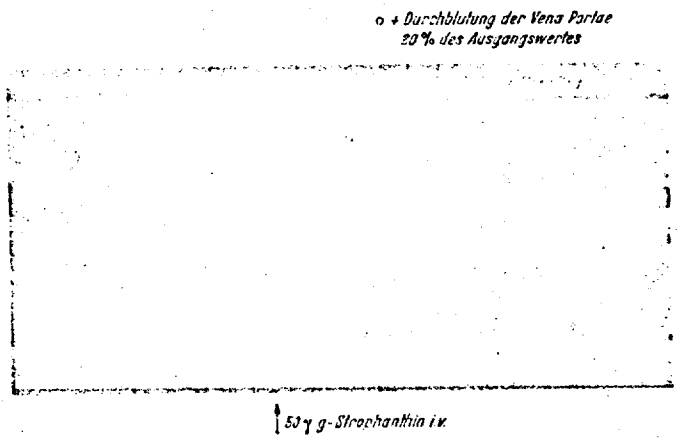
liver and kidney parenchyma. In the case of acetylcholine this may be a membrane effect; the excess production of pyruvic acid and lactic acid is due to increased metabolism; their vascular effect is capable of creating a state of equilibrium.

On the whole the vascular systems of the liver and kidney become more sensitive to drugs with a vasodilating effect, and also to those with a vasoconstricting effect in experimental inflammatory and degenerative alterations. Morphologically similar pictures are not necessarily indicative of the nature and direction of the inflammatory or degenerative reaction. Thus the Masugi kidney often reacts quite differently to Otrivin than the kidney following the administration of cantharidin. The degree and nature of damage to these organs modify the intensity of the vascular reaction to drugs as they do to endogenous substances; only in rare cases do inverse reactions also occur, in the liver after veratrine and added g-strophanthin in the form of increased perfusion, and in the cantharidin kidney after Otrivin also in the form of increased perfusion, in the Masugi kidney after adrenalin in the form of complete renal blockage and prolonged obstruction of perfusion, far beyond the usual degree. Sensitization of the vasodilating and perfusion-increasing processes nevertheless predominates in the earlier stages of inflammatory and degenerative liver and kidney diseases and is thus capable of counteracting the pathologic process. In inflammatory diseases, however, this favorable effect is limited to the first weeks; if sclerosis of the organ and interstitial connective tissue

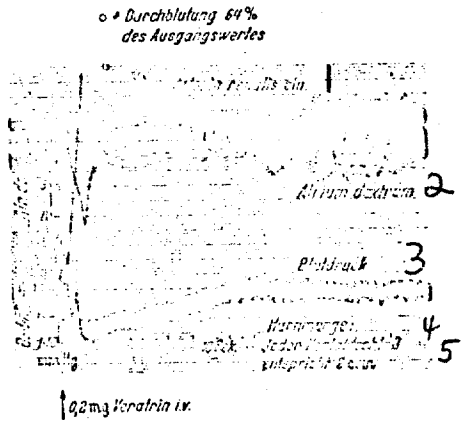
sclerosis exist, no live vessels compete with the disease any more, only a few cells travel with difficulty through the wasteland of the connective tissue scar. In degenerative liver and kidney diseases, in the absence of interstitial, collateral inflammatory processes, the phase of possible excess perfusion persists longer.

The study of the behavior of the vascular reactions is only a small part of the possibilities of influencing the progression of disease of these organs. It will remain for further vigorous research to pursue the problems of disturbances in protein metabolism and enzyme systems started by us. Nevertheless, perfusion, as the route to the organs, remains an important objective of therapy. In angina pectoris it is decisive and fateful, as was pointed out as early as 1923 by G. von Bergmann. In all inflammatory and degenerative diseases there are also chemical problems, and it may be said that our problem of the progression of disease, having reached one level, merely furnishes a view of other peaks to be scaled.

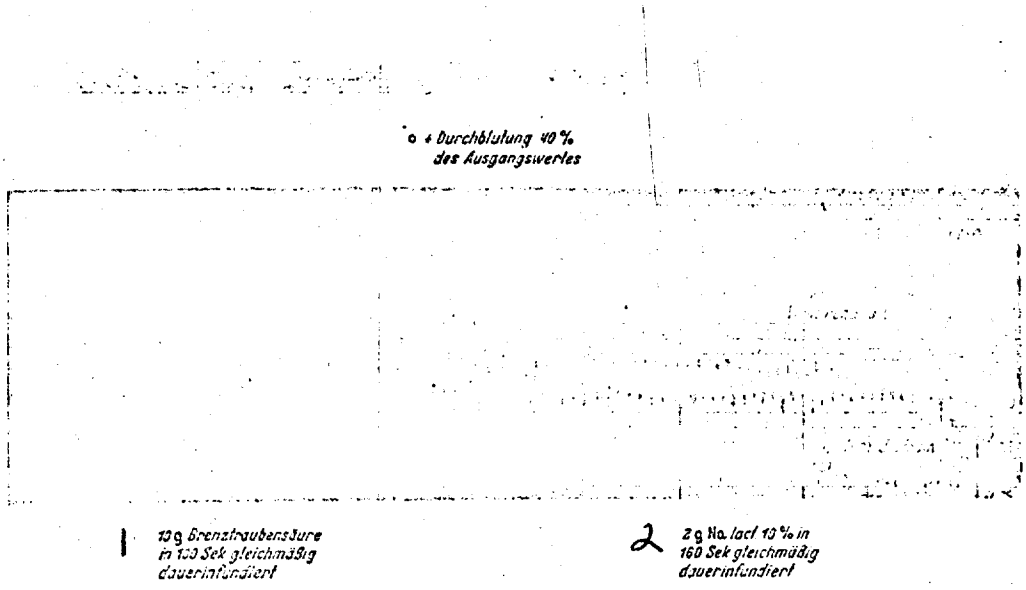
FIGURES



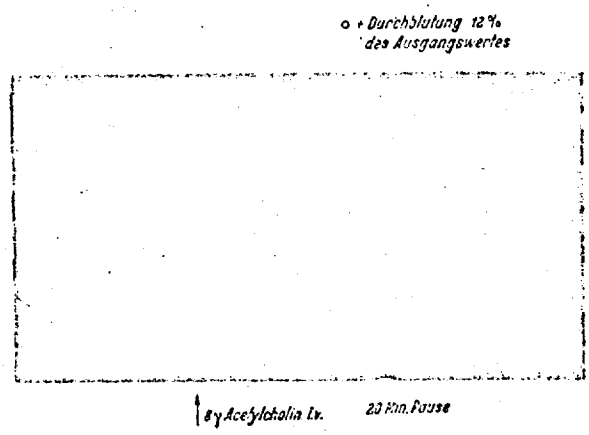
1. Effect of g-strophanthin on liver perfusion after sensitization with veratrine. 20 min previously, termination of the effect of i.v. administration of 0.5 mg veratrine. Key: o + perfusion of portal vein 20% of initial value.



2. Effect of 0.2 mg veratrine i.v. in a dog with Masugi nephritis. Key: o + perfusion 64% of the initial value. 1. Left renal artery. 2. right atrium. 3. Blood pressure. 4. Urine quantity. 5. Every contact impulse corresponds to 2cc.



3. Effect of pyruvic acid and sodium lactate on renal perfusion after damage with di-toxin. Key: o+ perfusion 40% of initial value. 1. 10 g pyruvic acid in 100 sec in an even continuous infusion. 2. 2 g Na lactate, 10% administered in 100 sec in even continuous infusion.



4. Comparison of perfusion of right coronary artery, hepatic artery and renal vein after acetylcholine in the di-toxin animal. Key: o+ perfusion 12% of initial value. 20 min pause.

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Arch. Exp. Path. Pharmakol., 203: 47-58, 1944

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Durchblutung der geschädigten Leber und Niere beim Fortschwelen von Organerkrankungen.

Von

Hansjürgen Oettel.

Mit 4 Textabbildungen.

(Eingegangen am 16. Oktober 1943.)

Bei der Untersuchung über die Entstehung endogener degenerativer Nierenerkrankungen ergab sich, daß die Nierenschäden durch körpereigene frische Leber- und Nierenextrakte an Hunden und Katzen oft für viele Monate bestehen bleiben. Hierbei sind nach bisherigen Befunden neben dem Eiweiß dieser Organe vornehmlich die Enzymsysteme entscheidend wirksam. Die Tatsache der Durchblutungssteigerung im Gebiete von Leber, Niere und Herz durch Stoffe, die bei der Tätigkeit des Organs eine Rolle spielen, scheint diesem Vorgang entgegen zu wirken. Untersucht man frische körpereigene Organextrakte auf die Durchblutung des Organs, dem sie entstammen, so sind Drosselungen der Blutzufuhr mit der Reinschen Stromuhr nicht festzustellen oder kommen nur sehr selten vor. Interessant ist es, daß auch beim unvorbehandelten Tier i. v. Gaben von 0,1 mg Cantharidin, 0,1 mg Aconitin, 0,5—2 mg Spartein und bis zu 1,0 mg Veratrin pro 10 kg die Durchblutungsgröße dieser lebenswichtigen Organe ebenfalls fast gesetzmäßig vermehren; diese als Modellsubstanzen verabfolgten Reizstoffe sind also in der Lage, die organansässigen Rezeptoren als Mittler einer günstigen Durchblutungssteigerung in Betrieb zu setzen; auch dieser Mechanismus sollte imstande sein, der Fortentwicklung von Organerkrankungen entgegenzuwirken, gleich, ob es sich um entzündliche oder degenerative Prozesse handelt. Die Dosen verstehen sich im allgemeinen für 10 kg.

Nach dem heutigen Stande sind die Stoffwechselvorgänge in der Zelle bei allen Organprozessen im Sinne der Krankheit als gesteigert bekannt. Wir dürfen somit einen Mehrbedarf an nutritiver Durchblutung annehmen und sind in der Lage, die Durchblutungsgröße als einen wesentlichen Faktor für das Schicksal und den Verlauf von krankhaften Erscheinungen an den Organen anzusehen. Für solche Betrachtungen ist es jedenfalls entscheidend, Organe in bezug auf ihre Durchblutung im möglichst intakten Organismus zu untersuchen, weshalb die schonende Registrierung der Durchblutungsgröße nach Rein für uns von entscheidendem Wert ist,

gerade auch für therapeutische Untersuchungen. Weiterhin ist es unerläßlich, krankhafte Organzustände möglichst nachzuahmen, um die Darreichung wirksamer Pharmaka wenigstens angenähert unter Berücksichtigung vorkommender Krankheiten vornehmen zu können.

Die Untersuchungen wurden an großen Hunden in Chloralosenarkose durchgeführt; auf natürliche Atmung wurde Wert gelegt. In der vorliegenden Arbeit sind Störungen an Leber und Niere betrachtet, mit denen sich unsere Untersuchungen zunächst für die Frage des Fortschwelens von Organerkrankungen besonders befassen; sie sind auf den jeweiligen Organzustand leichter kontrollierbar als andere Organsysteme, wenigstens was den Tierversuch anlangt, da dieser an den Abscheidungen in Galle und Harn zu testen ist. Die Prüfungen der Nierendurchblutung erfolgten durch Registrierung der Durchblutungsgröße vornehmlich der Vena renalis, die der Leberdurchblutung durch Registrierung der Durchflußmenge von Arteria hepatica und Vena Portae mit Hilfe der Reinschen Stromuhr. In jedem Falle wurde die Situation der hämodynamischen Verhältnisse durch laufende Schreibung von Vorhofdruck im rechten Vorhof, Blutdruck (Glasplattenmanometer) und die Messung der Durchblutung der Vena cava inferior definiert. — Untersucht wurden entzündliche und degenerative Schäden der beiden Organe.

A. Erkrankungen der Leber.

1. Entzündliche Leberschädigung.

In Anlehnung an Findlay, welcher entsprechende Versuche an Ratten und Meerschweinchen mit Manganochlorid durchführte, gelangte dies Gift zur Anwendung, welches Organsclerosen hervorruft, die vom gallenwegsumgebenden Bindegewebe ausgehen.

Die Tiere erhielten zweitägig 20 mg pro 10 kg Manganochlorid i. m. Zwei Tiere überlebten 4 Wochen, weitere zwei 8 Wochen, weitere zwei 16 Wochen. Bis zu einer Dauer von 10 Wochen der Behandlung mit Manganochlorid konnte eine Art von cholangitischer „Cirrhose" erzielt werden mit beginnender portaler Stauung.

Die früheren Versuche an lediglich narkotisierten Tieren hatten ergeben, daß vor allem Veratrin als besonders intensiv neurotrop wirkender Stoff auch die Leberdurchblutung stark erhöhen kann, wobei eine Erklärungsmöglichkeit in dem Effekt auf die erweiternden Gefäßnerven gegeben sein könnte. Weiter war von einer größeren Reihe untersuchter Körper als Produkt der Lebertätigkeit Harnstoff von einer Mehrung der Leberdurchblutung gefolgt befunden worden; in der gleichen Richtung wirkten Leberkonzentrate wie Pernämyl, Campolon, Hepartrat und Hepatopson.

Die Applikation dieser Körper, die uns als Modelle für die Wirkung von Reizstoffen, sowie von Tätigkeitsstoffen besonders interessierten, veranlaßte nun bei den entzündlich geschädigten Lebern wesentlich stärkere Steigerungen der arteriellen und portogenen Leberdurchblutung, als dies bei nichtgeschädigten Tieren der Fall war. Bei Dosen von 0,5 mg Veratrin, 1,0 g Harnstoff in 20 %iger Lösung und 0,5 ccm Pernämyl forte i. v. war die durchblutungssteigernde Wirkung in je zehn Versuchen um bis zu 40 % stärker gegenüber den Prüfungen an ungeschädigten Tieren, wobei die Dosen sich jeweils wie auch im folgenden jeweils auf 10 kg beziehen. Solche Überhöhungen des Durchblutungseffektes waren am stärksten bei den Tieren, welche 4 Wochen lang Manganochlorid erhalten hatten. Die

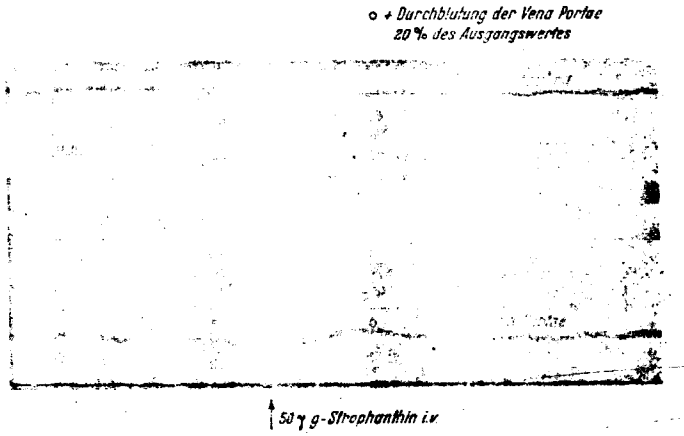

Abb. 1. Wirkung von g-Strophanthin auf die Leberdurchblutung nach Sensibilisierung mit Veratrin. 20 Minuten vorher Abklingen der Wirkung einer i. v. Gabe von 0,5 mg Veratrin.

in geometrisch steigenden Zeitdosen verabfolgten Darreichungen von Manganochlorid bis zu 16 Wochen ließen ein allmähliches Absinken des Überhöhungseffektes an Graden Durchblutungssteigerung erkennen. Dies Verhalten kann eine Interferenz zwischen Sclerose und Sensibilisierung der Gefäßbindegewebsapparate bei entzündlichen Organprozessen an der Leber für Reizstoffe, sowie für Tätigkeitsstoffe gegenüber der Möglichkeit fast aktiver Mehrdurchblutung darstellen.

Weitere Versuche an ebenfalls insgesamt sechs Hunden, von denen je zwei 4, weitere zwei 8 und endlich zwei 16 Wochen Manganochlorid erhalten hatten, befaßten sich mit der Darreichung von Körpern, welche die Durchblutung der Leber herabsetzen. Appliziert wurden je 20 γ Adrenalin und 20 mg Veritol i.v. Die Wirkungen im Sinne einer Durchblutungsdrosselung der Arteria hepatica und Vena Portae waren bis zu 28 % geringer als bei gesunden Tieren in Narkose. Das Maximum der Wirkungs-

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abschwächung lag ebenfalls bei den Tieren mit 4 Wochen Manganochloridbehandlung, bis zu 16 Wochen Manganochloridbehandlung war eine Minderung des Abschwächungseffektes auf die Durchblutungsdrosselung festzustellen.

Für die Herzglykoside haben wir in zahlreichen anderen Versuchen gefunden, daß sie arterielle und portogene Leberdurchblutung herabsetzen können. Es konnte noch nicht geklärt werden, ob dabei eine auch bei cardialer Dekompensation ins Gewicht fallende Umgehung dieses Gefäßgebietes vorliegt, wie sie zweckmäßig erscheinen könnte, da der Abbau der Herzglykoside zum Teil auch in der Leber stattfindet.

Um den Effekt entzündlicher Reize mit besonderer Sensibilisierung der Gefäßapparate zu reproduzieren, wurde in zehn Versuchen an vier Hunden die Applikation von 50 γ g-Strophanthin nach Applikation von 0,5 mg Veratrin vorgenommen, dessen akuter Effekt 20 Minuten zuvor abgeklungen war. Bei solchem Vorgehen konnten Steigerungen der Portadurchblutung bis zu 20 % der Ausgangswerte beobachtet werden. Reize mit Veratrin und vermutlich auch Entzündungsreize im Organ Leber sensibilisieren also für eine Mehrdurchblutung auf jeden erneuten Reiz hin.

2. Degenerative Leberschädigung.

Um degenerative Schäden am Leberparenchym hervorzurufen, wurden an acht Hunden 8 Tage lang täglich 2 ccm Chloroform pro 20 kg i. v. gegeben. Die nunmehr erfolgende Untersuchung der Durchblutungsmenge von Arteria hepatica und Vena portae ergab mit den hauptsächlich interessierenden Körpern Veratrin, Harnstoff und auch antianämisch wirksamen Leberextrakten in den genannten Dosen Steigerungen, die sich in den Bereichen der Wirkungen an gesunden Tieren hielt. Insbesondere war nach 4 mg Metasympatol i. v. eine Mehrung der Durchblutung sowohl von seiten der Arteria hepatica wie der Vena portae festzustellen, wenn vorher Schädigungen der Leberparenchymzellen experimentell durchgeführt worden waren. Wichtig ist die Feststellung, daß 10 g Brenztraubensäure und 2 g Na lact. an gesunden Tieren die Durchblutung der Leber nur wenig erhöhten, während bei degenerativen Leberschäden dies sowohl über Arteria hepatica wie Vena portae in hohem Grade der Fall ist.

B. Erkrankungen der Nieren.

1. Entzündliche Nierenschädigung.

Im allgemeinen läßt sich nach dem Vorgehen von Masugi eine der menschlichen Nephritis nicht unähnliche Affektion der Nieren hervorbringen, wobei wir freilich in Übereinstimmung mit Fahr und Sarre oft gleichzeitig Glomerulonephrose gesehen haben. Die Nierendurchblutung, gemessen an der Durchblutungsgröße der Vena renalis, war besonders gut

zu erhöhen bei unbehandelten Tieren durch Padutin 1 ccm, 4 g pro 10 kg Hund Dextrose und 2 mg Adenosintriphosphorsäure. An 10 Hunden, die mit Antihundenierentoxin in gleichen Dosen 12 Tage lang behandelt waren, wurde nach Ablauf dieser Zeit eine akute Glomerulitis histologisch gefunden. Das Antihundenierentoxin war von Katzen gewonnen worden.

Maßen wir nun auf die Darreichung von entzündlichen Reizen hin, die mit Antihundenierentoxin gesetzt wurden, die Nierendurchblutung, so blieb in Bestätigung der Daten von Sarre die Durchblutungsgröße der Nieren unverändert. Andere Versuche wurden so durchgeführt, daß zusätzliche Reizung mit 0,2 mg Veratrin i. v. bewerkstelligt wurde. Hierbei stellten sich erhebliche Mehrdurchblutungen der Nieren ein; entgegen den vorkommenden Anstiegen der Vorhofdrucke kam meist die Diurese bei solchem Vorgehen wieder in Gang. Wir möchten hiernach annehmen, daß nicht ein entzündlicher Reiz im üblichen Sinne durch Veratrin veranlaßt wird, sondern daß vornehmlich Gefäßnerven dilatorischer Art gereizt werden können. Gerade beim Vorliegen entzündlicher Vorgänge in der Niere ist nun eine Ansprechbarkeit der gefäßerweiternden Vorgänge offensichtlich in besonderem Maße vorhanden. Veratrin selbst ist nicht therapeutisch verwendbar, jedenfalls nicht in den genannten Dosen; doch ist anzunehmen, daß man sich den Vorgang der Sensibilisierung gerade gefäßdilatorisch wirksamer Nerven bei den entzündlichen Nierenkrankheiten sehr zunutze machen muß. Wir sind damit befaßt, in dieser Richtung besonders körpereigene Stoffe eingehenden Prüfungen zu unterziehen.

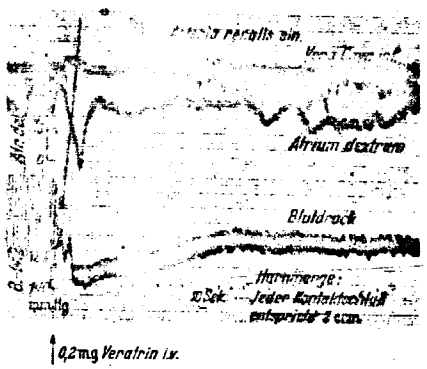

Abb. 2. Wirkung von 0,2 mg Veratrin i. v. bei einem Hund mit Masugi-Nephritis.

Hier sind weitere Versuche an 8 Katzen von Wichtigkeit, die in gleicher Weise wie die Hunde nach dem Vorgehen von Masugi nephritiskrank gemacht wurden. Bei ihnen wurde das Antinierentoxin von Hunden gewonnen. Nach den Feststellungen, die eine besondere Empfindlichkeit der Nierengefäße bei experimenteller nephrotoxischer Nephritis ergaben, war auch die Applikation von allgemein gefäßerweiternden Körpern von Wichtigkeit. Besonders deutliche Wirkungen konnten im Sinne einer Mehrdurchblutung der Nieren bisher von uns nach Applikation von 5 mg Eupaverin pro 5 kg Katze und 0,2 ccm Padutin pro 5 kg Katze i. v. gesehen werden. Adenosin (5 mg/5 kg), Natrium lacticum (0,2 g/5 kg) und 0,5 mg

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pro 5 kg Adenosintriphosphorsäure wirkten bei der Prüfung auf die Durchblutung der Nieren bei Katzen mit Masugi-Nephritis deutlich schwächer, als die erstgenannten Substanzen.

An weiteren 4 Katzen, ebenfalls mit Magusi-Nephritis, wurde die Wirkung von 10 γ/5 kg Adrenalin i. v. geprüft. Es ergab sich eine deutliche Sensibilisierung auch gegenüber diesen gefäßkontrahierenden Reizen bei der Magusi-Nephritis. Man wird demnach alle Mittel mit Gefäßwirksamkeit stets unter Berücksichtigung des Organzustandes verwenden müssen. Insbesondere gilt dies bei den entzündlichen Erkrankungen der Niere.

Hier sind schließlich Untersuchungen an 4 Hunden mit frischer Cantharidinschädigung der Nieren einzufügen. Bei diesen konnte das sonst stark gefäßkontrahierende Otrivin in Dosen von 5 mg/10 kg Hund in 8 von 10 Versuchen eine Gefäßerweiterung im Gebiet der Nieren hervorbringen. Dies ergibt für die Frage des Fortschwelens von Organerkrankungen, daß die entzündlichen Reize verschiedene Wertigkeit besitzen und wohl auch zu verschiedenen Verläufen Anlaß geben; insbesondere sind die Gefäßreaktionen bei verschieden ausgelösten entzündlichen Erkrankungen oft voneinander abweichend; die allergische Masugi-Nephritis sensibilisiert gegenüber der Wirkung von Phenylalkanolaminen im Sinne der Verengerung, die Schädigung nach Cantharidin ist eher von Mehrungen des Blutdurchflusses nach Applikation dieser Stoffe gefolgt. Solche Überlegungen spielen für die Erprobung von Pharmacis bei den verschiedenen Formen der Nephritis eine bedeutsame Rolle. Sogar die durch Pharmaka durchblutungsgedrosselte Niere kann durch Phenylalkanolamine eine Mehrung der Durchblutung im Sinne einer inversen Reaktion erfahren; verursacht man mit Pitressin (Versuche an 4 Katzen) in Dosen von 2 Einheiten pro kg Spasmen der Nierengefäße, wobei Durchblutung der Nieren und Diurese erheblich absinken, so läßt die Applikation von 10 γ Adrenalin pro kg Katze in 4 von 10 Versuchen die Nierendurchblutung bis über den Ausgangswert ansteigen, wobei auch die Diurese in Gang kommt, obwohl Hinterlappenhormon auch praerenal diuresehemmend wirkt.

Die Lage und Richtung der Gefäßwirkungen ist allgemein und ganz besonders an den Nieren nicht nur von dem Krankheitszustand des Organs, sondern von der Tonisierung der Gefäße durch bestimmte auch krankhafte Stoffe abhängig. Auch dies Moment wird sich bei dem Fortschwelen der Organerkrankungen bemerkbar machen, da die Wirkung vor allem organeigener Substanzen ein ganz wechselnd abgestimmtes Orchester auch der Gefäßreagibilität trifft; darüber hinaus werden therapeutische Maßnahmen auf Basis dieser Situation nicht immer vorhergesehen werden können; es ist vielmehr jeweils die Medikation nach der Art des Organ- und Gefäßzustandes unter Berücksichtigung der Intensität der Erkrankung erforderlich.

Zwar ist die Nierendurchblutung genau wie die Durchblutung der Kranzgefäße und der Arteria hepatica wie der Vena portae relativ unempfindlich. Jedoch sind entzündliche Nierenerkrankungen imstande, besondere Reaktionen gegenüber pressorischen Stoffen auszulösen, was mit den Untersuchungen von Enger über pressorische Stoffe aus der Niere selbst im Einklang steht.

2. Degenerative Nierenschädigung.

In eingehenden Untersuchungen an 16 Hunden wurden akute degenerative Schäden an den Nieren durch i. v. Gaben von 10 ccm 20 %igen körpereigenen frischen Leberextrakts pro 10 kg gesetzt. Die in einer anderen Arbeit, die früher entstand, an 30 Hunden dargelegten Untersuchungen lassen abweichende Gefäßreaktionen von gesunden Tieren nicht erkennen; nur sind die Wirkungen nicht selten etwas deutlicher als beim unvorbehandelten Tier.

In der vorliegenden Arbeit an 16 Hunden wurden die Leberextraktgaben 4 Tage vor dem Versuch appliziert. Die Untersuchungen der Durchblutungsgrößen der Nieren erfolgten unter Mitregistrierung von Blutdruck, Druck im rechten Vorhof und Durchblutung der Vena cava inferior.

Von Tätigkeitsstoffen waren, berechnet auf 10 kg Hund, 1 mg Adenosintriphosphorsäure und 50 γ Acetylcholin, von übergeordneten Substanzen 1—5 g Urea (20 %) und Gaben von Leberextrakten, von anderen körpereigenen Stoffen 1—5 g Dextrose (20 %) gut wirksam. Die Wirkungsintensität lag nach den früheren Untersuchungen 30 % über den Werten bei unvorbehandelten Tieren.

Die Hunde mit hepatogenen Diureseschäden boten degenerative Zeichen an den Harnkanälchenepithelien. Therapeutisch hatten die Darreichungen von Harnstoff nur geringe Wirkungen auf die Nierendurchblutung zur Folge. Die Gaben von Dextrose wirkten etwas stärker durchblutungssteigernd.

Die Durchbrechungen der hepatogenen Diuresehemmung, mit morphologisch degenerativen Veränderungen an den Nieren einhergehend, gelangen mit Dextrose und Harnstoff; der Effekt des Harnstoffs war manchmal deutlicher als der von Dextrose bei gleich hohen Dosierungen. Dies ermöglicht folgende Schlüsse: Auch die tubulären Nierenerkrankungen erfahren eine renale Diuresehemmung. Deren Beeinflussung ist nicht unbedingt gebunden an die Durchblutung. Die Organerkrankung degenerativer Art an den Nieren heilt aber um so eher, je früher die Diurese in Gang kommt. Dies konnte an 10 Meerschweinchen gezeigt werden, bei welchen die gleichen Maßnahmen im Sinne hepatogener Nierenschädigung mit 1 ccm 20 %igem körpereigenem Leberextrakt in identischer Weise durchgeführt wurden. Die eine Serie zu 5 Tieren erhielt täglich 0,5 g Harnstoff in 10 %iger Lösung durch Schlundsonde zweimal verabreicht,

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die andere blieb unbehandelt. Von den unbehandelten Tieren starben zwei; die Tiere, welche Harnstoff erhalten hatten, erholten sich rasch, während die unbehandelten überlebenden Tiere kachektisch wurden, genau wie wir dies für Hunde, die ebenfalls mit Leber- oder Nierenextraktgaben behandelt waren, in einer weiteren Arbeit über das Fortschwelen von Organerkrankungen darlegen werden. Bei der Obduktion 10 Tage nach der Operation zeigten die Tiere mit Harnstoffgaben nur selten, und zwar im Höchstfall, tropfige Entmischung der Zellen, dagegen die unbehandelten Tiere schwerste vakuoläre Degeneration und erhebliche degenerative Verfettung. Diese Feststellungen weisen darauf hin, daß bei den Erkrankungen der Organparenchyme der Abtransport der Abbaustoffe auch vermöge der Sekretion nach außen vordringlich ist.

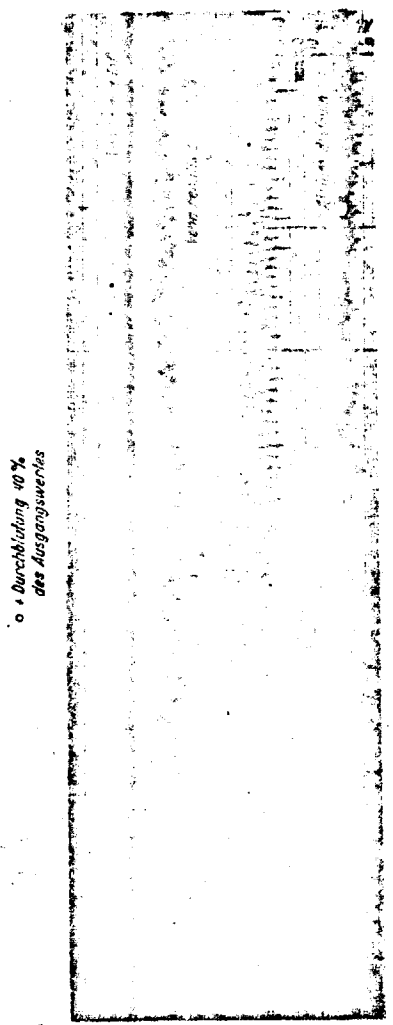

Abb. 3. Wirkung von Brenztraubensäure und Natrium lacticum auf die Nierendurchblutung nach Schädigung mit Di-Toxin.

Die Tatsache der Anregung der Sekretion von drüsigen Parenchymen durch physiologische Mittel veranlaßt eine raschere Ausheilung und verhindert womöglich Übergang in die Stadien der Zellnekrose. Diese Wege sind nun nicht in Abhängigkeit von der Durchblutung; freilich besteht eine Verwandtschaft zu diesem physiologischen Ausgleichsmechanismus, denn der Traubenzucker mit stärkerer positiver Wirkung auf die Nierendurchblutung wirkt beim degenerativen Nierenschaden in 2 von 20 Versuchen intensiver als der Harnstoff, wenn die veranlaßten Schäden sehr intensiv sind und zur Zellnekrose

überleiten. Von Körperklassen, die im intermediären Stoffwechsel eine Rolle spielen, wurden hier geprüft Brenztraubensäure und Milchsäure. Beide vermögen in geringem Maße die Harnsekretion anzuregen. Jedoch wirkt Brenztraubensäure, wie wir an 4 Hunden zeigen konnten, erheblich stärker bei degenerativen Nierenschäden als bei Normaltieren. Die Hunde wurden unter Chloralosenarkose im akuten Versuch nach Applikation von 0,1 ccm Di-Toxin i. v. untersucht. Bei Normaltieren ist die Wirkung von Brenztraubensäure auf die Nierendurchblutung gering, während die vorliegende degenerative Schädigung für deren Wirkung anscheinend sensibilisiert. Wichtig ist weiter, daß nach vorheriger Darreichung von Brenztraubensäure der Effekt von Milchsäure auf die Nierendurchblutung bei Normaltieren wie bei solchen mit degenerativen Nierenschäden recht gering ausfällt.

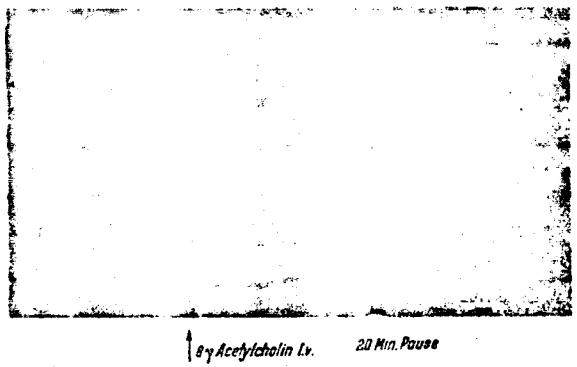

Abb. 4. Vergleich der Durchblutung von Arteria coronaria dextra, Arteria hepatica und Vena renalis nach Acetylcholin am Di-Toxin-Tier.

Nach allem hat es den Anschein, daß bei den degenerativen Erkrankungen der Niere wie auch der Leber Brenztraubensäure und Milchsäure die Durchblutung verbessern und somit in physiologischer Weise dem Fortgang der Störung entgegenwirken. Es ist sicher, daß bei den Steigerungen des Organstoffwechsels im Verfolg einer degenerativen Erkrankung besonders viel an intermediären Produkten nicht aufoxydiert wird. Dieses Manko führt aber der kranken Zelle ein Mehr an ernährendem Blut zu, so daß vielleicht Ausgleichsvorgänge sich anbahnen.

Um Anhaltspunkte über den Grad der Empfindlichkeit der Gefäßreaktionen von Leber, Niere und Herz zu gewinnen, wurden an 4 Hunden nach akuter i. v. Vorbehandlung mit 0,1 ccm/10 kg Di-Toxingaben von 8 γ Acetylcholin verabfolgt. Während diese Dosis Acetylcholin sonst fast blutdruckunwirksam ist, erfolgten hier recht deutliche Senkungen

Dementsprechend konnte das Gleichbleiben der Coronardurchblutung und die leichte Steigerung der Arteria hepatica-Durchblutung als relative Mehrdurchblutung dieser Gefäßgebiete angesprochen werden. Die Nierendurchblutung wurde im Vergleich hierzu noch deutlicher gesteigert.

Wir dürfen demgemäß die Nierendurchblutung als besonders deutlich sensibilisierbar durch krankhafte Ereignisse ansprechen. Auch Leber und Herz erfahren durch Di-Toxin degenerative Schäden, doch wird ihr Gefäßapparat nicht im gleichen Grade empfindlicher.

Daß sonst unterschwellige Gaben von Acetylcholin bei Organschäden wirksam sind, darf ein Hinweis darauf sein, daß der humorale Überträgerstoff des Vagus unter krankhaften Umständen intensiver wirkt als dies bei normalem Organverhalten stattfindet. Das Di-Gift ändert übrigens auch die Gefäßreaktion auf Acetylcholin im allgemeinen, wie aus dem Blutdruckeffekt auf sonst unterschwellige Dosen gefolgert werden muß.

Zusammenfassung und Besprechung.

Die Prüfung der Beeinflußbarkeit der Durchblutung der geschädigten Leber und Niere durch Pharmaka ergibt zunächst die Möglichkeit, krankhafte Reaktionen am Gefäßbindegewebsapparat und an den Parenchymzellen zu trennen.

Die entzündlichen Vorgänge lassen eine besonders starke Sensibilisierung gegenüber Einflüssen erkennen, welche die Durchblutung zu erhöhen, oder auch zu mindern imstande sind. So ist an der Leber wie an den Nieren beim Vorliegen von entzündlichen Affektionen eine besonders starke allgemeine Erhöhung des Blutdurchflusses nach i. v. Gaben von Veratrin festzustellen; dies scheint bei manganochloridgeschädigten Lebern und antinierentoxingeschädigten Nieren vornehmlich die Gefäßnerven dilatorischer Art zu reizen. Ein solcher Effekt des Veratrins wird aus der Umkehrung sonst eher mindernder Wirkung von Herzglykosiden auf die Leberdurchblutung besonders deutlich. Bei Tieren mit Masugi-Nephritis veranlaßt Veratrin so eklatante Steigerungen der Durchblutung dieses Organs, daß die Diurese wieder in Gang kommt. Ganz allgemein ist bei entzündlichen Erkrankungen von Leber und Niere die Sensibilisierung der gefäßerweiternden Nerven eine Möglichkeit, dem krankhaften Prozeß entgegenzuwirken. Auch gegenüber Produkten der Organe, wie Harnstoff und eiweißfreien Leberextrakten bei der Leber und Brenztraubensäure bei den Nieren, gilt diese Tendenz, auf gefäßerweiternde Stoffe mit Zunahme der Durchblutung zu reagieren. Adenosin und Adenosintriphosphorsäure sind bei der Masugi-Niere ebenfalls stärker wirksam als an gesunden Nieren. Wichtig ist hierbei, daß die Richtung der Gefäßreaktion bei den entzündlichen Erkrankungen der Niere von der Norm, wie bisher feststellbar, insofern abweicht, als an der Masugi-Niere Adrenalin

zwar besonders stark wirkt, Otrivin beispielsweise aber nach Cantharidinschädigung inverse Wirkungen hervorrufen kann. Die entzündlichen Erkrankungen erfordern allgemein eine Wahl des Medikamentes und der Dosis je nach Organ- und Gefäßzustand; die Intensität der Erkrankung modifiziert die Dosis.

Auch degenerative Affektionen von Leber und Niere lassen eine erhöhte Bereitschaft erkennen, auf durchblutungssteigernde Pharmaka zu reagieren. Dies gilt bei Leber und Niere für den Reizstoff Veratrin, besonders aber für die Tätigkeitssubstanzen Harnstoff, Dextrose und in besonderem Maße bei der Niere für Brenztraubensäure. Hierbei ist die Harnstoffwirkung bei endogenen degenerativen Nierenschäden nach Applikation von körpereigenem Leberextrakt mit rascherer Heilung vergesellschaftet und mit der Durchblutung im gewissen Zusammenhang stehend. Bei Leber und Niere sind die Aufrechterhaltungen der Sekretion im Falle der Erkrankung der Epithelzellen maßgebend für eine Erholung der Zelltätigkeit. Je intensiver die degenerativen Schäden sind, um so stärker ist die Intakthaltung der Sekretion abhängig von der Steigerung der Durchblutung: nur der stärker durchblutungserhöhende Traubenzucker wirkt manchmal noch diuretisch bei beginnender Epithelnekrose der Nieren.

Brenztraubensäure, Milchsäure sowie Acetylcholin wirken besonders durchblutungssteigernd bei Parenchymerkrankungen von Leber und Niere. Beim Acetylcholin mag dies ein Membraneffekt sein; Brenztraubensäure und Milchsäure verdanken ihre übermäßige Entstehung der Stoffwechselerhöhung; ihre Gefäßwirkung vermag einen Ausgleich zu schaffen.

Im ganzen werden die Gefäßapparate von Leber und Niere bei experimentellen entzündlichen und degenerativen Veränderungen gegenüber Pharmacis mit gefäßerweiternder Wirkung, aber auch gegenüber solchen mit gefäßverengernder Wirkung gegenüber empfindlicher. Morphologisch gleichartige Bilder müssen nicht immer für die Art und Richtung der entzündlichen oder degenerativen Reaktion maßgebend sein. So ist die Masugi-Niere im Verhalten auf Darreichungen von Otrivin z. B. oft recht verschieden von der Niere nach Applikation von Cantharidin. Grad und Art einer Organschädigung modifizieren die Intensität der Gefäßreaktion auf Pharmaca wie auf körpereigene Stoffe; nur in seltenen Fällen kommen auch inverse Reaktionen vor, an der Leber nach Veratrin und zusätzlich g-Strophanthin im Sinne der Steigerung der Durchblutung, an der Cantharidinniere nach Otrivin ebenfalls im Sinne der Steigerung, an der Masugi-Niere nach Adrenalin im Sinne völliger Nierenblockade und langwährender Durchblutungsdrosselung, die weit über das sonst übliche Maß hinausgeht. Die Sensibilisierung der gefäßerweiternden und durchblutungssteigernden Vorgänge überwiegt trotz

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allem in den früheren Stadien entzündlicher wie degenerativer Leber- und Nierenerkrankungen und ist in diesem Sinne imstande, dem krankhaften Prozeß entgegenzuwirken. Bei den entzündlichen Erkrankungen beschränkt sich jedoch dies günstige Verhalten auf die ersten Wochen; ist erst eine Organsclerose und eine interstitielle Bindegewebssclerose vorhanden, so wetteifern keine lebendigen Gefäße mehr mit der Krankheit, nur noch wenige Zellulationen durchstreifen mühsam das Ödland der bindegewebigen Narbe. Bei degenerativen Leber- und Nierenerkrankungen bleibt beim Fehlen interstitieller, kollateral entzündlicher Vorgänge die Phase der möglichen Mehrdurchblutung länger erhalten.

Die Betrachtung des Verhaltens der Gefäßreaktionen ist nur ein kleiner Teil bei der möglichen Beeinflussung des Fortschwelens von Organerkrankungen. Es wird lebendigste Forschung sein, die begonnenen Fragen auch der Störungen im Eiweißstoffwechsel und Enzymhaushalt für solche Fragen weiterzugehen wie wir begonnen haben. Immerhin ist die Durchblutung als Weg zu den Organen wichtiges Ziel der Therapie. Bei der Angina pectoris ist sie entscheidend und Schicksal, worauf bereits 1923 von G. von Bergmann hingewiesen wurde. Bei allen entzündlichen und degenerativen Erkrankungen kommen chemische Probleme hinzu, und es darf gesagt werden, daß unser Problem der Fortentwicklung von Organerkrankungen beim Gewinnen eines Ausblicks vorerst stets nur neue Gipfel zu betrachten gestattet.

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Role of liver in regulating distribution and excretion of manganese[1,2]

Amer. J. Physiol. 211: 211-216, 196

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PAPAVASILIOU, P. S., S. T. MILLER, AND G. C. COTZIAS. *Role of liver in regulating distribution and excretion of manganese.* Am. J. Physiol. 211(1): 211–216. 1966.—Rectal obstruction abolished the total body loss of Mn^{54} in both manganese-loaded and jaundiced rats. Bile duct obstruction only diminished the excretion of the radioisotope, proving that several gastrointestinal routes excrete manganese. Excretion was exceedingly fast if the isotope was injected intraportally, but then the effect of bile ligation became enhanced. Furthermore, biliary ligation induced a rise followed by a decline in the concentration of Mn^{54} in the liver, whereas that of the natural metal (Mn^{55}) increased. Ancillary experimentation linked these phenomena to absorption and rapid reexcretion of the metal. This enterohepatic circulation participated in regulating the excretion of manganese. The customary acceleration of excretion following moderate loads with manganese was abolished by blocking the bile duct. Acceleration could be induced in bile-ligated animals provided they were overloaded with metal. The sum of the data indicated that bile formation constitutes the main regulatory route under ordinary conditions, but, under conditions of overloading, auxiliary gastrointestinal routes participate.

Isotopes; neutron activation analysis; homeostatic regulation; bile

SELECTIVE EXCRETION by the kidney is a major mechanism by which the concentrations of many minerals are regulated in mammalian tissues, but significant excretion also occurs via the gastrointestinal tract. The latter route is especially important in the elimination of alkaline earth and metals of the periodic table's first transition group, of which one, the essential element manganese, appears to be almost totally excreted into the gastrointestinal lumen (8).

Mammalian liver and pancreas, both of which originate from the primordial gut (1, 19), have been reported to excrete manganese (6, 13). It is not known however, whether one or both of these organs regulate the concentration of this metal in mammalian tissues, and if so, whether the gut participates in this regulation.

Received for publication 8 October 1965.
[1] This work was supported by the U.S. Atomic Energy Commission and, in part, by National Institutes of Health Grant OH 00013.
[2] A preliminary report has been published (11).

The accompanying papers (5, 15) indicated that the turnover of manganese depends extensively on the dietary supply of this metal, whereas the tissue concentrations tend to remain stable primarily by virtue of excretion of excess metal. The present studies examine the effect of obstruction of the rectum and of the bile flow on the distribution and excretion of this nutrient. The findings suggest the existence of a rapid enterohepatic circulation which regulates the excretion of manganese as one among several gastrointestinal routes.

METHODS

Animals. Male, albino Sprague Dawley rats, weighing 200–250 g, were observed for 1–2 weeks prior to experimentation. They were housed in steel cages, some of which permitted separate collection of urine and feces. Purina laboratory chow (32.5 μg Mn^{55}/g) and tap water were offered ad lib., except that some groups were fed milk containing approximately 36 μg Mn^{55}/liter as determined by neutron activation analysis (10, 12, 18). Manganese sulfate was added as noted. The results on 325 of the animals studied are reported.

Operations. Laparotomies were performed under ether anesthesia. In some groups, the common bile duct was ligated with 5-0 silk at the juncture of the middle to the proximal third; in a few of these, double ligatures were applied also to the lower segment of the rectum. In another series, the liver was prevented from receiving Mn^{54++} by clamping the hepatic artery and the portal vein with a polyvinyl-tipped mosquito clamp for the 10 min which followed the intravenous injection of the isotope (2 and Fig. 4). The control animals of this series received the Mn^{54} after the release of the clamp, at which time the viscera had begun resuming their normal appearance.

All but two animals were decapitated by guillotine. Surgery seemed to affect the distribution of manganese and it was suspected that the agonal stages might do so as well. To vary the agonal stages one animal was killed by electrocution and one by oxygen deprivation.

Isotopes. The $Mn^{55}SO_4 \cdot H_2O$ (J. T. Baker Chemical Co.) was added to the milk and was given by stomach tube or injected intravenously in 0.1 ml 0.9% NaCl solution. In one experiment this salt was first incubated

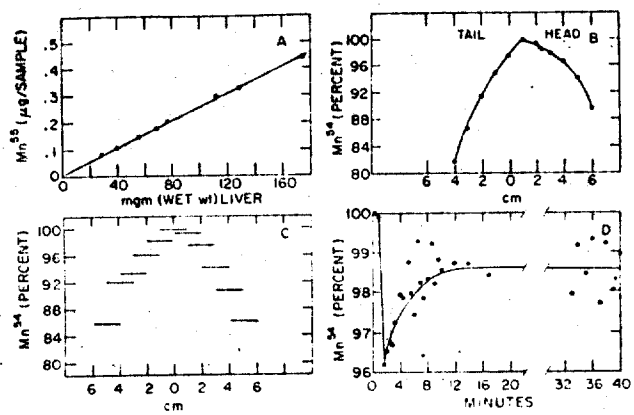

FIG. 1. *A:* determination of Mn^{55} in 8 samples of varying weight from the same rat liver. (Note that wet weights are reported in contrast to Figs. 5 and 6.) *B:* change of counting rate of tagged rat, when the midpoint of its trunk was moved parallel to the crystal. 100% Represents the counting rate when the midpoint was over the center. *C:* same as *B* but for the kidney of this animal. *D:* change in total-body counting rate with passing time in a rat injected with Mn^{54++}. The first point (100%) was determined at the completion of the injection of Mn^{54++}.

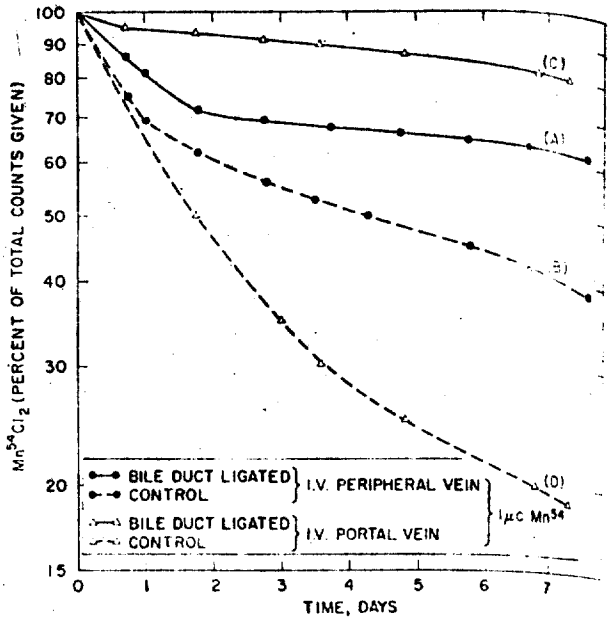

FIG. 2. Total-body retention curves of rats injected with Mn^{54} as indicated.

with rat serum aerobically at 20 C for 1 hr and the serum was injected intravenously.

The $Mn^{54}Cl_2$ ($T_{1/2}$=314 days) was the carrier-free isotope used in earlier work (5, 15). Standardized doses of 1.0–1.5 μc in 0.1 ml 0.9% NaCl were injected.

The operated animals were given the radioisotope either into the vena cava or into the portal vein; the intact animals were injected into a leg or a tail vein. Animals were excluded if the success of these steps was in doubt.

Neutron activation analysis. The methodology has been discussed earlier (10, 12, 18). The precautions in obtaining the samples and the reasons for not computing "specific activities" of Mn^{54} are indicated in the preceding paper (15). Figure 1, curve A, shows that the measurement of Mn^{55} was independent of sample size, the variance of which had been suspected to affect the metal's activation in the nuclear reactor.

Measurement of radioactivity. One-half hour or 2 hr after injection of the isotope each rat was placed in a Bollman cage (4) which held the midpoint of the animal's trunk fixed 6 cm above the center of a flat NaI-Tl crystal measuring 11 x 8.8 cm. Figure 1, curve B, shows the diminution in recorded radioactivity when the animal's midpoint was moved from the center of this crystal. The crystal was connected to the circuitry discussed elsewhere (9). The total-body radioactivity and the Mn^{54} of the tissues were counted in the manner stated in the second paper of this series (15). There was some dependence of the counts recorded on the position of a given organ in relation to the center of the crystal (Fig. 1, curve C). Hence this center was utilized as much as possible for the measurement of all radioactivities. Because the various organs approximated a point source better than did intact animals, the total-body radioactivity was again (5) invariably lower by 10–17% than the sum of its fractions recovered with isolated organs. By comparison to the position of animals or organs over the crystal, the changes in counting geometry which followed the distribution of the injected tracer in a given animal were minor indeed (Fig. 1, curve D).

Typical protocol. A rat was weighed, anesthetized, and operated on according to one of the procedures discussed above. The tracer was then injected intravenously and the animal was placed over the crystal 20 min later. The first count served as the 100% retention for the subsequent ones and the decline of the radioactivity was plotted semilogarithmically as a function of time. After a period of observation, the animal was decapitated and autopsied: the liver, kidneys, thoracic and abdominal viscera, and the remainder of the carcass were dissected and counted separately on the apparatus used for the intact animal. If the bile duct was ruptured, the animal was excluded from the study.

RESULTS

Abolition of the excretion of Mn^{54} by rectal ligation. Normally, Mn^{54} is excreted into the gastrointestinal tract but this remained to be shown in jaundiced and manganese-loaded animals. Groups of four rats with rectal obstruction were given Mn^{54} intravenously and manganous sulfate by stomach tube (3.2, 6.4, and 12.8 mg Mn^{++}/animal). The total-body radioactivity remained unchanged during the subsequent 5 days of observation. A similar almost total retention of the isotope was observed in four animals after both the common bile duct and the rectum were ligated. Analyses of the urine of these latter animals and analyses of both urine and feces from three animals with biliary ligation showed that the urine contained almost no measurable radioactivity, whereas the fecal Mn^{54} accounted for the total-body loss.

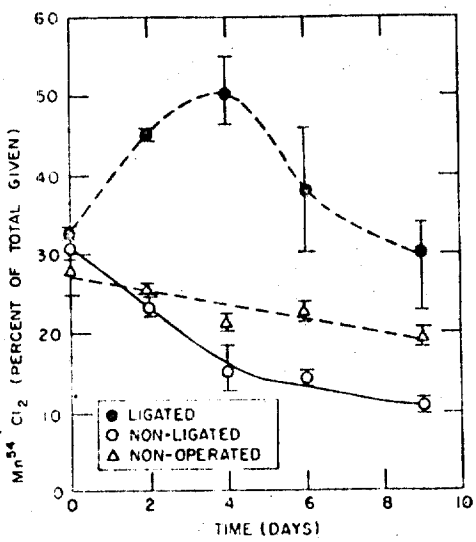

FIG. 3. Comparison of the retention curves of Mn^{54} in livers of animals indicated. Vertical lines denote the total envelope of the data.

Diminished excretion of Mn^{54} following biliary ligation. The previous experiments confirmed the gastrointestinal excretion of manganese. The contribution of the bile was studied thereafter by means of biliary ligation in animals receiving the isotope into either a peripheral or the portal vein. The total-body retention curves of the ensuing four groups of three animals are shown in Fig. 2. Biliary ligation diminished but did not abolish the excretion of this isotope whether or not it had been injected intravenously or intraportally. The two routes of injection reflected themselves in the relative rates by which this isotope became excreted: intraportal injection was followed by more rapid excretion or by more marked retention, depending on whether the bile duct was open or shut. These experiments indicated that, although the liver was only one of the excretory routes, it excreted its isotope very rapidly, possibly because it received significant amounts of metal from the gut.

The diminished excretion of Mn^{54} following biliary ligation was expected to produce a rise of the isotope's concentration in the liver. The unexpected findings and their explanation are dealt with below.

Fifteen animals with biliary obstruction were dissected at various intervals in parallel with 2 control groups of 15 laparotomized and 15 intact animals. Figure 3 shows that the radioactivity rose sharply and then declined in the livers of obstructed animals. This differentiated the obstructed group from its two control groups. Surprisingly, the livers of all operated animals differed from the nonoperated ones. The effects of surgery were not restricted to the liver, since the kidneys of these animals showed a significantly higher rate of loss than did the nonoperated controls. The other tissues studied did not show striking differences among these groups.

Several possible mechanisms could account for the striking rise and subsequent fall of the radioactivity in these livers. The first to be investigated was that the

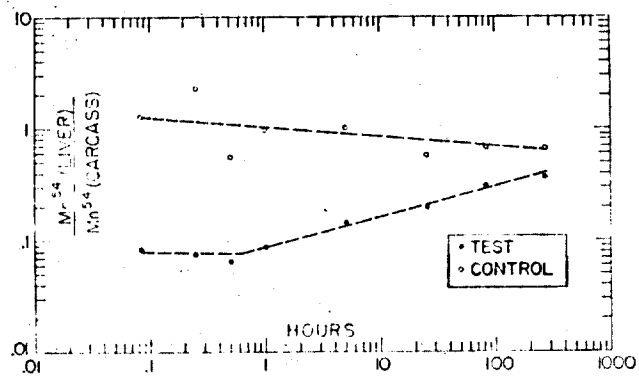

FIG. 4. Changes in the ratio of concentration of Mn^{54} in liver and carcass of rats as a function of time. A clamp had been applied to the hepatic vessels of all animals. Intravenous injection of Mn^{54++} was given to the test animals before, and to the control animals after the release of the clamp.

transport of Mn^{54} from the tissues to the liver might assume the form of a wave when the tracer's flow into the bile was blocked. Hence a different method of blocking was tried: A single intravenous injection of Mn^{54} was given to each of 34 rats either before or after the application of a clamp to the hepatic blood vessels for the 10 min during which 90% of the isotope is cleared (2 and Fig. 4). Five minutes later, the livers of the clamped animals contained 2–3.5% of the injected Mn^{54}, whereas those of the corresponding controls contained 32%. Eleven days later, the respective values were 18 and 24%. These results are summarized in Fig. 4 as liver-to-carcass ratios (14, 15). The total-body retention of Mn^{54} was measured in the animals sacrificed on the 5th and 11th days (4 groups of 3 animals each). The animals that had received the isotope with the clamp on showed identical total-body retention curves of Mn^{54}, as had those with biliary ligation (Fig. 2, curve C). Those that had received the isotope after the release of the clamp behaved identically to the sham-operated animals (Fig. 2, curve D). Evidently, some Mn^{54} was being excreted via extrahepatic routes even when the bile duct was patent; transport from the tissues to the liver accounted only for the rise shown in Fig. 2, but not for the fall.

A second possibility was that cellular damage (3) diminished the manganese uptake and thus induced the decline under investigation. This was tested in 18 bile-ligated, 13 sham-operated, and 6 control animals, all of which were given 1 μc Mn^{54} intravenously 2 hr prior to sacrifice. Sacrifice took place after the following time intervals: 2 hr (6 ligated, 6 sham operated, 6 controls); 1 day (6 ligated, 2 sham operated); 5 days (6 ligated, 5 sham operated). The percent uptake by the liver was computed for each animal. The data were paired on all permutations and subjected to statistical analysis (t test). No significant differences were found between groups: the ligated group as a whole showed liver uptakes with a mean and standard deviation of 26.5 ± 8 while the corresponding numbers for the other two groups were 29.1 ± 7 and 21.4 ± 3, respectively.

Now it became probable that manganese absorbed from the diet accumulated in the obstructed liver and

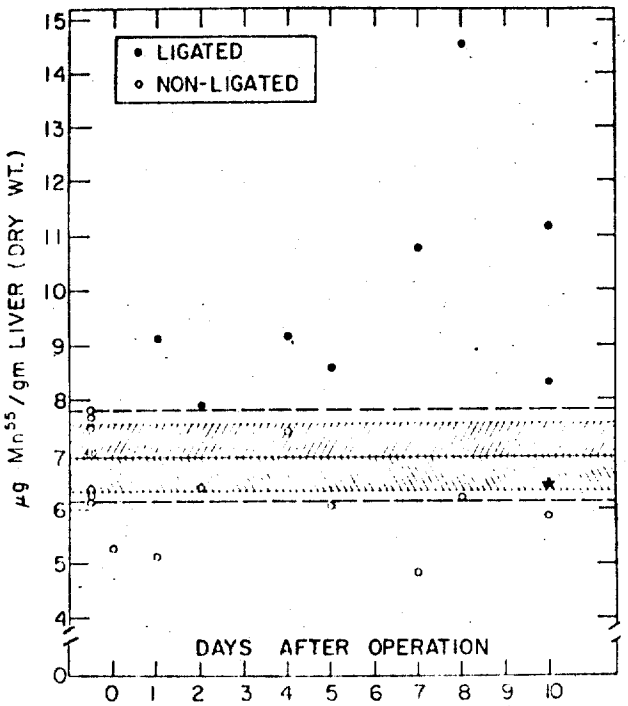

FIG. 5. Changes in concentration of Mn^{55} in livers of rats. Data which precede zero time represent intact animals, the remainder laparotomized ones. Among the horizontal lines, dashed ones indicate the total envelope, solid ones the mean and 1 SD each, on the data obtained on the nonoperated animals. Asterisk represents one rat in which no evidence of obstruction was elicited in spite of ligation of the bile duct.

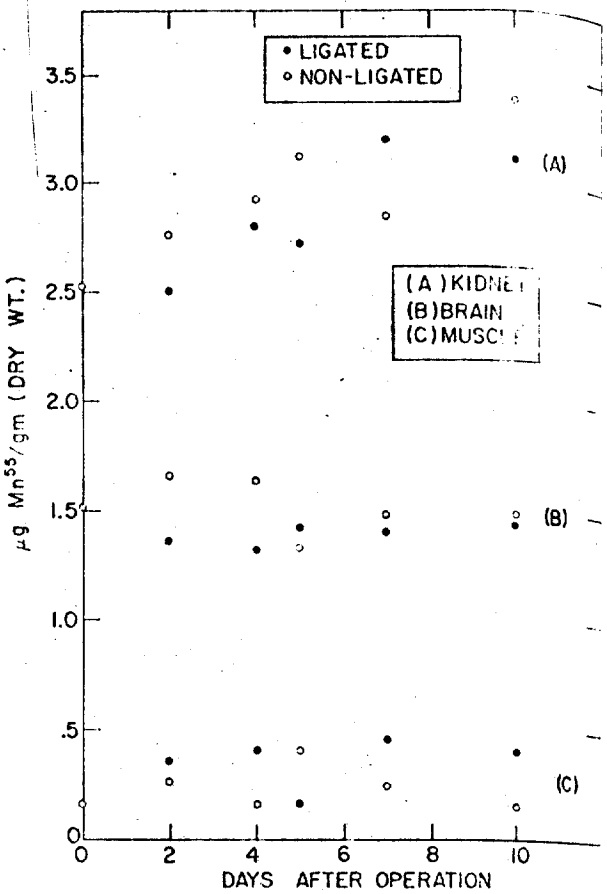

FIG. 6. Changes in the concentration of Mn^{55} in selected tissues of laparotomized animals.

eventually displaced the radioisotope. This could be shown only by quantifying the stable isotope Mn^{55} present in foods and not by injecting an artificial radioisotope. Figure 5 shows the concentration of manganese in eight livers from intact and laparotomized animals, with and without biliary obstruction. The expected rise of this element's concentration did occur following biliary obstruction but it is less certain whether this rise was a temporary one. In addition, some of the nonobstructed animals showed a diminution of the Mn^{55} concentration in the liver. These findings agreed with the results obtained with Mn^{54} (Fig. 3) in that biliary ligation and surgery had again affected the metabolism of this metal in opposite directions.

The concentrations of Mn^{55} in tissues other than the liver are shown in Fig. 6. Effects of biliary obstruction reflected themselves only in muscle, with a significant elevation of Mn^{55}. On the other hand, surgery resulted in progressively increasing concentration of Mn^{55} in the kidneys from both operated groups. Two similar groups had shown a rapid initial loss of Mn^{54} from the kidneys by comparison to intact animals.

This increased concentration proximally to the obstruction of one among several excretory routes did not indicate whether the obstructed pathway simply permitted the flow or whether it also regulated the excretion rate of the metal. This was investigated by varying the excretion rate while the bile duct was either open or shut.

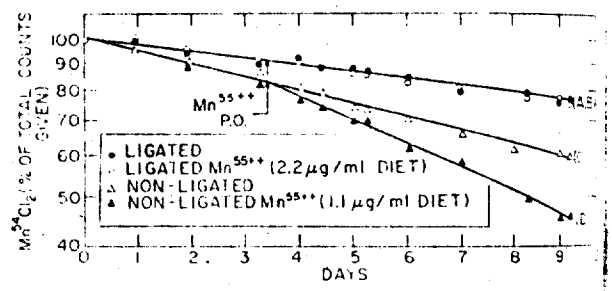

FIG. 7. Total-body retention curves of injected Mn^{54} laparotomized rats with (*A,B*) and without (*C,D*) biliary ligat At the arrow *groups B* and *D* received the indicated amounts Mn^{55++} in the milk.

Regulation of the excretion rate of manganese. Sixteen ra eating milk for 1 week were laparotomized, injected w tracer, and the bile duct was ligated in half of them. T ligated group showed again a distinctly slower rate loss of Mn^{54} from their bodies (Fig. 7). On the 3rd of observation 2.2 μg stable Mn^{55++} were added milliliter of milk in four of the obstructed anim whereas four of the sham-operated rats were offere diet to which only 1.1 μg/ml had been added. The r men of the remaining animals was not changed. Des a larger dietary $MnSO_4$ supplement, the bile d

TABLE 1. *Distribution of injected dose of Mn^{54} at end experiment summarized in Fig. 7, as percent of initial dose given*

	Liver	Viscera	Carcass
Biliary obstruction (milk)	45 (44–48)	21 (20–22)	22 (19–24)
Biliary obstruction (milk + $MnSO_4$)	27 (26–27)	42 (41–43)	23 (19–26)
No biliary obstruction (milk)	27 (24–29)	19 (18–20)	23 (21–24)
No biliary obstruction (milk + $MnSO_4$)	9 (6–14)	20 (17–25)	23 (21–25)

Values are means, with ranges in parentheses. Under viscera here is listed the sum of the determinations of the radioactivity of the gastrointestinal tract, kidneys, spleen, pancreas, testes, adrenals, and thoracic organs for each animal.

TABLE 2. *Percents of total-body Mn^{54} remaining 5 days after injection as a function of single oral doses of manganese carrier*

Mn^{55} Given, μg	No. of Animals	Mean	Range	No. of Animals	Mean	Range
	Ligated			*Nonligated*		
Controls	11	79.5	79.0–80.5	9	75.5	75.0–76.0
5×10	3	78.0	75.2–80.5	3	57.0	51.2–63.2
1×10^2	2	82.5	81.5–83.5	3	56.0	49.5–63.2
2×10^2	3	76.0	66.4–85.0	3	52.7	47.2–58.6
4×10^2	3	86.0	78.6	3	59.8	52.0–67.5
8×10^2	3	66.8	62.0–72.0	3	40.5	26.2–56.0
1.6×10^3	6	54.2	46.0–63.0	6	20.8	19.5–22.1
3.2×10^3	1	46.0		3	21.5	17.5–25.5
6.4×10^3	6	46.5	38.5–55.0	7	21.8	11.5–32.5
12.8×10^3	5	32.5	28.2–37.0	6	16.5	9.2–24.0

obstructed animals showed no discernible increase in the rate of total-body loss of Mn^{54}, whereas a marked enhancement of the excretion was exhibited by the controls (Fig. 7).

This striking retention could have been a reflection of diminished dietary (manganese) intake rather than of excretory consequences of biliary ligation. Therefore evidence for isotopic exchange was sought in the distribution of the radioisotope among the organs of these rats (Table 1). Again (2, 5), the carcasses of all these animals showed similar retention of this isotope regardless of jaundice or dietary manganese. However, the percent of the Mn^{54} administered was smaller in the liver, after the manganous supplement was given. Biliary obstruction again induced a relative increase of the radioactivity remaining in the liver, regardless of the manganous supplement. The pooled viscera showed the same percent retention of the administered Mn^{54} in all groups, except for the one that had both biliary obstruction and the manganous supplement: here the viscera (including the gastrointestinal tract) contained the balance of the radioactivity lost by the livers.

These findings ruled out diminished food intake as the only reason for the insensitivity of the Mn^{54} turnover to manganese loading. Still, this insensitivity could have been feigned by diminished mobility of the intestines of these jaundiced rats. If this were the case, the ligated animals should remain insensitive to even larger amounts

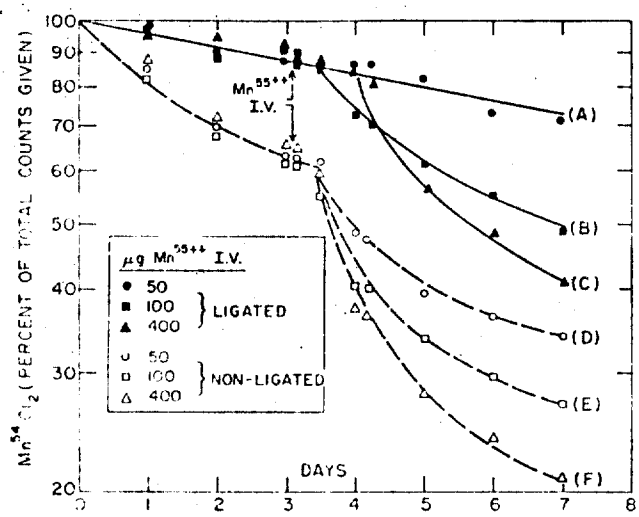

FIG. 8. Total-body retention curves of injected Mn^{54++} in laparotomized rats with (*A,B,G*) and without (*D,E,F*) biliary obstruction. At the arrow all animals were injected intravenously with the indicated amounts of Mn^{55++}.

of manganese as well as to parenterally injected carrier. Administration of graded doses of metal, orally and parenterally, were preferable to fractionation of the Mn^{54} between feces and intestinal wall, since washings extracted significant amounts of radioactivity.

The amount of orally administered carrier was varied as follows: Rats maintained on milk for 1 week were divided into sham-operated and ligated groups. These were injected iv with calibrated doses of Mn^{54} and were further divided into subgroups of three. Each was given by stomach tube 1 ml of water or graded amounts of $MnSO_4$ solution and were sacrificed after recording their total-body radioactivities for 5 days. These radioactivities are shown as percents of the six animals killed 2 hr after tagging, in Table 2. Biliary ligation resulted in slower excretion of Mn^{54} regardless of how much natural manganese was given.

The above experiments would have proved that the bile flow can be bypassed under conditions of heavy manganese loading, if the following were true: that the isotope lost by these animals had been actually excreted, rather than exchanged across their intestinal mucosas. Hence, in the experiments shown in Fig. 8, the manganous salt was injected intravenously to eliminate isotopic exchange across the gut.

The rate of loss of Mn^{54} by the animals with biliary ligation was similar to the corresponding ones on the milk diet (Fig. 7), whereas the sham-operated controls showed a faster rate of loss. The obstructed ones showed accelerated excretion of Mn^{54} only with doses higher than 50 μg Mn^{55++}/animal, whereas the controls responded to all doses given. When 200 μg Mn^{55++} were given intravenously with rat serum or saline as the vehicle, the responses obtained were less pronounced than those found in the corresponding animals receiving 400 μg Mn^{55++} (Fig. 8) but the vehicle induced no differences.

DISCUSSION

The first demonstration to emerge of this work was that the intestine and some of its tributaries constitute a system of multiple excretory routes for manganese. This system contrasted to the kidney which excreted negligible amounts of metal even during jaundice and after loading with manganese. The routes which did excrete this metal have as their common origin the primordial gut. This embryological consideration evoked interest in the relative effectiveness of these routes, as discussed in a companion paper (2).

Additional findings pertained to several effects of surgery. These were generally opposite in direction to those induced by biliary obstruction. Hence, in the present experiments, the normal contribution of the liver to the excretion of manganese had been underrated because of the surgery employed. While attempting to explain these manifestations of stress, one might note the effects of administering cortisol or ACTH (15), since all were in the same direction.

The biphasic change of the concentration of Mn^{54} in obstructed livers became important after it led us to show the central role of the liver in the distribution of this metal: the initial rise indicated movement of manganese from the tissues to the liver, which was confirmed by excluding the liver from receiving initially the injected radioisotope. Transport in the opposite direction was shown by the evidence summarized in Table 1. In addition, some of the absorbed metal must be normally transported promptly into the bile, in view of the decline of radioactivity and increased manganese concentration which followed biliary obstruction (Figs. 3 and 5).

Several intriguing observations pertained to the regulation of excretion: rectal obstruction abolished the excretion, whereas biliary obstruction impaired [illegible]arily the sensitivity with which excretion was norm[illegible] regulated. Furthermore, preventing the liver from [illegible]ving the initial bulk of tracer did not prevent the an[illegible] from excreting it, although their livers were acc[illegible]ulating instead of losing radioactivity. Consequently, although these results proved the existence of more th[illegible] one excretory route (6, 13), they raised valid questi[illegible] regarding the identity of the tissues drained by each.

The sum of these and some earlier findi[illegible] indicates that the absorbed manganese reaches the liver, becomes localized in its mitochondria (14, 16, 17, 20), and, although some metal becomes distributed to the tissues, a significant fraction is discharged into the bile. It remains to be seen to what extent this circulation is completed by reabsorption, whether its pump is located in the mitochondria and, if so, of which tissues. The latter is worth ascertaining since the liver was shown by these investigations to be instrumental in the homeostasis of this metal.

The authors thank Mr. Alexander R. Nicholson for securing data on distribution and turnover and for their statistical analysis, and Miss Judith Edwards for analysis of some of the tissues.

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J Neur Path & Exp't Neurol 22: 488-489, 1963

EXPERIMENTAL MANGANESE ENCEPHALOPATHY IN MONKEYS

A Preliminary Report*†

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Since 1837, when Couper (1) described manganese encephalopathy in 5 laborers who inhaled great quantities of powder containing manganese dioxide, manganese has been of great interest to the neurologist because it affects the extrapyramidal system selectively. Despite numerous investigations of the past, the manner in which this metal injures the central nervous system still remains a mystery. Attempts to reproduce manganese encephalopathy in animals (Mella (2), van Bogaert and Dallemagne (3), and others) were not successful. In addition, improved protection in manganese mills decreased the number of human cases and interest in research on the problem. The latter is regrettable since the elucidation of the neuropathology and pathogenesis of manganese encephalopathy, which clinically differs from paralysis agitans as well as from Wilson's disease (for more clinical details see Pentschew (4)), would undoubtedly contribute to a better understanding of the diseases related to the extrapyramidal motor system.

METHODS AND MATERIAL

Unlike van Bogaert and Dallemagne, who used the respiratory pathway for the introduction of insoluble manganese dioxide, we injected olive oil suspensions of this manganese compound into the muscles. In order to obtain a larger surface for absorption, the dose was injected in at least 5 different sites. Since previous experiments in cats had shown that intramuscular deposits of manganese dioxide tended to abscess with autoclaved suspensions, the monkeys were also given antibiotics.

Five mature rhesus monkeys were used. In the course of 9 to 24 months after the beginning of the experiment all 5 monkeys developed similar unusual neurological symptoms. In this paper we shall report primarily on the one monkey (AFIP Acc. 1010594) that was sacrificed; the other 4 were still under observation at the time of reporting‡.

This monkey was given 2000 mg. of MnO_2 on 6 May 1960, followed by 3500 mg. on 6 July

* This investigation was supported by Research Grant NB-02275, from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland.

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** Dedicated in friendship and admiration to Julius Hallervorden on the occasion of his eightieth birthday.

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‡ One of these animals has since been sacrificed (24 months after exposure).

1960. Each dose was injected in 8 different sites. The animal was sacrificed by sodium pentobarbital on 21 July 1961, 14½ months after the first injection. A complete post mortem examination was performed, and sample sections of the viscera and the entire brain and spinal cord were fixed in 10 per cent buffered formalin. No gross changes in any of the viscera or the central nervous system were noted. Blocks from coronal sections of the brain were cut and stained with Nissl (cresyl echt violet), Klüver-Barrera, Holzer, PAS, Gomori's method for iron, hematoxylin and eosin, and van Gieson preparations. In addition, frozen sections were prepared for Cajal's gold sublimate and Hortega's silver carbonate methods.

CLINICAL SIGNS

The first symptoms of intoxication were noticed in February, 1961, about 9 months after the first injection. The animal became very excitable, especially when confronted or approached by people. It jumped, hitting its head against the top of the cage. At rest, the right arm and left leg were held in flexion; in motion this was less apparent. During the following 5½ months the symptoms steadily became more intense. In jumping, the animal often fell on its heels or on its buttocks instead of on its feet. In addition, there was general clumsiness. In moving about there was a resemblance to a moderate inebriation. No cog-wheel phenomenon, tremor or involuntary movements were observed. The general condition of the animal otherwise was good, despite the continuous state of excitability. Appetite was good and there was no loss of weight.

PATHOLOGIC FINDINGS*

1. Symmetrical Selective Lesions in the Grisea: The *subthalamic nucleus* was one of the most severely damaged of all the involved grisea. A very large number of densely packed glial cells rendered this nucleus conspicuous even at low magnification (fig. 1). At least one half of the glial cells consisted of bizarre, delicate, transparent, naked nuclei, containing 3 to 8 very small chromatin particles connected by tiny bridges. These nuclei were usually oval, but sometimes lobulated, and were 2 to 3 times larger than the dark hyperchromatic nuclei representing the other half of the glial population (figs. 2 and 3). We tentatively designated these bizarre glia as "immature" glial cells†, meaning thereby "immature" astrocytes and eventually oligodendrocytes. Interestingly enough, the same glial pattern extended to the substantia innominata, including the nucleus basalis of Meynert. Holzer preparations revealed a significant astrogliosis of the subthalamic nucleus (fig. 4), also extending to the comb system, *i.e.*, the connections of the pallidum with the subthalamic nucleus and the thalamus. It was particularly interesting that the Holzer positive glial fibers did not appear to be connected with glial cells. In fact, some of the "immature" glia cells seemed to have only a limited ability to form glial fibers. Klüver-Barrera slides revealed pronounced demyelination (fig. 5) also extending to the comb system. Another important finding was the disappearance of nerve cells; the few remaining were atrophic or deformed.

The medial segment of the *pallidum* (*medial pallidum*) was as severely affected as the subthalamic nucleus (fig. 6). The numerous "immature" glial cells in this nucleus were larger than in the subthalamic nucleus and displayed a greater variety of forms (fig. 7). The Holzer method revealed significant astrogliosis (fig. 4), mostly due to glial fibers not connected to glial cells. Some of the "immature" glial cells showed ability to produce scanty fibers, however (fig. 8). The neuronal loss was almost complete. The Klüver-Barrera slides demonstrated pronounced demyelination inclusive of ansa lenticularis.

Conspicuously different from the changes in the medial pallidum were those in the

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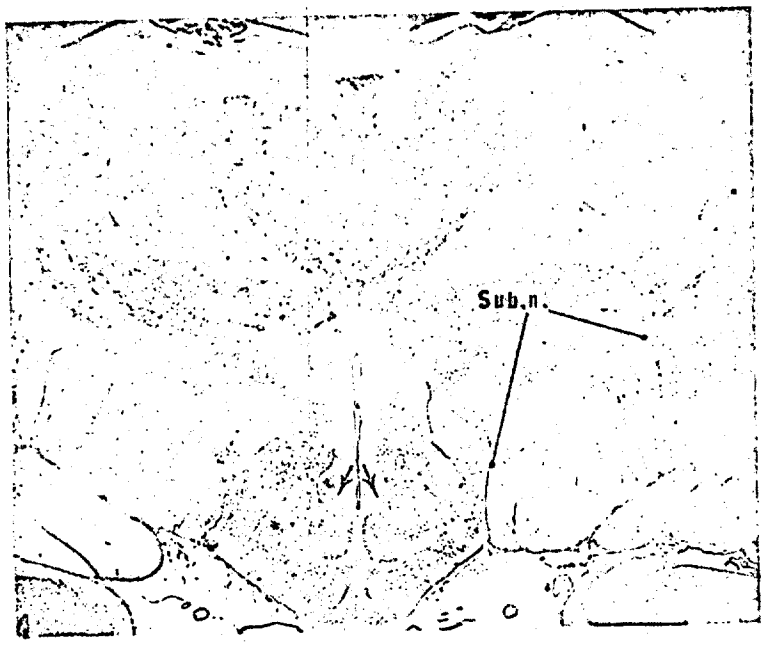
* A control monkey of the same age was sacrificed at the same time and processed in a manner identical to the experimental animal. All changes noted have been carefully compared to corresponding sections in this control.

† The subject of the production of "immature glia" as a result of intoxication will be discussed in detail in a later publication.



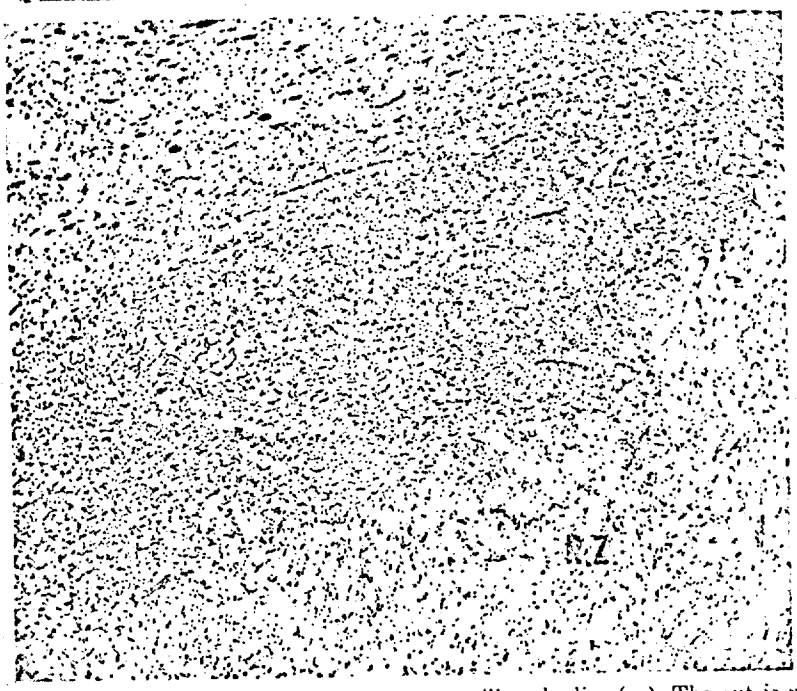
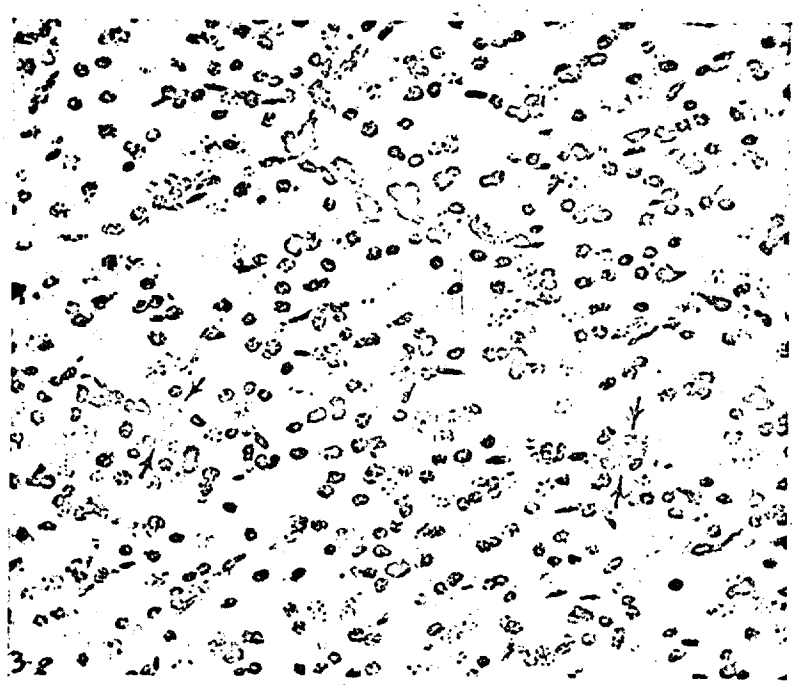


FIG. 1. Coronal sections at the level of the mammillary bodies (→). The cut is not quite symmetrical. Subthalamic nucleus (sub.n.) very conspicuous even at low magnification because of the tremendous cellularity. Nissl stain; × 6½. Neg. 61-6597.

FIG. 2. Tremendous increase of the glia population with nearly complete loss of neurons of subthalamic nucleus. The bordering red zone of substantia nigra (r.z.) shows only slight glial proliferation. Zona incerta (z.i.) normal. Nissl stain; × 50. Neg. 61-6303.



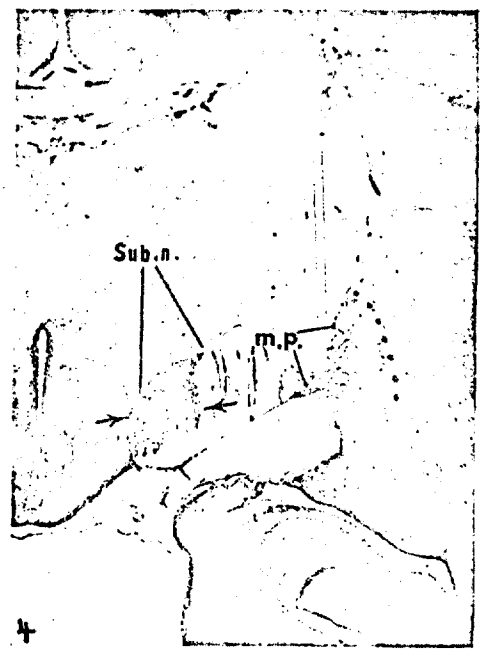
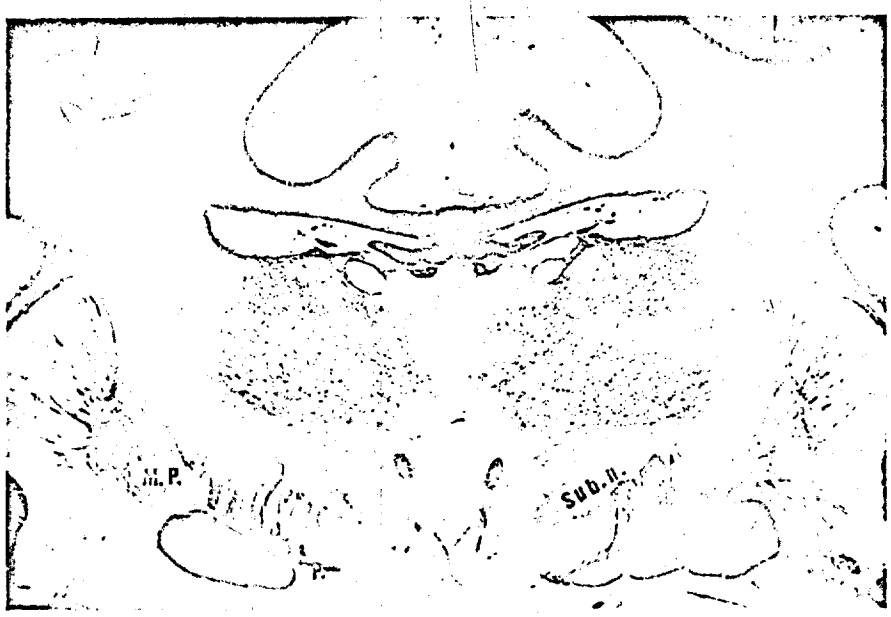


FIG. 3. Same as Figure 2 at higher magnification. At least one half of the glia population consists of medium "immature" astrocytes, characterized by extremely light, large, oval, naked nuclei containing quite a few small chromatin crumbs. The other cells, characterized by hyperchromatic nuclei, are presumably normal astrocytic nuclei. Spare atrophic nerve cells (→). Nissl stain; × 300. Neg. 61-6294.

FIG. 4. Coronal section at the same level as Figure 1, showing conspicuous Holzer gliosis of subthalamic nucleus (sub.n.) and the fiber bundles connecting globus pallidus with subthalamic nucleus and thalamus crossing the internal capsule. The medial pallidum (m.p.) also shows significant Holzer gliosis. The dotted line indicates the border line between pallidum and striatum. The dark staining of the red zone of substantia nigra (→), as well as that of some other grisea, does not indicate astrogliosis but is due to insufficient differentiation of the preparation. Holzer method; × 4. Neg. 61-6600.



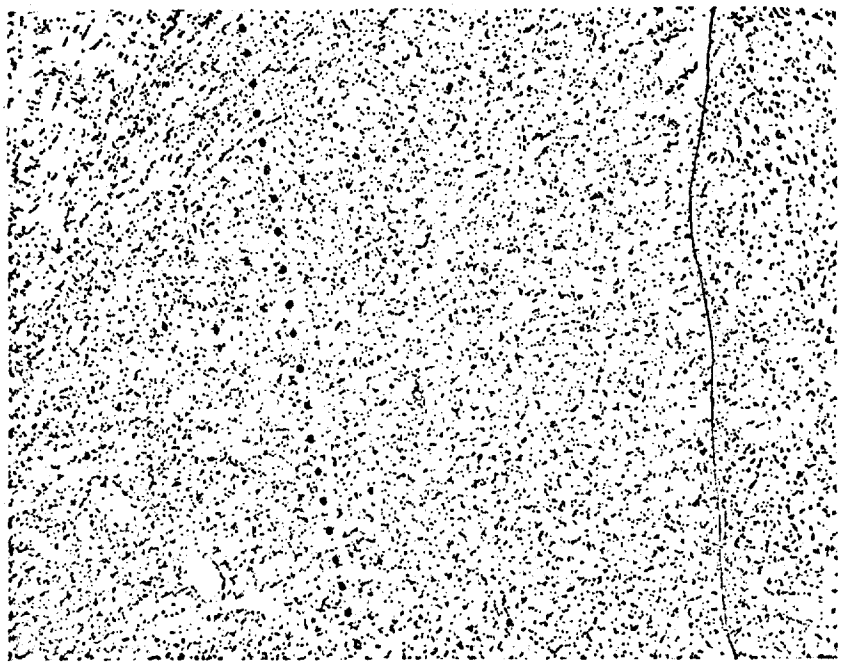
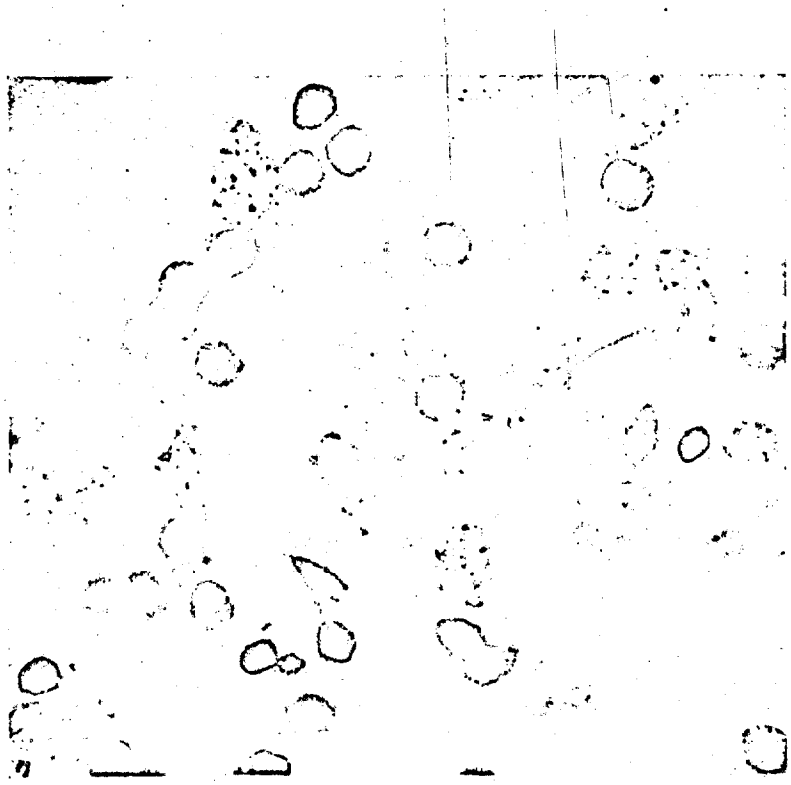


FIG. 5. Coronal section at the same level as Figure 1. Severe demyelination of subthalamic nucleus (sub.n.), comb system, and medial pallidum (m.p.). The cut is not quite symmetrical. Klüver-Barrera stain; × 4. Neg. 61-6698.

FIG. 6. Pallidum shows significant increase of the glial population. Considerable neuron loss in medial pallidum. Note the smallness of the neurons of lateral pallidum in contrast to medial pallidum. This is due partly to the atrophy of the neurons in the former, partly to the fact that normally they are smaller than the neurons in medial pallidum. The unbroken line indicates the border line between striatum and pallidum, the dotted line that between medial and lateral segment of pallidum. Nissl stain; × 50. Neg. 61-6302.



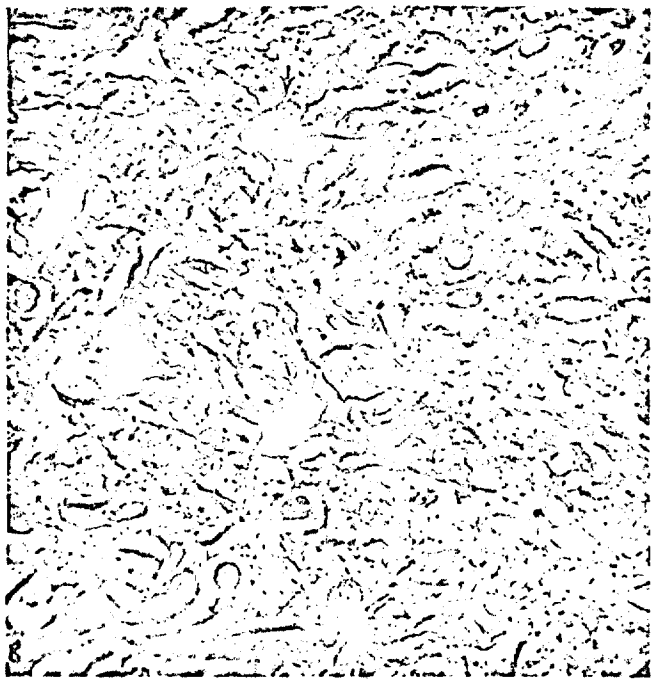


FIG. 7. Pallidum, medial segment. Note the variety of shapes of the large "immature" astrocytes. Nissl stain; × 900. Neg. 62-2602.

FIG. 8. Pallidum, medial segment. Large "immature" astrocytes forming scanty fibers (→). Holzer method; × 900. Neg. 61-6315.

lateral pallidum (fig. 6). The "immature" astrocytic nuclei were smaller, oval or uniform (fig. 9). Furthermore, they were less densely packed, except for a narrow zone around small vessels. The neuron loss here was slight to moderate, with the majority of the nerve cells atrophic, being about half the size of normal nerve cells (figs. 10 and 10a)*.

We were unable to reach a definite decision as to the involvement of the *red zone of the substantia nigra* since it normally contains numerous astrocytes resembling "immature" glia. In general, under pathological conditions, this nucleus reacts in a manner similar to the pallidum.

A great increase in the glial cell population was found in the *field H of Forel*; the majority of these cells were small and medium "immature" glia. Holzer preparations revealed a circumscribed astrogliosis. The neurons present were intact and there was no apparent neuron loss.

A peculiar finding in field H of Forel also involving the neighboring zona incerta and a small area of the adjacent ventral surface of the thalamus was the presence of PAS-positive, small astrocytes. Another structure in which these astrocytes were found was the comb system.

The *magnocellular portion of the lateral habenular nucleus* (Olszewski (6)) was the second of the grisea with conspicuous proliferation of small and medium "immature" glial cells and apparently without neuronal involvement (fig. 11). Holzer preparations were not available. The nerve cells looked perfectly normal. No appreciable neuron loss was present.

2. Diffuse Changes: Cerebrum, brain stem, and cerebellum were the sites of extensive alterations involving the neuroglia of both the grisea and the white matter. The cerebral cortex and the white matter of the hemispheres and striatum were less involved. The changes consisted of slight to moderate proliferation of small "immature" glial cells.

The most striking diffuse proliferation of the neuroglia, with numerous small and medium "immature" glial cells, was in the midbrain, pons, and medulla oblongata. There was an irregular increase in the glial population; the black zone of the substantia nigra, the locus caeruleus, and the inferior olive showed the most pronounced density.

In the cerebellum the glial alterations were restricted to the white matter and the subcortical nuclei. Both small and medium "immature" glial cells were present.

3. Focal Lesion: The hippocampal formation at the level of the medial geniculate body was the site of an asymmetrical, elongated focal lesion, embracing alveus, pyramidal layer, and adjacent white matter (fig. 12). This lesion was characterized by the presence of densely packed small "immature" astrocytes. In the involved area of the pyramidal layer there was conspicuous loss of neurons, and those remaining were shrunken (fig. 13).

4. Spinal Cord: The spinal cord was normal; the glial alterations stopped abruptly at the anatomical boundary of this structure.

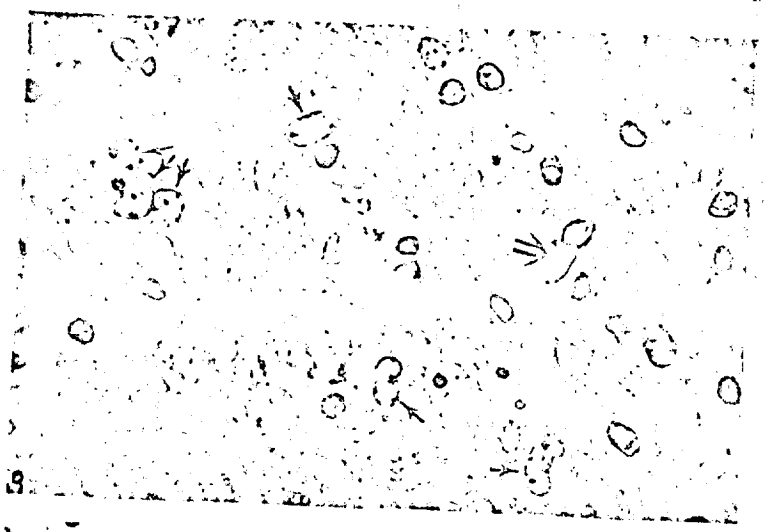
5. Body Organs: The liver showed hemosiderosis of the Kupffer cells. No changes in the kidney, adrenals, heart, and lungs were noted. The spleen was not available for examination.

DISCUSSION

The neuropathological findings in this monkey exposed to manganese dioxide for about 14½ months can be classified as holotopistic† and diffuse. The holotopistic changes involved the subthalamic nucleus and pallidum. The most

* In evaluating the size of the neurons of the pallidum it should be kept in mind that according to Mehler (5) about 99 per cent of the neurons in the lateral pallidum are significantly smaller than the great majority of the neurons in the medial pallidum, the rest being small neurons.

† Symmetrical lesions characterized by initial spotty cell involvement of a topistic unit followed by generalized cell destruction were referred to by C. and O. Vogt (7) as "holotopistic" (holos: whole; topistic: a topistic unit or griseum). We also use "holotopistic" in a broader sense; *i.e.*, when the alterations concern the glia alone without neuronal involvement.



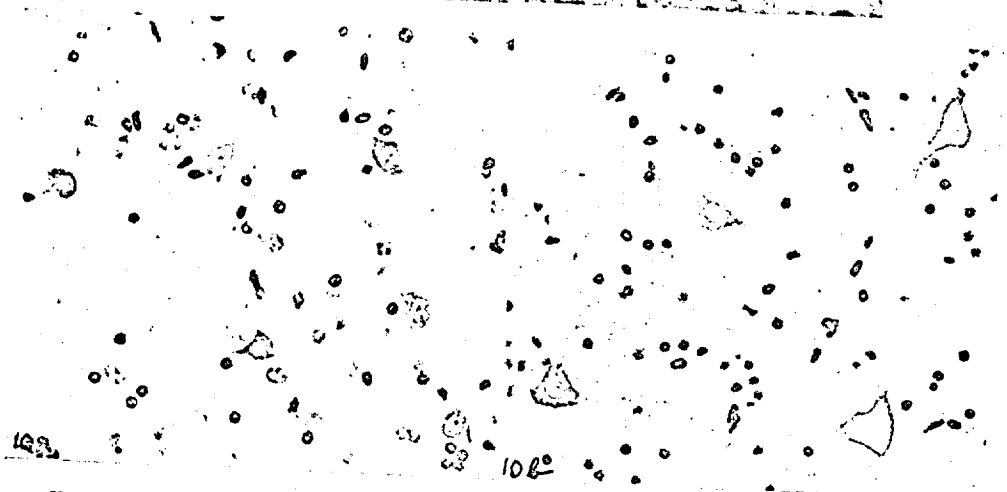
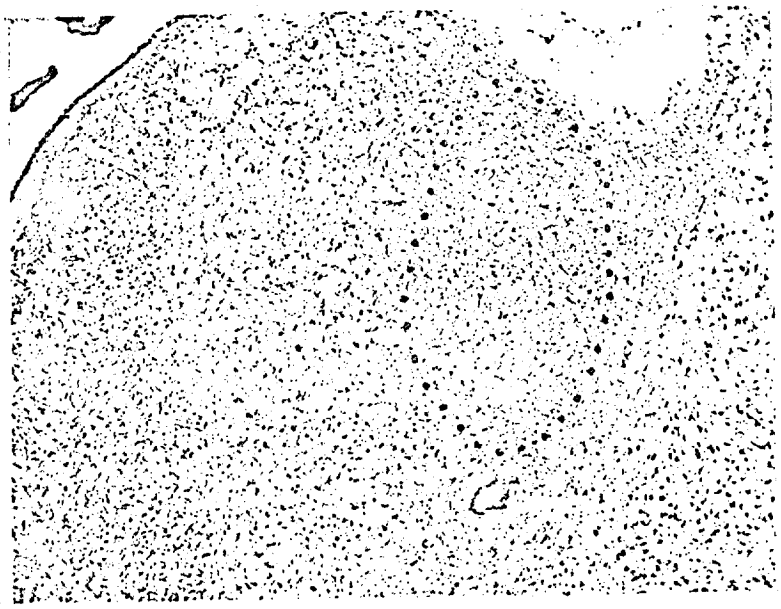


FIG. 9. Pallidum, lateral segment. Medium "immature" astrocytes (→). A single neuron (⇒). PAS; × 900. Neg. 61-6606.

FIG. 10. Pallidum, lateral segment. Atrophy of the nerve cells with poor staining of the dendrites. In comparison to the control (fig. 10a) they are closer together because of the general atrophy of pallidum. Note also "immature" middle-sized astrocytes. Nissl stain; × 300. Neg. 62-334.

FIG. 10a. Lateral pallidum of control rhesus monkey of about the same age as experimental monkeys, shown for comparison. Note the difference in the neuronal size and in the glial pattern. Nissl stain; × 300. Neg. 62-335.

prominent feature was the proliferation of bizarre glial cells. The neuronal loss in the subthalamic nucleus and medial pallidum was almost complete. In the lateral pallidum it was slight to moderate, with the majority of the nerve cells atrophic. In the magnocellular portion of the habenular nucleus and in field H of Forel the "immature" astrocytes were so densely packed and so circumscribed that the alterations in these nuclei deserved the appellation "holotopistic" in the broad sense.



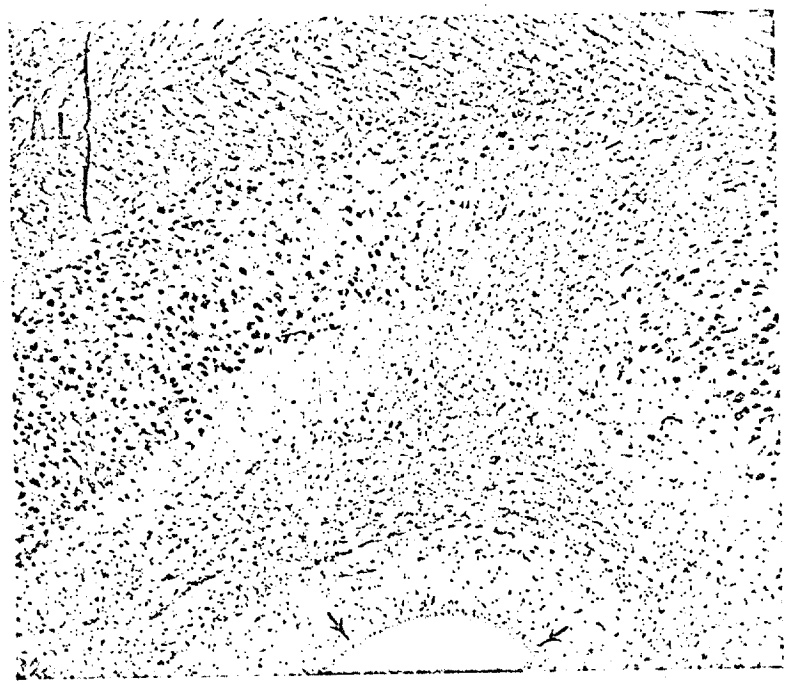


FIG. 11. Conspicuous increase of the glial population in the *magnocellular portion* of the lateral *habenular nucleus* due to the presence of medium "immature" astrocytes. The dotted line indicates the boundary of the damaged nucleus on the left of the habenular nucleus; on the right, the dorso medial nucleus of the thalamus (d.m.th.). The unbroken line indicates the border of the medial habenular nucleus. Nissl stain; × 50. Neg. 61-6305.

FIG. 12. Plaque-like circumscript lesion in the hippocampus spreading out indiscriminately to the alveus (al.), the H_1 sector of the pyramidal layer, and the adjacent white matter. The lesion is characterized by a tremendous proliferation of "immature" glial cells and by considerable neuron loss. Dentate gyrus (→). Nissl stain; × 50. Neg. 61-6304.

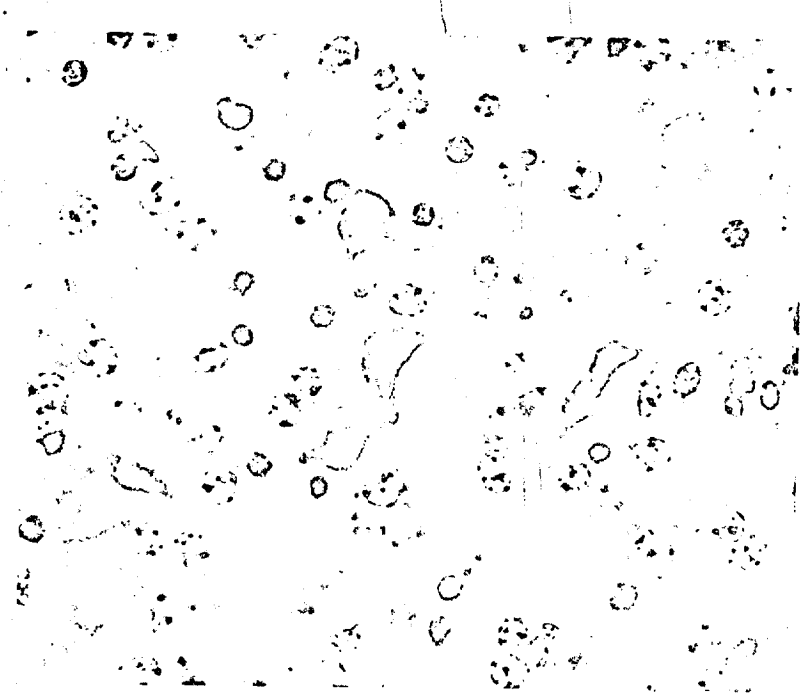


FIG. 13. Same as Figure 12, showing the lesion within the realm of the pyramidal layer at higher magnification. The great majority of the glial cells are small to medium-sized "immature" glial cells. Considerable neuron loss, the remaining neurons shrunken. Nissl stain; × 900. Neg. 61-6487.

In addition to the holotopistic alterations, diffuse changes consisting of an ubiquitous metamorphosis to small and medium-sized bizarre astrocytes were found in about half of the glial population of the cerebrum, brain stem, and cerebellum.

The bizarre glial cells were of three sizes: small, medium, and large. The small cells could be compared in size and shape to the "liver" astrocytes (see Greenfield *et al.* (8)); the medium and large were comparable to Alzheimer II astrocytes. (We have provisionally classified the bizarre cells as "immature" glial cells, meaning thereby an ontogenetic dedifferentiation to "immature" astrocytes.) A great number of cigar-shaped cells in the white matter, especially in the corpus callosum and the anterior commissure, were considered to be "immature" oligodendrocytes. In a second manganese monkey* such cells were quite numerous.

To date, only 3 human cases of manganese encephalopathy have been examined neuropathologically in a more or less satisfactory manner. These are the cases of Ashizawa (9), Stadler (10), and Parnitzke and Peiffer (11). In all cases the most prominent neuropathological findings were located in the *globus pallidus* and consisted of neuron loss, especially in its medial segment.

* This monkey (AFIP Acc. 1034382), the second of the group to be sacrificed, has been only partially examined thus far.

In addition, significant astrocytic proliferation in this griseum was found in the cases of Stadler and of Parnitzke and Peiffer. Parnitzke and Peiffer were the only authors who mentioned the subthalamic nucleus, which they described as showing focal neuron loss. Degenerative changes of the large neurons of the striatum, with eccentric displacement of the nucleus, were also noticed.

The neuropathological findings in the manganese monkey showed such striking similarities with those in human cases of manganese encephalopathy *that they could be said to be identical.* The additional findings in the monkey, not described in human cases, do not preclude their existence in these cases. In comparing the brain alterations of the human cases to those in the manganese monkey, one should keep in mind that none of the three authors, having the opportunity of examining human cases, had an entire brain at their disposal and that the routinely used Nissl method would not demonstrate the finer histological alterations such as the peculiar glial pattern, since the brain pieces had been in formalin too long, in the case of Parnitzke and Peiffer, for several years. Of no less importance is the fact that the human cases involved patients who survived several years after the appearance of neurological symptoms and withdrawal from the manganese mills, in the case of Parnitzke and Peiffer, 24 years. The importance of the stage of development of the process is best illustrated by the following: The eccentric displacement of the nucleus of the large cells of the striatum mentioned in all human cases was missing in the first manganese monkey, which survived until sacrificed 14½ months after exposure to manganese, whereas this finding was very conspicuous in the second manganese monkey, which survived 24 months before sacrifice. In addition, neuronal depletion in the lateral pallidum in the second animal was nearly complete, in contrast to the slight neuron loss in the first animal.

The selective elimination by the application of manganese of two closely associated nuclei belonging to a specific unit within the extrapyramidal motor system, leaving the remaining nuclei of this system intact is without precedent. This poses many theoretical and possibly certain practical problems. For instance, the feasibility of using manganese as a therapeutic agent in paralysis agitans and postencephalitic Parkinsonism, for which surgical destruction of the pallidum has proved successful, might be considered.

SUMMARY

1. Five rhesus monkeys, treated with repeated intramuscular injections of insoluble manganese dioxide suspended in olive oil, developed similar neurological disturbances suggesting dysfunction of the extrapyramidal motor system in the course of 9 to 24 months.

2. The monkey sacrificed 14½ months after the beginning of the experiment showed spectacular alterations in the brain identical to human case of manganese encephalopathy. The alterations were classified as holotopistic and diffuse. In the animal sacrificed 24 months after exposure, the alterations in the brain were similar, but more advanced.

The *holotopistic* alterations involved the subthalamic nucleus and the medial

and lateral pallidum. They were most severe in the subthalamic nucleus and medial pallidum and were characterized by a tremendous proliferation of bizarre glial cells provisionally classified as "immature" glia. In both nuclei the neuron loss was nearly complete. In the lateral pallidum the pattern of "immature" glia was different from that of the medial pallidum; the neuron loss was slight, but the nerve cells were atrophic. The alterations in the magnocellular portion of the lateral habenular nucleus and in the field H of Forel were considered holotopistic in the broader sense; they consisted of a significant increase of the glial population with the appearance of "immature" glial cells, but without neuronal involvement.

In addition, *diffuse* alterations of the cerebrum, the brain stem, and cerebellum were observed. They consisted of proliferation of "immature" glial cells in both the gray and the white matter.

A single, asymmetrical, circumscribed lesion was found in the hippocampus. It was characterized by accumulation of small "immature" astrocytes with involvement of the neurons.

3. While manganese is capable of producing an almost ubiquitous metamorphosis of the neuroglia of the brain, which we interpret as an ontogenetic dedifferentiation*, the most outstanding feature of manganese encephalopathy in man and primates appears to be the severe, selective damage to the subthalamic nucleus and pallidum.

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* This subject will be more extensively discussed in a second paper.

April 14, 1962 Vol. 194

Nature, Lond. 194: 188 (1962)

Hypoglycæmia induced by Manganese

PLANT extracts have long been used as home remedies for diabetes mellitus[1-3], and have been investigated for their hypoglycæmic effect[4,5]. However, there has been little experimental or clinical proof of their efficacy[6].

Recently it has come to our notice that infusions of lucerne (alfalfa, *Medicago sativa*) are being used in the treatment of this disease by certain diabetics in South Africa. We have had the opportunity of studying a young male diabetic who gives a marked hypoglycæmic response following the oral administration of an aqueous extract of this plant (Fig. 1). The patient is twenty-one years of age, having been a diabetic for the past seven years. A diabetic glucose tolerance curve has been demonstrated on repeated occasions with fasting blood sugar-levels varying from 150 mgm./100 ml. to 250 mgm./100 ml. He has been treated intermittently with insulin, requiring large doses (100–200 units daily) for even moderately satisfactory control. He himself claimed that lucerne infusions gave better control. He often exhibits the feature of marked hyperglycæmia even when under treatment with insulin with little increase in plasma or urinary ketones. Routine investigations failed to reveal evidence of thyrotoxicosis, Cushing's disease, phæochromocytoma or chronic pancreatitis.

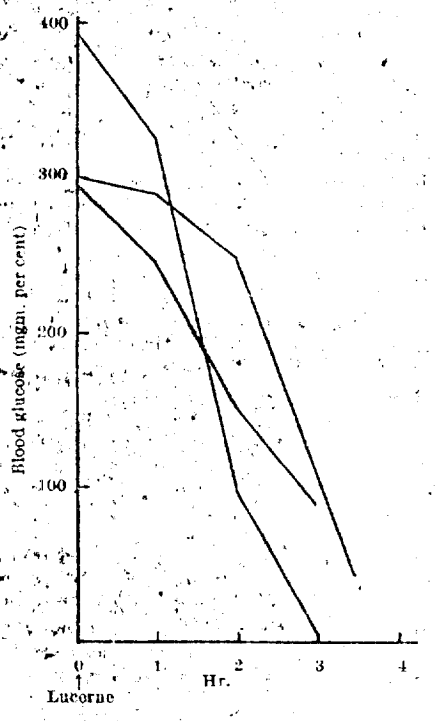

Fig. 1. Blood sugar-levels following the oral administration of lucerne extract

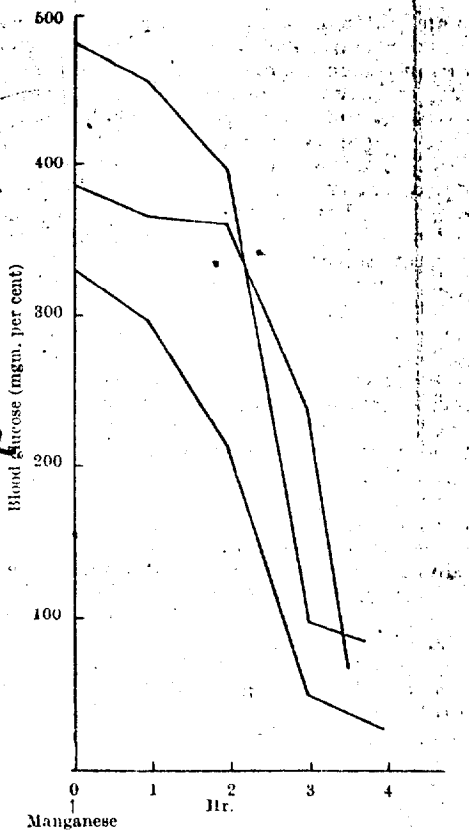

Fig. 2. Blood sugar-levels following the oral administration of 5 mgm. manganese (as manganese chloride)

There was no hypoglycæmic response to the administration of the amino-acid *l*-leucine[7,8]. Parents and sibling have normal glucose-tolerance curves.

Lucerne was noted to contain relatively large concentrations of manganese[9]. Accordingly, manganese as manganese chloride was administered many times orally in doses of 3–5 mgm. and intravenously on one occasion in a dose of 20 mcgm. to the patient. The effect on the blood glucose-level was a consistent fall often to severely hypoglycæmic levels (Fig. 2). Both lucerne and manganese when given orally modified the glucose tolerance curve (Fig. 3).

The oral administration of manganese to four middle-aged diabetics, three juvenile diabetics and one case of diabetes resulting from chronic pancreatitis did not affect the blood sugar-level.

Although manganese is well known to be a component and co-factor in certain enzyme systems including some concerned in the intermediary metabolism of carbohydrates[10], a hypoglycæmic effect of manganese has not previously been reported. The time-interval between administration of manganese and its effect on the blood glucose-level is consistent with the possibility of enzyme activation[10]. Further work on the metabolism of manganese is being done.

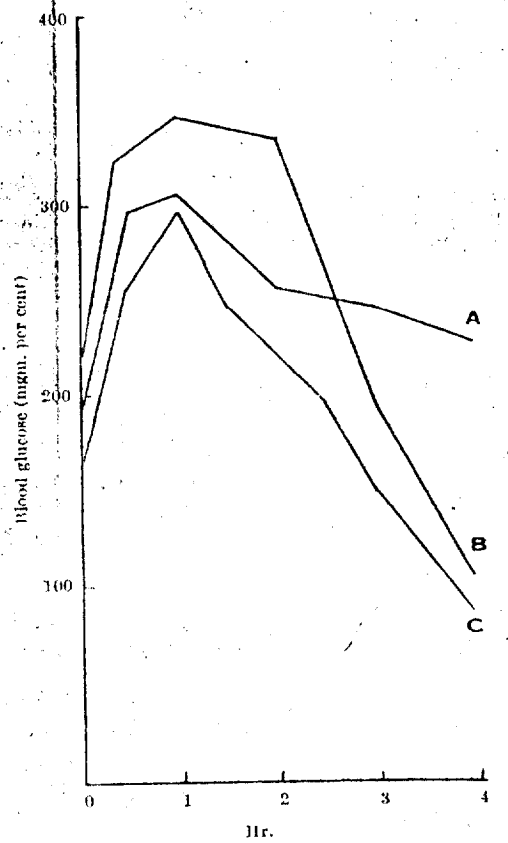

Fig. 3. Glucose tolerance curves: *A*, after 100 gm. glucose orally; *B*, after 100 gm. glucose and lucerne; *C*, after 100 gm. glucose + 5 mgm. manganese (as manganese chloride) orally

We thank Prof. B. J. P. Becker and Prof. H. B. Stein for their assistance and interest.

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Arch. Sci. Biol. 16: 141-159, 1931

PHARMACOLOGICAL INVESTIGATIONS OF MANGANESE. XII: effects of manganese chloride, carbonate and phosphate.

By Professor L. Sabbatini[1].

I. Object of the research and testing method.

When a study of the pharmacological action of manganese salts is intended, the most suitable of all is undoubtedly chloride, on account of its chemical and chemical-physical characteristics, and because its effect is harmless. However, as soon as we started the test and injected the substance into live animals, it was suspected that the chloride immediately converts into other saline forms of the manganous carbonate and phosphate, the solubility of which is minor. Therefore the effects observed by us should not be attributed exclusively to the injected chloride; instead, they should be ascribed to those other salts which form as soon as (the chloride) encounters the alkaline carbonates and phosphates in the blood circulation[2].

For the above reason I undertook, besides researching the chloride effect, a comparative study concerning the manganese carbonates and phosphates as well. When determining the technical testing method, I attempted to approximate as closely as possible the chemical conditions found by us in the live animal.

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1) This work, ready for printing, was found in the Community Library of Imola after the decease of the lamented Professor L. Sabbatini. Professor I. Simon completed its notes only. I am pleased to publish it in memory of the renowned biologist.

2) This was already demonstrated by Seremin (Archives of Experimental Pathology and Pharmacology, 1924, 105, 49) concerning the soluble salts of lead which convert into phosphocarbonate, and by the research work of Messini (Archives of Experimental Pathology and Pharmacology, 1928, 135, 346) indicating that the ferrous salts transform into phosphate.

Regarding the general testing conditions, I likewise attempted to obtain results with said salts comparable to the results previously obtained by me with sodium permanganate and with the bisodium of colloidal manganese[1]. Therefore the final aim of the research work at issue is the detection of the ultimate and constant forms always achieved by manganese in the organism, regardless of the diversity of the preparations originally used. The ultimate and constant chemical forms are the direct cause of the general pharmacological effect of the manganese, which effect could be defined as real and fundamental, insofar as it is not subject to further modification by the chemical and biological factors pertaining to specific preparations; said effect is instead characteristic for all of them.

For these tests I almost always used colloidal solutions of $MnCL_2$, $MnCO_3$ and $Mn_2(PO_4)_2$ with 0.025 g-atoms of manganese per liter[2]. I always used them for intravenous injections, and always in rabbits. I calculated the doses per kg of body weight, and the injection velocity in g-atoms of the manganese.

I performed <u>five test series</u> with manganese chloride, carbonate and phosphate.

For the <u>first test series</u> I used crystallized pure manganese chloride for analyses, from the business firm C. Erba.

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1) L. Sabbatini: Pharmacological action of sodium permanganate. This publication: ARCHIVES, 1928, <u>12</u>, 50.

2) This concentration does not differ much from the concentration of the g-atom of Mn 0,017, used for the tests with sodium permanganate, nor does it differ much from (the g-atom) of Mn 0,020, used for the tests with bisodium of collodial manganese.

(For Footnote 3) see page 3 of translation)

For the second test series I used manganese carbonate: but I encountered some technical difficulty immediately after the preparation of the solutions. I needed a solution with 0,025 g-mol per liter; instead, the solubility of the carbonate is much below 0.000445 g-mol per liter at 25°.

Mixing two equimolar solutions of manganese chloride and sodium carbonate, so as to obtain a solution with 0,025 g-mol per liter, immediate precipitation of the manganese carbonate and manganese hydroxide is achieved; its composition varies depending on dilution and temperature; I failed in all attempts made to prevent the precipitation and to obtain a collodial form.[1)]

The safest method for obtaining manganese carbonate is the use of two solutions of $MnCl_2$ and Na_2CO_3, both saturated with CO_2. Working as indicated, a mixture can be obtained which corresponds to 0,025 g-mol per liter; it remains quite limpid for ½ hour and the subsequent precipitation is slow. The half-hour while said mixture remains limpid is more than sufficient for testing it in animals. From a chemical viewpoint, its composition is uncertain and varies from the moment of the mixing to the appearence of the precipitate. But from a biological viewpoint the solution at issue is closer to the real facts which we intend to study: if we assume that a manganese carbonate can form within the animal organism, it will always and exclusively form from bicarbonates while excess CO_2 is present, and not from neutral carbonates which do not exist in the blood.

For the reasons cited above and intending to experiment with manganese carbonate under conditions approximating as far as feasible those real biological conditions under which the pharmacological test is performed, I used mixtures made

1) (Footnote 5 on page 142 of original text)
For reference see the article "Manganese" by A. Miolati in Abegg, Handbook of Inorganic Chemistry, 1913, 4,(2),611.

from equal volumes of $MnCl_2$ and CO_2 ; on each occasion, I prepared them a few minutes before injecting them into the vein of the animals.

Proceeding as described, we did perform in vitro a few minutes before the injection what can occur immediately after the injection in the blood when manganese chloride is injected and encounters sodium bicarbonate and free carbonic acid there.

When, for brevity's sake, reference is made in this memorandum to manganese carbonate specified as $MnCO_3$, it is understood that I will do so under the aforementioned reservations.

For the <u>third test series</u> I used manganese phosphate; I was, however, faced with technical difficulties even greater than those related to the carbonate, concerning the preparation, the very minor solubility[1], the extremely unstable nature of the collodial solutions which I could produce.

Already when the first samples were obtained, I was compelled to abandon the preparation methods which would have supplied pure phosphate but which failed to provide the same in a collodial solution and with the desired concentration.

I had to be satisfied with the improvised preparation of collodial solutions using those reactive solutions for said purpose which were suitable for the biological test, applying a technology most adequate for the highly unstable colloid.

When manganese chloride is mixed with surplus bisodium phosphate, $Mn_3(PO_4)_2$ is obtained as amorphous precipitate; it is useless for us. Excess bisodium phosphate simultaneously remains in the liquid and acid phosphate appears, the pharmacological effect of which is certainly not negligible.

1) See Abegg, l.c.762.

When mixing manganese chloride and trisodium phosphate solutions, with a g-mol content of 0,1 per liter, in equivalent volumes, pure $Mn_3(PO_4)_2$ should be obtained, and sodium chloride only should remain in the liquid: sodium chloride could in no way alter the pharmacological experiment[1]. But under these conditions the manganese phosphate precipitates immediately and all attempts made to keep it in the collodial state failed.

On the other hand, when the same solutions saturated with CO_2 are mixed, a fine collodial solution forms immediately; however, its life is extremely short: it floculates and precipitates within 10 seconds, at the most.

Working as stated we are in fact doubtful whether trisodium phosphate does really form exclusively, or whether instead a mixture of phosphate and carbonate results, or some complex salt. But it is timely to recall that while--from a chemical viewpoint--these conditions are not the most suitable for obtaining a pure product, they are--from a biological viewpoint--those which come closest to the real pharmacological facts studied by us.

When, as initially mentioned, I thought of experimenting with manganese phosphate, I did so because I doubted that the manganese chloride injected into the veins would encounter phosphates in the blood, forming collodial phosphate with the latter. It should now be pointed out that if such a reaction does in fact occur, it will develop with the reagents present in the blood, i.e. with bisodium phosphate in the presence of alkaline bicarbonates and free carbonic acid. These are the same reagents which I have in the two solutions used for the preparation of manganese phosphate, because using a solution of trisodium phosphate saturated with CO_2 is like using a solution consisting of a mixture of bisodium phosphate, of sodium bicarbonate and of carbonic acid.

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1) It is even useful for the tonicity of the solution.

On the basis of all these ideas, intending to prepare a collodial solution corresponding to standard g-mol 0,050 per liter of $Mn_3(PO_4)_2$, I used two solutions with 0,1 standard g-mol per liter of $MnCl_2$ and of Na_3PO_4, both saturated with CO_2.

However, the collodial solution thus obtained is highly unstable and floculates within approximately 10 seconds. I eliminated this very serious disadvantage by building two confluent burettes in a tube at Y, towards the cannula through which the intravenous injection is given.

When the burettes are respectively filled with the two above-mentioned solutions, the mixing is performed by an automatic agitator, placed at the confluence point of the solutions; accordingly, the reaction and the formation of the colloidal phosphate occur step by step as the liquids arrive to the injection cannula[1]. A short period of a few sec. can elapse between the preparation of the solution and its entry into the vein. Then the solution is injected while its dispersiveness is still safely high-grade.

The <u>fourth test series</u> was performed as follows: instead of mixing the two solutions of manganese chloride and of trisodium phosphate (both saturated with CO_2) before injecting them into the vein, I injected them simultaneously and separately in equivalent volumes into the two jugular veins. With this method, the mixing, the reaction and the formation of the collodial manganese phsophate occured directly in the blood.

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1) L. Sabbatini: Burettes for the pharmacological study of highly unstable colloids. <u>Bulletin of the Italian Experimental Biology Association</u>, 1928, <u>3</u>, 205. How highly unstable colloids can be pharmacologically tested. <u>Biochemistry and Experimental Therapy</u>, 1929, <u>15</u>, issue no.4.

(For Footnote 2) see page 7 of translation)

During the fifth test series I likewise injected the two above-mentioned solutions into the veins; but I injected the phosphate solution first, and after a certain period of time, the manganese solution followed. In this way, I studied how a preventive phosphate injection could modify the pharmacological effect of the manganese.

II. Tests with manganese chloride.

I performed 25 tests with manganese chloride. After some preliminary sampling[2] I adopted a solution which had 0,025 g-atoms per liter, and subsequently used it for all other tests. They are compiled on Table I; they indicate that the minimal dose which is immediately and unquestionably lethal is the dose having 0,00039 g-atoms of manganese per kg of rabbit body weight.

Results vary largely with doses between 0,00039 and 0.00023 g-atoms; the animals either succumb immediately, or only after a certain period of time, or they survive without evidence of any immediate or delayed perceptible disorder.

Finally, with doses of less than 0,00023 g-atoms per kg of body weight, the animals certainly survive.

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2) (page 145 of original text)

Test	Concentration of the solution used, in g-atoms per l. (liter)
1	1,000
2	0,100
4	0,050
10	0,020

TABLE I.

Manganese chloride injected into the veins of rabbits.

1 Esperienza	2 Coniglio di kg	3 Soluzione di $MnCl_2$ iniettata: 4 g-atomo per L	5 in cc	6 in minuti	7 g atomi di Mn × 10^6: 8 iniettati	9 per kg corporeo	10 per kg e minuto	11 Osservazioni
1	1,000	1,000	1,3	0,30	1500	1500	3000	muore immediatamente 12
2	1,100	0,100	7,5	0,55	750	682	760	» »
3	1,100	0,025	25	3,20	625	568	171	» »
4	1,000	0,050	9,6	1,20	480	480	370	» »
5	1,100	0,025	20,2	3,27	505	459	135	» »
6	1,070	0,025	18,8	3,2	470	439	146	» »
7	0,920	0,025	15,5	2,27	387	421	175	» »
8	1,120	0,025	17,9	2,38	447	400	134	sintomi gravissimi, sopravvive 13
9	1,200	0,025	18,7	1,51	467	390	216	muore immediatamente 12
10	1,070	0,020	20	2,33	400	374	150	nessun disturbo, sopravvive 14
11	1,370	0,025	19,7	2	490	359	180	muore immediatamente 12
12	1,100	0,025	15,4	1,42	385	350	206	nessun disturbo, sopravvive 14
13	1,000	0,025	14	1,47	350	350	194	muore dopo più di 8 ore 15
14	0,970	0,025	13,2	3,23	330	340	100	nessun disturbo, sopravvive 14
15	1,320	0,025	17,3	2,22	433	327	136	muore in meno di 24 ore 16
16	1,150	0,025	15	1,46	375	326	181	muore immediatamente 12
17	1,030	0,025	13,1	1,55	328	321	169	» »
18	0,900	0,025	11,2	1,44	280	311	183	» »
19	1,170	0,025	14	3,36	310	265	76	nessun disturbo, sopravvive. 14
20	0,950	0,025	9,5	1,17	237	250	190	muore dopo alcune ore 17
21	1,020	0,025	9,8	1	245	240	240	nessun disturbo, sopravvive 14
22	1,020	0,025	9,4	0,45	235	230	330	muore dopo alcune ore 17
23	1,220	0,025	10,8	0,41	270	221	316	nessun disturbo, sopravvive 14
24	1,220	0,025	10,2	0,48	255	209	261	» » »
25	1,150	0,025	9,2	0,33	230	200	363	» » »

1) Test
2) Rabbit of kg . .
3) Injected $MnCl_2$ solution
4) with g-atoms per liter
5) in cc
6) in minutes
7) g-atoms of Mn x 10^6
8) injected
9) per kg of body weight
10) per kg and minute
11) Remarks
12) dies immediately
13) very serious symptoms, survives
14) no disorder, survives
15) dies after more than 8 hours
16) dies within less than 24 hours
17) dies after a few hours

Whenever death occurs immediately after high or average doses, it is the result of a direct and intense toxic action of the manganese chloride affecting the heart. It causes a slow-down of the heartbeat frequency, reduces arterial pressure, and finally a sudden heart arrest occurs (Fig. 1.).

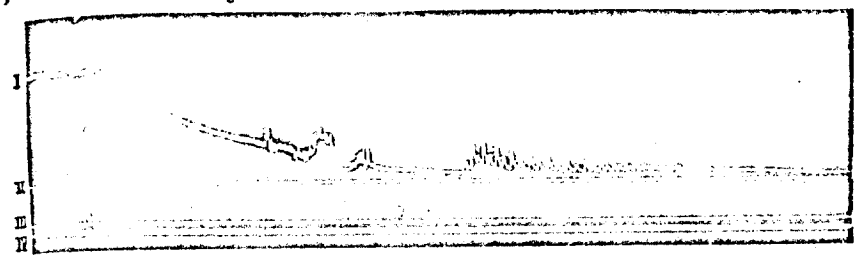

Fig. 1.

I. Tracing of the pressure at the carotide.
II. Pressure zero.
III. Indication of the intravenous injection: A start; B end.
IV. Time in minutes, seconds.

Anemia of the nerve centers then trigger general convulsive seizures, after which the animal drops in general paralysis. Respiration stops and artificial respiration proves useless; the animal expires. When immediately dissected, the heart is found arrested in diastole and only slight fibrillating movements of the heart occur.

Whenever death is delayed after average doses, it is still the result of a toxic action affecting the heart. During the injection the animals show no disorder whatsoever, or at the most, a transient and slight reduction of the heartbeat frequency. They are entirely sound after the termination of the injection; they are lively and feed; but a few hours later they are stricken by general tonic convulsions the nature of which (convulsions) is asphyxiate; they are followed by the respiration arrest.

In cases when the animals safely survice after receiving doses of less than 0,000230 g-mol, no disorder whatsoever is perceptible either during or after the injection.

I performed three tests[1] to detect how the manganese chloride modifies the heart function and arterial pressure in rabbits. During these tests I recorded the manometric tracing at the right carotide while injecting at a constant speed rate the usual solution of $MnCl_2$ with 0,025 g-atoms per liter into the left jugular, and followed the injection until heart arrest took place.

The obtained results coincide; in Figure 1. I therefore report the tracing of Test 27 only. It shows that as soon as the injection begins, the pressure declines abruptly from mm 129 (normal) to mm 90. While the injection proceeds, the pressure remains stable at said value for a certain period of time, presumably because an equilibriam is immediately reached between the action of the manganese arriving into the circulation at a constant velocity and the specific automatic defense potentials of the organism.

Following the subsequent course of the injection, a point is reached when these defensive agents begin to fail and the pressure starts a second slow decline which is gradual and continuous. The heartbeat simultaneously continues to slow down. Very low values are thus obtained ; they are incompatible with life. Violent convulsive shocks occur, after which the heart stops. Gasping movements take place; after these everything seems to be finished and the animal can be considered dead. In fact, however, the heartbeat resumes very slowly after approximately forty seconds, and its rythm has a certain regularity. This lasts for approximately two and a half minutes with a slow and gradual decline until the final heart arrest occurs and the animal is irreversibly dead.

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1) Test 26. Rabbit weighing 2,210 kg, 19 cc of the solution in 61 seconds; 0,000430 g-atoms per kg. - Test 27. Rabbit weighing 2,250 kg; 20 cc of the solution in 154 seconds; 0.000444 g-atoms per kg. -Test 28. Rabbit weighing 2,050 kg; 12.3 cc of solution in 49 seconds; 0,000300 g-atoms per kg.

TABLE II

Manganese carbonate injected into the veins of rabbits.

Esperienza 1		4 Coniglio di kg	5 Soluzione di $MnCO_3$ iniettata					11 g-atomi di Mn × 10^6			Osservazioni
2 numero	3 numero d'ordine		6 numero	7 a g-atomo per L	8 preparata da minuti	9 in cc	10 in minuti	12 iniettati	13 per kg corporeo	14 per kg e minuto	15
1	24	1,100	V	0,025	10	18,5	1,40	462	420	247	morì subito 16
2	18	1,320	II	0,025	30,30	21,0	2,20	525	398	173	» »
3	25	1,400	V	0,025	20	21,8	1,45	545	389	229	» »
4	22	0,900	VI	0,025	23	13,7	1,20	342	380	293	sopravvisse 17
5	30	1,450	VII	0,025	2	21,7	2, 4	544	375	181	»
6	31	1,450	VII	0,025	30	21,7	2,12	543	375	170	»
7	17	1,870	II	0,025	15,30	27,7	1,26	692	370	264	»
8	16	1,370	I	0,020	5,48	25,0	2,17	500	365	160	»
9	28	0,900	IV	0,025	35	12,8	2, 9	320	355	169	morì subito 16
10	34	1,250	VIII	0,025	17	17,5	1,57	437	350	175	» »
11	35	1,370	IX	0,025	10	18,8	2,27	470	343	140	sopravvisse 17
12	29	1,400	VI	0,025	19	19,0	2,29	457	339	186	morì entro 48 ore 18
13	36	1,170	IX	0,025	18	15,7	1,35	392	335	245	morì subito 16
14	33	1,000	VIII	0,025	32	13,2	1,10	330	330	282	sopravvisse 17
15	23	1,100	V	0,025	30	14,2	1,47	355	323	180	morì subito 16
16	32	1,000	VIII	0,025	40	12,6	1,23	315	315	252	» »
17	26	1,215	VI	0,025	18	15,4	1,32	385	310	207	sopravvisse 17
18	20	1,050	III	0,022	13	14,3	1,36	318	303	190	»
19	38	1,230	XI	0,025	15	14,5	5,40	362	295	52	morì entro 20 ore 19
20	21	1,120	III	0,022	21	14,3	1,21	318	284	218	sopravvisse 17
21	39	1,250	XI	0,025	22	14,0	1, 4	320	280	261	»
22	19	0,810	III	0,022	6	10,1	0,49	224	267	334	»
23	37	1,400	X	0,025	23	14,6	2,48	365	261	93	»
24	27	1,070	VI	0,025	6	10,7	1, 2	267	250	250	morì entro 40 ore 20
25	40	1,100	XII	0,025	8	10,6	0,43	265	241	314	sopravvisse 17
26	41	1,150	XII	0,025	13	10,6	0,57	265	230	256	»
27	42	1,250	XII	0,025	17	11,0	0,42	275	220	314	»

1) Test 2) No. 3) Serial number 4) Rabbit weighing kg . .

5) Injected $MnCO_3$ solution . .

6) No 7) with g-atoms per liter

8) prepared for minutes . .

9) in cc 10) in minutes. .

11) g-atom of Mn x 10^6

12) injected 13) per kg body weight

14) per kg and minute 15) Remarks

16) Died immediately

17) Survived .

18) Died within 48 hours

19) Died within 20 hours

20) Died within 40 hours.

The animal can even be saved if it is aided with artificial respiration when the heartbeat resumes.

The automatic defense potentials of the organism against the effect of the manganese chloride are believed to consist, as we will see below, in the alkaline phosphates reacting with the manganese chloride; the reaction yields an insoluble collodial phosphate which has no immediate toxicity; instead, its toxicity is slow and delayed. The recovery of the heart after a first arrest could explain why the results obtained with excessively high doses are uncertain, including doses between 0,00039 and 0,00023 g-atoms per kg.

III. Tests with manganese carbonate.

I performed 27 tests with manganese carbonate, almost always using a solution with 0,025 g-atoms per liter[1].

The tests are compiled on Table II; they indicate that the minimal immediately and certainly lethal dose has 0,000389 g-atoms of manganese per kg of rabbit body weight.

A wide variety of results is obtained with doses within the range of 0,000389 and 0,000250 g-atoms; the animals either die immediately, or they succumb only after a certain period of time, or they survive without any immediate or delayed perceptible disorder.

Finally, with minor doses of 0,000225 g-atoms per kg of body weight the animals survive safely.

The symptomatology, either in cases when death occurs immediately, or when it is delayed, is always similar to lethal symptoms described in conjunction with manganese chloride.

IV. -Tests with manganese phosphate.

1. - Colloidal manganese phosphate.

I performed 16 tests with colloidal manganese phosphate, using solutions with 0,0250 manganese g-atoms per liter.[2]

1) For Tests 18,20 and 22 only did I used a solution with 0,022 g-atoms per liter; for Test 8, the solution used contained 0,020 g-atoms per l.
2) For two tests only, solutions with 0,0125 manganese g-atoms per liter were used.

For Test,4 the age of the collodial solutions was 4" ; for Test 1, it was 2" and 8/10, and 2" 2/10 for Test 2; for all other tests it was always within the range of 1" and 2".

These tests are compiled on Table III. They indicate to us that the phosphate of colloidal manganese never causes immediate death; the animal dies only 19 hours after the injection, even when an extremely high dose is applied, with 0,0001006 g-atoms per kg of body weight (Test 1).

The minimal certainly lethal dose after a very variable period of time from 19 hours to 15 minutes, has 0,00042 g-atoms per kg of body weight.

A wide variety of results is obtained with doses within the range of 0,000420 and 0,0003000 g-atoms; the animals succumb after one or two days, or survive the test .

Finally, the animals survive safely with minor doses of 0,000300 g-atoms per kg of body weight.

The symptomatology is identical with the symptomatology related to manganese chloride and carbonate, when the animals die some time after the injection only.

TABLE III

Manganese phosphate injected into the veins of rabbits.

1 Esperienza	2 Coniglio di kg	3 Soluzione di $Mn_3(PO_4)_2$ iniettata: 4 a g-atomo di Mn per L × 10^4	5 in cc	6 in minuti	7 dell'età di minuti secondi	g-atomi di Mn × 10^6 8: 9 iniettati	10 per kg corporeo	11 per kg e minuto	12 Osservazioni
1	1,250	250	50,3	4,42 0,24	2, 0,80	1257	1006	214	muore dopo 19 ore dall'iniezione 13
2	1,250	250	38,9	2,50 0,76	2, 0,19	972	778	273	» 12 ore circa 14
3	1,150	125	63,3	3,15 0,40	1, 0,43	854	742	228	» 19 ore circa 15
4	1,150	125	63,4	3,2	4, 0,01	792	689	230	» pochi minuti dall'iniezione 16
5	1,300	250	29,2	1,34 0,54	1, 0,29	730	561	357	» 10 minuti 17
6	1,270	250	26,3	1,28 0,54	1, 0,70	657	517	351	» minuti 17 ½ 18
7	1,100	250	19,8	1,12 0,90	1, 0,47	495	450	375	» 18 ore 19
8	1,500	250	25,2	1,59 0,51	1, 0,90	630	420	210	» 15 minuti 20
9	1,600	250	16,3	0,51 0,30	1, 0,57	407	400	464	sopravvive, cessa l'osservazione dopo 17 giorni 21
10	1,120	250	17	1,15 0,40	1, 0,77	425	379	301	sopravvive, cessa l'osservazione dopo 16 giorni 22
11	1,600	250	14	0,44 0,80	1, 0,28	350	350	466	muore dopo 36 ore circa 23
12	1,250	250	16,3	0,43 0,19	1, 0,32	407	326	453	sopravvive, cessa l'osservazione dopo 16 giorni 24
13	1,270	250	15,2	0,58 0,70	1, 0,52	380	300	306	muore dopo 48 ore circa 25
14	0,970	250	10,8	0,29	1, 0,71	270	278	556	sopravvive, cessa l'osservazione dopo 16 giorni 26
15	1,120	250	11,6	0,29	1,	290	259	555	» » »
16	1,200	250	11,6	0,30 0,30	1, 0,04	290	242	484	» » »

1) Test
2) Rabbit weighing kg. .
3) Injected $Mn_3(PO_4)_2$ solution
4) g-atom of Mn per l(liter) x 10^4
5) in cc
6) in minutes
7) aged, in minutes, seconds
8) g-atoms of Mn x 10^6
9) injected
10) per kg of body weight
11) per kg and minute
12) Remarks
13) dies 19 hours after the injection
14) " 12 hours " " " (approximately)
15) " 19 hours " " " " "
16) " a few minutes after the injection
17) " within 10 minutes " "
18) " " 17½ " " "
19) " 18 hours " "
20) " 15 minutes " "
21) survives, observation ceases after 17 days
22) survives, observation ceases after 16 days
23) dies after approx. 36 hours
24) survives, observation ceases after 16 days
25) dies after approx. 48 hours
26) survives, observation ceases after 16 days.

2. Sodium phosphate and manganese chloride. Simultaneous injections.

For the preceding tests the collodial manganese phosphate was prepared by mixing two reactive solutions at the moment when the injection was applied. For the injections at issue here, on the other hand, I simultaneously injected into the jugular veins equivalent volumes of the two solutions, i.e. of the manganese chloride and of the sodium phosphate; with this method, their admixture, the chemical reaction and the formation of the manganese phosphate had to occur in the circulating blood. Under the above conditions the manganese phosphate which forms in the blood plasma is likewise collodial and does not yield precipitates, encrustations or coagulations which could obstruct pharmacological observation for mechanical reasons.

TABLE IV.

Simultaneous intravenous injections of Na_3PO_4 and $MnCl_2$, in rabbits.

1 Esperienza	2 Coniglio di kg	3 Entrambe le soluzioni iniettate			7 g-n-mol × 10⁶ di fosfato di sodio e di cloruro di manganese			11 Osservazioni	
		4 g-n-mol per L	5 in cc	6 in minuti	8 iniettati	9 per kg di coniglio	10 per kg e minuto		
1	0,800	0,100	20,6	13,57	2060	2575	185	muore alla fine dell'iniezione	12
2	1,500	0,050	50,0	10.	2500	1666	166	» dopo parecchie ore	13
3	1,170	0,100	17,9	2,27	1790	1530	624	» immediatamente	14
4	1,480	0,050	35,0	4,19	1750	1182	274	» dopo parecchie ore	13
5	1,300	0,050	25,0	2,33	1250	962	378	» immediatamente	14

1) Test
2) Rabbit weighing kg . .
3) Both solutions injected
4) with g-n-mol per liter
5) in cc
6) in minutes
7) g-n-mol x 10^6 of sodium phosphate and of manganese chloride
8) injected
9) per kg of rabbit
10) per kg and minute
11) Remarks
12) Died when the injection ended
13) " after several hours
14) " immediately.

The result of these tests which are compiled on Table IV agrees well with the results obtained with colloidal manganese phosphate, provided that the above statements concerning confluent liquids blending in the veins are taken into consideration. Here the two solutions enter through the two jugular veins, blend with the blood and flow to the heart, and there, through the vortices which form, the mixing and the chemical reaction will be completed. However, the possibility cannot be excluded that a thin stream of the still separate liquid could act on the right heart (chamber) with the direct and immediate effect of manganese chloride. This explains why cases of immediate death occurred in three tests (1,3,5); extremely high doses of manganese g-atoms were always involved in said cases, i.e. ($x\ 10^6$), 1287,5-765-481 per kg of rabbit.

3. Preventive injections of sodium phosphate versus those of manganese chloride.

While in the course of the preceding tests two reactive solutions of manganese chloride and sodium phosphate were simultaneously injected into the veins, the procedure at issue here involves the preliminary injection of the phosphate, followed by the injection of the manganese which could thus convert into phosphate because it finds sodium phosphate present in the circulation.

The tests in question are compiled on Table V.

The first two tests fully confirm the results obtained with colloidal manganese phosphate and with the simultaneous injections of phosphate and manganese. The other tests, performed with a constant quantity of sodium phosphate, show the correlation which develops between the antidotal preventive action of the sodium phophate against the manganese chloride and the time which elapses between the injections.

Table V. Successive injections of Na_3PO_4 and $MnCl_2$ into the veins of rabbits.

TABELLA V.

Iniezioni successive di Na_3PO_4 ed $MnCl_2$ nelle vene dei conigli.

1) Esperimento	2) Coniglio di kg	3) I. iniezione fosfato: 9) a g-n-mol per l.	in cc	10) in minuti	4) II. iniezione manganese: 9) a g-n-mol per l.	in cc	10) in minuti	5) Intervallo fra le iniezioni in minuti	6) g-n-mol × 10^6 per kg di coniglio: 11) fosfato	manganese	12) differenza	g atomo × 10^6 di manganese per kg di coniglio	7) Esito 8)
1	1,250	0,050	50	3,51	0,050	30	2,52	1,10	2083	1250	- 833	625	muore dopo più di 12 ore 13)
2	1,250	0,050	25	2,33	0,050	25	2,50	1,15	1042	1042	0	521	muore dopo più di 30 ore 14)
[*	1,000	—	—	—	—	—	—	0	1300	2080	+ 780	1040	muore immediatamente] 15)
3	0,970	0,050	25	2,40	0,050	23	2,5	5	1289	1186	— 103	593	» »
4	0,800	0,100	10,4	1,3	0,100	9	1,20	10	1300	1125	— 175	562	» »
5	0,820	0,100	10,7	1,35	0,100	9,1	1,30	15	1305	1110	- 195	555	» »
6	1,200	0,100	15,6	0,97	0,100	18,1	1,28	30	1300	1092	- 208	546	» »
[**	1,000	—	—	—	—	—	—	∞	1300	780	— 520	390	» »]

*) — Valori calcolati dalla somma della dose minima, sicuramente ed immediatamente letale (esp. 9, tabella I), più una quantità stechiometricamente uguale al fosfato, iniettato preventivamente.

**) — Valori calcolati dalla dose minima, sicuramente ed immediatamente letale (esp. 9, tabella I), supponendo che, trascorso un tempo lunghissimo dall'iniezione preventiva di fosfato, l'animale sia tornato del tutto normale.

1) Test no.
2) Rabbit weight, in kg.
3) I. Phosphate injection
4) II. Manganese injection
5) Intervals between injections, in min.
6) Gram-mole x 10^4 per kg rabbit
7) Gram-atoms x 10^6 of manganese per kg. rabbit
8) Death
9) Gram-mole/l
10) In minutes
11) Phosphate
12) Difference
13) Died after more than 12 hrs.
14) Died after more than 30 hrs.
15) Died immediately

*) - Values calculated from the sum of the minimum dose known to cause immediate death (test #9, Table I) and an amount stoichiometrically equal to phosphate, injected as a preventive measure.

**) - Values calculated from the minimum dose known to cause immediate death (test #9, Table I), assuming that after a very long time following preventive injection of phosphate, the animal is in a totally normal condition.

The elimination of the phosphate begins immediately after the preventive injection. Proportionately to the lengthening of the interval between the injections, the residue decreases. The manganese quantity which is transformable into phosphate will decrease proportionately to the increased length of time. It follows that the minimal surely and immediately lethal dose of manganese chloride will become continuously smaller.

Tests 3 to 6 on Table V and the graph shown in Figure 2., recording the interval between the two injections on the axis marked with A, while the $MnCl_2$ dose is recorded in g-n-mol on the axis under Y, demonstrate the variations of the immediately lethal dose of manganese as a function of the time elapsed from (the start of) the preventive and constant injection of 0,001300 g-n-mol sodium phosphate.

It can be logically assumed that whenever the interval between the two injections is lengthy, the entire phosphate would already have been eliminated, and the animal could recover to completely normal conditions, as if it had not received anything at all. It is then assumed that the certainly lethal dose for the animal in question would be the experimentally determined dose of 0,000390 g-atoms per kg; this is the limit towards which the function and the curve tend after an infinite period of time.

On the other hand, when it is assumed that the interval between the two injections is reduced to zero, in which case it seems as if the two injections were simultaneously applied, the immediate toxicity of the manganese chloride becomes practically zero; we have been able to verify the above experimentally. The manganese chloride is in this case chemically and pharmaceutically neutralized by the phosphate. Since the injected phosphate volume is 0,001300 g-n-mol, the animal would theoretically be able to tolerate 0,001300 g-n-mol of manganese unharmed.

In order to exterminate said animal, it would be necessary to inject additional manganese, as if it were a normal rabbit. Accordingly, the additional volume of 0,000780 g-n-mol would have to be injected, which is precisely the dose causing certain and immediate death when given to a sound rabbit weighing 1 kg. We can therefore calculate theoretically that after the preventive injection of 0,001300 g-n-mol phosphate and when the interval between injections is reduced to zero, the minimal immediately and certainly lethal dose of manganese chloride would be g-n-mol (0,001300+0,000780 = 0,002080) 0,002080; the function and the graph point to this value when the interval between the injections tends towards zero.

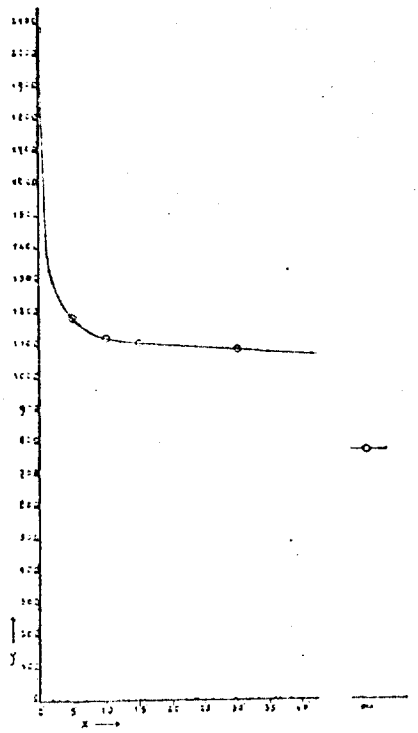

Fig. 2.

FIGURE 2.

Logically, the conclusion must be reached from these tests that as the manganese chloride entering the circulation reacts with the sodium phosphate which it finds there and which we introduced preventively, the same reaction occurs also with the physiologically pre-existent phosphate which it encounters there. We injected chloride;but the latter converts into manganese phosphate. The standard alkaline phosphates accordingly will act as natural antidotes against the manganese which could be lethal only after it exhausted said defensive chemical-physiological reserve in the animal. At the same time,it is logical to assume that when the organism is deprived of natural alkaline phosphates which convert into collodial manganese phsophate, a new toxicity factor will result from the latter which joins the toxicity factors inherent in the manganese.

V. Comparisons and conclusions.

For the synthetic purpose at which all these manganese studies are aimed and when summarizing results obtained with chloride, carbonate and phosphate, it is useful to compare them with the results as well which I obtained in the course of previous work with sodium permanganate[1] and with bisodium of manganese[2].

The toxicity of all these preparations, deduced from lethal doses and from the course of the intoxication,are summarized comparatively on Table VI. The lethal doses with all these preparations vary much less than could be expected, considering the considerable differences between them regarding their chemical structure and solubility.

1) L. Sabbatini: c.l, page 2.
2) Work in course.

TABLE VI.

Comparative toxicity of manganese compounds.

1 Esito dell' animale			2 Con g-atomo di Mn per kg di coniglio				
			3 permanganato di sodio	4 biossido di manganese	5 cloruro di manganese	6 carbonato di manganese	7 fosfato di manganese
8 Esito	9 sicuro, letale	12 immediatamente con dosi non minori di .	—	—	0,000390	0,000389	—
		13 dopo qualche tempo con dosi non minori di	0,000230	0,000230	—	—	0,000420
	10 variabile	14 immediatamente letale	—	—	0,000390	0,000389	
		15 letale dopo qualche tempo	—	—	—	—	0,000420
		16 non letale			0,000230	0,000250	0,000300
	11 sicuro,	17 non letale con dosi minori di	0,000230	0,000230	0,000230	0,000250	0,000300

1) Death of the animal

2) With g-atoms of Mn per kg of rabbit, of

3) sodium parmanganate
4) bisodium of manganese
5) manganese chloride
6) manganese carbonate
7) manganese phosphate

8) Death

9) certain, lethal

12) immediate, with doses not below. .
13) after some time, with doses not below . .

10) variable

14) immediately lethal . .
15) lethal after some time . .
16) non-lethal . . .

11) certain

17) non-lethal with doses below . .

Doses. - The minimal certainly lethal dose is the same, i.e. 0,00023, with bisodium of manganese as with permanganate which converts into bisodium at the moment when it is intravenously injected; it is a little higher, i.e. 0,00039, with carbonate and with chloride; it is slightly higher still, i.e. 0,00042, with phosphate into which the chloride and the carbonate presumably convert during the intravenous injection, encountering the alkaline phosphates in the blood.

The limit of the certainly non-lethal doses is similar to the first three: 0,00023; it is slightly higher with carbonate: 0,00023; and it is a little higher still with phosphate: 0,00030.

It results from the above that a certainly lethal dose is found with sodium permanganate and with bisodium of manganese; said dose indicates a definite limit between lethal and non-lethal doses: an interval exists between the certainly lethal and the certainly non-lethal doses of manganese chloride, carbonate and phosphate; the test result within said limit is not constant, but it varies from animal to animal. The interval at issue diminishes jointly with the decrease of the solubility of the salts: from chloride to carbonate and to phosphate.

Course of the intoxication. -

As a rule, death is never immediate with permanganate, bisodium and phosphate of manganese, even when doses are extremely high, because the two last-mentioned substances are collodial and the first converts into the collodial form of the bisodium at the first contact with blood during the intravenous injection.

Immediate death always results with manganese chloride and with manganese carbonate, which are soluble.

Symptomatology . - During the first moment, the permanganate develops its direct oxidizing effect in the blood , while it is reduced into manganese by hemolysis, hemoglobinemia, hemoglubinuria and anemia. During a second moment its action is identical with the action of the collodial bisodium of manganese. It seems that this (substance) as such has no perceptible effect; it reduces slowly and converts into the saline form. Its delayed paralyzing effect on the heart must be attributed to the manganous ion. The chloride, the carbonate and the phosphate have a paralyzing effect on the heart which is related to the manganous ion. It can occur immediately with soluble salts, with chloride and carbonate; with the latter, the minimal ionic concentration sufficient for causing paralysis is easily reachable. It always occurs late with collodial phosphate the solubility of which is very minor. With the latter, the minimal ionic concentration sufficient for causing paralysis can only be attained slowly.

The pharmacological action of all these preparations is therefore fundamentally very similar regarding dosage, course and symptomatology of the intoxication. This is due to the fact that all tend to convert into the single form of manganous phosphate in the animal organism.

Toxicity factors. - The chemical changes to which said manganese preparations are subject in the organism are schematically represented in Figure 3. It exactly summarizes all observed facts and indicates the factors with pharmacological effect, as well as their succession.

The factors at issue are: the oxidizing active and immediate factors of the permanganate; their effects on the local activity of the blood is considerable. They are the slower oxidizing factors of the manganese bisodium, and their effects are not very conspicuous. They depend on the Mn^{++}cation, and they manifest themselves with immediate and very serious effects, or with slow and delayed effects, depending on the concentration reached. The effects are immediate

and very serious with the highly soluble chloride and with carbonate which has a fairly good solubility. The effects occur slowly and with delay in conjunction with permanganate , bisodium and with phosphate: the ionic concentration of the manganese is always very low with these substances.

Other factors must be added to these direct factors of the pharmacological activity; they are indirect and secondary. Such factors are: the destruction of the red corpuscles caused by the permanganate and the subsequent hemoglobinemia, hemoglobinuria and anemia; the phosphate withdrawal which is finally caused by all preparations, with the exception of manganese phosphate. The latter always proves to be the least toxic among them. The above factors include the modification of the ionic equilibrium which follows the withdrawal of the phosphates.

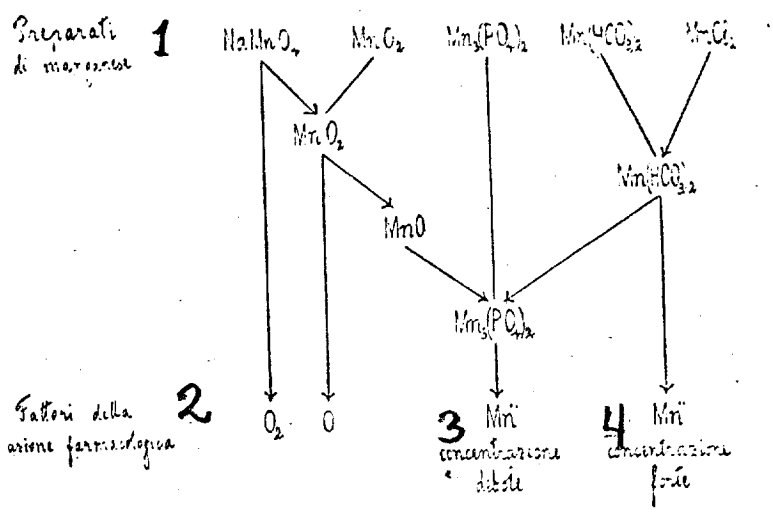

FIGURE 3.

1) Manganese preparations
2) Factors of the pharmacological action
3) Concentration, weak
4) Concentration, strong

Defense potentials of the organism. - Some of the above-mentioned facts which, in correlation with manganese, are toxicity factors, become means of defense of the organism through which it achieves the neutralization of the poison.

The reducing effect of the red corpuscles on the permanganate acts as an antidote against the permanganate's oxidizing effect. Permanganate injected into the veins decomposes on the red corpuscles, which constitutes a direct and intense local action in the blood; but it becomes impossible for the same reason to obtain a general, true and exact permanganate action.

The immediate toxicity of the soluble manganese salts is due to the cation which, jointly with said salts, can rapidly reach a high degree of concentration; but the alkaline phosphates of the organism, reacting with them, convert them into a phosphate having minor solubility which (phosphate) does not have an immediate toxic effect. Toxicity will result only when the means of defense are exhausted.

Arch. Sci. Biol. 16: 141-159, 1931

RICERCHE FARMACOLOGICHE SUL MANGANESE. XII: Azione comparata del cloruro, del carbonato e del fosfato di manganese.

Del prof. L. SABBATANI [1].

1. Scopo delle ricerche e metodo sperimentale.

Volendo studiare l'azione farmacologica dei sali manganosi, il più adatto di tutti è indubbiamente il cloruro per le sue caratteristiche chimiche e fisico-chimiche e per la innocuità dell'azione. Ma appena incominciamo gli esperimenti e lo iniettiamo negli animali sorge il dubbio che il cloruro passi immediatamente ad altre forme saline poco solubili di carbonato e fosfato manganoso, e quindi gli effetti che osserviamo non siano da riferire esclusivamente al cloruro iniettato, ma a questi altri sali, che si formano appena incontra nel circolo sanguigno i carbonati e fosfati alcalini [2].

Per questo accanto all'azione del cloruro ho studiata comparativamente anche quella del carbonato e del fosfato di manganese. E nel fissare le modalità tecniche dell'esperimento ho cercato di stare per quanto era possibile vicino alle condizioni chimiche che incontriamo nell'animale vivo. Ho cercato altresì che per le condizioni generali dell'esperimento i risultati ottenuti con questi sali fossero comparabili con quelli che ho precedentemente ottenuti col perman-

[1] Questo lavoro, già pronto per la stampa, è stato trovato nella Biblioteca Comunale di Imola dopo la morte del compianto prof. L. SABBATANI. Il prof. I. SIMON ne ha completato solamente le note. Mi è gradito dovere pubblicarlo come omaggio alla memoria dell'illustre biologo. F. B.

[2] Questo è già stato dimostrato da SEREMIN (*Archiv. f. exper. Pathologie u. Pharmakologie*, 1924, **105**. 89) per i sali solubili di piombo che passano a fosfocarbonato e dalle ricerche di MESSINA (*Archiv. f. exper. Pathol. u. Pharmak.* 1928, **135**. 3) si risulta che passano a fosfato i sali ferrosi.

ganato di sodio e col biossido di manganese colloidale [1]. E ciò perchè lo scopo ultimo cui mirano tutte queste ricerche è di trovare le forme chimiche ultime e costanti a cui arriva sempre il manganese nell'organismo, qualunque sia la diversità dei preparati da cui si parte. Forme chimiche ultime e costanti, che sono la causa diretta di quella azione farmacologica generale del manganese, che potremmo dire vera e fondamentale, in quanto non è più modificata da fattori chimichi e biologici contingenti a speciali preparati, ma è comune a tutti.

In queste esperienze comparative ho adoperato quasi sempre soluzioni di $MnCl_2$, $MnCO_3$ ed $Mn_3(PO_4)_2$ colloidale a g-atomo di manganese 0.025 per litro [2]: le usava sempre per iniezione endovenosa e sempre nei conigli. Calcolava le dosi per kg corporeo e le velocità d'iniezione in g-atomo di manganese.

Col cloruro, col carbonato e col fosfato manganoso ho fatte *cinque serie di esperienze*.

In una *prima serie di esperienze* ho adoperato del cloruro di manganese cristallizzato, puro per analisi della Ditta C. Erba.

In una *seconda serie di esperienze* ho adoperato del carbonato di manganese: ma ho incontrata qualche difficoltà tecnica già subito per la preparazione delle soluzioni. A me occorreva una soluzione a g-mol 0.025 per litro, il carbonato invece ha una solubilità molto più bassa di g-mol 0.000445 per litro a 25°.

Mescolando due soluzioni equimolecolari di cloruro di manganese e di carbonato sodico, in modo da ottenere una soluzione a g-mol 0.025 per litro, si ha un precipitato immediato di carbonato e idrossido di manganese, la cui composizione varia colla diluzione e colla temperatura: ogni tentativo per impedire la precipitazione e per ottenere una forma colloidale mi è riuscito vano [3].

Il modo più sicuro per ottenere il carbonato di manganese è di adoperare due soluzioni di $MnCl_2$ e di Na_2CO_3, sature entrambe di CO_2. Operando così si riesce ad ottenere una miscela che corrispende a g-mol 0.025 di $MnCO_3$ per litro, che resta limpidissima per circa una mezz'ora e che precipita poi solo lentamente. Nella mezz'ora in cui questa miscela resta limpida c'è tempo più che sufficiente per poterla sperimentare sugli animali: dal punto di vista chimico la costituzione sua è incerta e varia dal momento della miscela alla comparsa del precipitato: ma dal punto di vista biologico questa soluzione è più vicina alla realtà dei fatti che vogliamo studiare, perchè se pensiamo che nell'organismo animale si possa formare un carbonato di manganese, sarà sempre e solo da bicarbonati in presenza di CO_2 in eccesso, e non da carbonati neutri, che nel sangue non esistono.

Per tutto questo, volendo sperimentare col carbonato di manganese e stare per quanto è possibile vicini alla realtà delle condizioni biologiche nelle quali si svolge l'esperimento farmacologico, ho adoperate delle miscele fatte con volumi uguali di $MnCl_2$ ed Na_2CO_3 a g-mol 0.050 per litro, sature entrambe di CO_2, e le preparava sempre di volta in volta, pochi minuti prima di iniettarle nella vena degli animali.

Operando così, in realtà facciamo in vitro pochi minuti prima dell'iniezione quello che subito dopo l'iniezione e nel sangue può avvenire, quando si inietta del cloruro di manganese e vi incontra del bicarbonato sodico e dell'acido carbonico libero.

Quando per brevità in questa nota parlerò del carbonato di manganese, e scriverò $MnCO_3$, s'intende che lo faccio colle riserve ora dette.

In una *terza serie di esperienze* ho adoperato del fosfato di manganese, ma ho incontrate delle difficoltà tecniche, ancora maggiori che per il carbonato, nella preparazione, nella solubilità sua estremamente piccola [1], nella instabilità grandissima delle soluzioni colloidali che può dare.

Fin dai primi saggi dovetti abbandonare quei metodi di preparazione che mi avrebbero dato un fosfato puro, ma non mi permettevano di averlo in soluzione colloidale ed alla concentrazione necessaria.

Dovetti accontentarmi di preparare estemporaneamente le soluzioni colloidali con quei reattivi che meglio si adattavano all'esperimento biologico, e con quella tecnica che meglio si adattava alla instabilità grandissima del colloide.

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[1]) SABBATANI L.: Azione farmacologica del permanganato di sodio. Questo *Archivio*, 1928, 12, 50.

[2]) Questa concentrazione non è molto diversa da quella di g-atomo di Mn 0.017, usata nelle esperienze col permanganato di sodio e da quella di g-atomo di Mn 0.020, usata nelle esperienze col biossido di manganese colloidale.

[3]) Vedasi per i richiami l'articolo « Manganese » di A. MIOLATI in Abegg-*Handbuch der Anorganischen Chemie*, 1913, 4, (2), 811.

[1]) MIOLATI, l. c., pag. 702.

Mescolando del cloruro di manganese con del fosfato bisodico in eccesso, si ottiene del $Mn_3(PO_4)_2$ come precipitato amorfo, insolubile per noi; contemporaneamente resta nel liquido un eccesso di fosfato bisodico e compare del fosfato acido, di azione farmacologica non certo trascurabile.

Mescolando delle soluzioni a g-nor-mol 0,1 per litro di cloruro di manganese e di fosfato trisodico in quantità equivalenti dovremmo ottenere del $Mn_3(PO_4)_2$ puro, e nel liquido dovrebbe restare solo del cloruro di sodio, che non potrebbe alterare in nessun modo l'esperimento farmacologico [1]; ma il fosfato di manganese in queste condizioni precipita immediatamente e tutti i tentativi fatti per farlo rimanere allo stato colloidale sono riusciti vani.

Mescolando invece le stesse soluzioni sature di CO_2 si forma immediatamente una bella soluzione colloidale, che però ha una vita brevissima ed in 10 secondi al massimo flocula e precipita.

Operando così non siamo affatto sicuri che si formi proprio esclusivamente del fosfato trisodico, o non piuttosto una miscela di fosfato e carbonato, o qualche sale complesso, ma è qui opportuno ricordare che se dal punto di vista chimico queste condizioni non sono le più adatte per ottenere un prodotto puro, dal punto di vista biologico sono quelle che meglio si avvicinano alla realtà dei fatti farmacologici che stiamo studiando.

Quando, come ho detto in principio, pensai di sperimentare col fosfato di manganese, era perchè dubitava che, iniettando nelle vene del cloruro di manganese, questo incontrasse nel sangue dei fosfati e formasse con essi un fosfato colloidale. Ora è opportuno ricordare che, se questa reazione avviene realmente, si formerà coi reattivi che abbiamo nel sangue, cioè con del fosfato bisodico in presenza di bicarbonati alcalini e di acido carbonico libero. Questi sono gli stessi reattivi che ho nelle due soluzioni con cui preparo il fosfato di manganese, perchè adoperare una soluzione di fosfato trisodico satura di CO_2 è come adoperare una soluzione mista di fosfato bisodico, di bicarbonato sodico e di acido carbonico.

Per tutte queste considerazioni, volendo preparare una soluzione colloidale che potesse corrispondere a g-norm.-mol 0,050 per litro di $Mn_3(PO_4)_2$, ho usate due soluzioni a g-norm.-mol 0,1 per litro di $MnCl_2$ e di Na_3PO_4, sature entrambe di CO_2.

[1]) È anzi utile per la tonicità della soluzione.

La soluzione colloidale che così si ottiene è però instabilissima e flocula in 10 secondi circa. A questo gravissimo inconveniente ho rimediato, costruendo due burette confluenti in un tubo ad Y, verso la cannula con cui si fa l'iniezione endovenosa.

Riempite le burette rispettivamente colle due soluzioni sopradette, la miscela viene fatta da un agitatore automatico, posto nel punto di confluenza delle soluzioni; la reazione e la formazione del fosfato colloidale avviene così mano mano che i liquidi arrivano alla cannula d'iniezione [1], e fra la preparazione della soluzione e l'entrata nella vena può intercorrere un tempo brevissimo di pochi secondi. Si inietta allora la soluzione quando ha sicuramente ancora un alto grado di dispersità.

In una *quarta serie di esperienze*, invece di mescolare le due soluzioni di cloruro di manganese e di fosfato trisodico (sature entrambe di CO_2) prima di iniettarle nella vena, le iniettava contemporaneamente e separatamente in quantità equivalenti nelle due vene giugulari. Così la miscela, la reazione e la formazione del fosfato di manganese colloidale avveniva direttamente nel sangue stesso.

In una *quinta serie di esperienze* iniettava nelle vene ancora le due soluzioni sopradette, ma prima quella del fosfato e poi dopo un certo tempo quella del manganese, e studiava così come una iniezione preventiva di fosfato modificasse l'azione farmacologica del manganese.

II. - Esperienze col cloruro di manganese.

Col cloruro di manganese ho fatte 25 esperienze. Dopo alcuni saggi preliminari [2]) adottai una soluzione a g-atomo 0,025 per litro e con questa feci poi tutte le altre esperienze. Sono riassunte nella tabella I e da esse vediamo che la dose minima immediatamente e sicuramente letale è di g-atomo 0,00039 di manganese per kg corporeo di coniglio.

[1]) Sabbatani L.: Burette per lo studio farmacologico di colloidi instabilissimi. *Bollettino della Società It. di Biologia sper.*, 1928, **3**, 205. Come si possono sterilizzare farmacologicamente dei colloidi instabilissimi. *Biochimica e Terapia sper.*, 1929, **15**, fasc. 4°.

[2])

Esperienza	Concentrazione della soluzione usata in g atomo per L
1	1,000
2	0,100
4	0,050
10	0,000

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TABELLA I.

Cloruro di manganese iniettato nelle vene dei conigli.

Esperienza	Coniglio di kg	Soluzione di $MnCl_2$ iniettata: a g-atomo per L	in cc	in minuti	g atomi di Mn × 10^6: iniettati	per kg corporeo	per kg e minuto	Osservazioni
1	1,000	1,000	1,3	0,30	1500	1500	3000	muore immediatamente
2	1,100	0,100	7,5	0,55	750	682	760	» »
3	1,100	0,025	25	3,20	625	568	171	» »
4	1,000	0,050	9,6	1,20	480	480	370	» »
5	1,100	0,025	20,2	3,27	505	459	135	» »
6	1,070	0,025	18,8	3,2	470	439	146	» »
7	0,920	0,025	15,5	2,27	387	421	175	» »
8	1,120	0,025	17,9	2,38	447	400	134	sintomi gravissimi, sopravvive
9	1,200	0,025	18,7	1,51	467	390	216	muore immediatamente
10	1,070	0,020	20	2,83	400	374	150	nessun disturbo, sopravvive
11	1,370	0,025	19,7	2	490	359	180	muore immediatamente
12	1,100	0,025	15,4	1,42	385	350	206	nessun disturbo, sopravvive
13	1,000	0,025	14	1,47	350	350	194	muore dopo più di 8 ore
14	0,970	0,025	13,2	3,23	330	340	100	nessun disturbo, sopravvive
15	1,320	0,025	17,3	2,22	433	327	136	muore in meno di 24 ore
16	1,150	0,025	15	1,46	375	326	181	muore immediatamente
17	1,020	0,025	13,1	1,55	328	321	169	» »
18	0,900	0,025	11,2	1,44	280	311	183	» »
19	1,170	0,025	14	3,36	310	265	76	nessun disturbo, sopravvive.
20	0,950	0,025	9,5	1,17	237	250	190	muore dopo alcune ore
21	1,020	0,025	9,8	1	245	240	240	nessun disturbo, sopravvive
22	1,020	0,025	9,4	0,45	235	230	330	muore dopo alcune ore
23	1,220	0,025	10,8	0,41	270	221	316	nessun disturbo, sopravvive
24	1,220	0,025	10,2	0,48	255	209	261	» » »
25	1,150	0,025	9,2	0,33	230	200	363	» » »

Con dosi comprese fra g-atomo 0,00039 e 0,00023 si ha una grande variabilità di risultati, e gli animali o muoiono immediatamente, o muoiono solo dopo un certo tempo, o sopravvivono senza presentare nessun disturbo apprezzabile, immediato o tardivo.

In fine con dosi minori di g-atomo 0,00023 per kg corporeo gli animali sopravvivono sicuramente.

Quando colle dosi alte e medie la morte avviene immediata coll'iniezione stessa, è dovuta ad un'azione tossica diretta ed intensa che il cloruro di manganese spiega sul cuore. Provoca un rallentamento della frequenza dei battiti, una diminuzione della pressione arteriosa, ed infine il cuore bruscamente si arresta (fig. 1). Allora

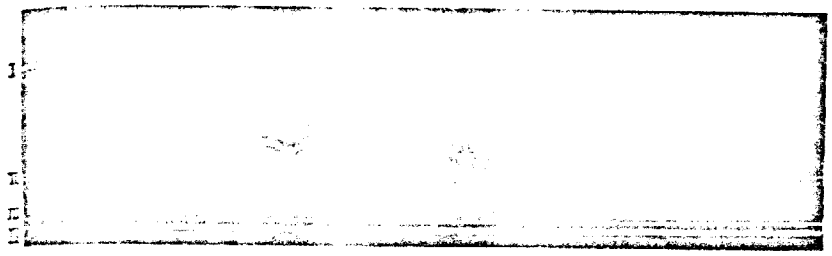

Fig. 1.

I. - Tracciato della pressione alla carotide.
II. - Pressione zero.
III. - Segnale della iniezione endovenosa: *A* principio, *B* fine.
IV. - Tempo in minuti secondi.

per l'anemia dei centri nervosi scoppiano accessi convulsivi generali, violenti, dopo i quali l'animale cade in paralisi generale. Il respiro si arresta ed a nulla giova la respirazione artificiale: l'animale muore. Sezionato subito, si trova che il cuore è fermo in diastole, o fa solo lievi movimenti fibrillari.

Quando la morte avviene tardiva colle dosi medie è ancora dovuta ad un'azione tossica sul cuore. Durante l'iniezione gli animali non presentano nessun disturbo, o tutt'al più una fugace e leggera diminuzione nella frequenza dei battiti cardiaci. Finita l'iniezione stanno in tutto benissimo, sono vivaci e mangiano, ma poi dopo alcune ore improvvisamente sono colti da convulsioni generali, toniche a carattere asfittico, dopo le quali il respiro si arresta.

Quando colle dosi inferiori a g-mol 0,000230 gli animali sopravvivono sicuramente, non si nota mai nè durante l'iniezione, nè dopo alcun disturbo apprezzabile.

Per vedere come il cloruro manganoso modifichi l'attività del cuore e la pressione arteriosa nei conigli ho fatte tre esperienze [1])

[1]) Esp. 26. Coniglio di kg 2,210, cc 19 di soluzione in 64 secondi, g-atomo 0,00039 per kg. — Esp. 27. Coniglio di kg 2,250, cc 20 di soluzione in 154 secondi, g-atomo 0,000444 per kg. — Esp. 28. Coniglio di kg 2,050, cc 12,3 di soluzione in 40 secondi, g-atomo 0,00290 per kg.

nelle quali, mentre iniettava con velocità costante nella giugulare sinistra la solita soluzione di $MnCl_2$ a g-atomo 0,050 per litro, prendeva il tracciato manometrico alla carotide destra, e seguitava la iniezione fino a che il cuore s'arrestava.

I risultati ottenuti sono concordi, e qui perciò riporto nella Fig. 1 solo il tracciato dell'esperienza 27. Da esso vediamo che appena comincia l'iniezione la pressione cade bruscamente da mm 129 (normale) a mm 90. Mentre l'iniezione continua, la pressione resta poi ferma per un certo tratto a questo valore, probabilmente perchè si è immediatamente raggiunto l'equilibrio fra l'azione del manganese che arriva in circolo con velocità costante e particolari poteri di difesa automatici dell'organismo.

Seguitando ulteriormente l'iniezione, s'arriva ad un punto in cui questi mezzi di difesa cominciano a fare difetto e la pressione comincia una seconda discesa lenta, graduale, continua; contemporaneamente il cuore batte sempre più lentamente. S'arriva così a valori molto bassi, incompatibili colla vita: compaiono violenti scosse convulsive asfittiche, dopo le quali il cuore è fermo. Si hanno movimenti di boccheggiamento, dopo i quali tutto pare finito, e l'animale si può considerare morto: ma in realtà, trascorsi quaranta secondi circa, il cuore riprende a pulsare molto lentamente, ma con una certa forza e regolarità di ritmo, e questo dura per circa due minuti e mezzo con una lenta e graduale degradazione, fino a che poi il cuore s'arresta definitivamente e l'animale è morto irremissibilmente.

Se mentre il cuore riprende a battere si aiuta l'animale colla respirazione artificiale, può anche campare.

I poteri di difesa automatici che l'organismo ha contro l'azione del cloruro di manganese consisterebbero, come vedremo in seguito, nei fosfati alcalini coi quali reagisce il cloruro di manganese, forma un fosfato insolubile, colloidale, che non ha tossicità immediata, ma solo molto lenta e tardiva. La ripresa del cuore dopo un primo arresto potrebbe dare ragione della incertezza dei risultati che si hanno con dosi non troppo alte, comprese fra g-atomo 0,00039 e 0,00023 per kg.

TABELLA II.

Carbonato di manganese iniettato nelle vene del coniglio.

Esperienza		Coniglio	Soluzione di $MnCO_3$ iniettata					g-atomi di $Mn \times 10^3$			Osservazioni
numero	numero d'ordine	di kg	numero	a g-atomo per L	preparata da minuti	in cc	in minuti	iniettati	per kg corporeo	per kg e minuto	
1	24	1,160	V	0,025	10	15,5	1,10	462	120	217	morì subito
2	18	1,620	II	0,025	30,30	21,0	2,20	525	398	173	» »
3	25	1,400	V	0,025	20	21,8	1,45	546	380	229	» »
4	22	0,900	VI	0,025	23	13,7	1,20	342	380	293	sopravvisse
5	20	1,450	VII	0,025	6	21,6	0,74	541	375	151	»
6	31	1,160	VII	0,025	30	21,7	2,12	543	468	150	»
7	17	1,870	II	0,025	15,30	27,7	1,26	692	370	264	»
8	16	1,370	I	0,020	5,43	25,0	2,17	500	365	160	»
9	23	0,900	IV	0,025	95	12,8	2,9	320	355	109	morì subito
10	34	1,250	VIII	0,025	17	17,6	1,57	437	350	175	» »
11	35	1,370	IX	0,025	10	18,8	2,27	470	343	140	sopravvisse
12	29	1,400	VI	0,025	19	19,0	2,29	457	339	136	morì entro 48 ore
13	36	1,170	IX	0,025	18	15,7	1,85	392	335	215	morì subito
14	33	1,000	VIII	0,025	32	13,2	1,10	330	330	282	sopravvisse
15	26	1,100	V	0,025	30	14,2	1,47	355	323	180	morì subito
16	32	1,000	VIII	0,025	40	12,6	1,33	315	315	252	»
17	28	1,240	VI	0,025	13	15,4	1,32	385	310	207	sopravvisse
18	30	1,050	III	0,022	13	14,3	1,66	318	303	190	»
19	38	1,280	XI	0,026	15	14,5	5,40	362	295	52	morì entro 20 ore
20	21	1,120	III	0,022	21	14,3	1,91	318	284	218	sopravvisse
21	39	1,250	XI	0,026	22	14,0	1,4	320	256	261	»
22	19	0,840	III	0,022	6	10,1	0,49	224	267	331	»
23	37	1,400	X	0,026	23	14,6	2,48	365	261	93	morì entro 40 ore
24	27	1,070	VI	0,025	6	10,7	1,2	267	250	250	sopravvisse
25	40	1,100	XII	0,025	8	10,6	0,43	265	241	344	»
26	41	1,150	XII	0,025	13	10,6	0,57	265	230	256	»
27	42	1,250	XII	0,025	17	11,0	0,42	275	220	314	»

III. - Esperienze col carbonato di manganese.

Col carbonato di manganese ho fatte 27 esperienze, quasi sempre adoperando una soluzione a g-atomo 0,025 per litro [1].

Sono riassunte nella tabella II, e da esse vediamo che la dose minima, immediatamente e sicuramente letale è di g-atomo 0,000389 di manganese per kg corporeo di coniglio.

Con dosi comprese fra g-atomo 0,000389 e 0,000250 si ha una grande variabilità di risultati: e gli animali o muoiono immediatamente, e muoiono solo dopo un certo tempo, o sopravvivono senza presentare nessun disturbo apprezzabile, immediato o tardivo.

In fine con dosi minori di g-atomo 0,000225 per kg corporeo gli animali sopravvivono sicuramente.

La sintomatologia, sia quando la morte avviene immediata, sia quando avviene tardiva, è sempre uguale a quella descritta per il cloruro di manganese.

IV. - Esperienze col fosfato di manganese.

1. - *Fosfato di manganese colloidale.*

Col fosfato di manganese colloidale ho fatte 16 esperienze, adoperando soluzioni a g-atomo di manganese 0,0250 per litro [2]. L'età delle soluzioni colloidali era nell'esperienza 4 di 4'', nella 1 di 2'' ed 8/10, nella 2 di 2'' e 2/10 ed in tutte le altre era sempre compresa fra 1'' e 2''.

Queste esperienze sono riassunte nella tabella III. Da esse vediamo che col fosfato di manganese colloidale non si ottiene mai la morte immediata, ed anche colla dose altissima di g-atomo 0,001006 per kg corporeo (esperienza I) l'animale morì solo 19 ore dopo la iniezione.

La dose minima sicuramente letale, dopo un tempo variabilissimo, da 19 ore a 15' minuti, è di g-atomo 0,00042 per kg corporeo.

Con dosi comprese fra g-atomo 0,000420 e 0,000300 si ha una grande variabilità di risultati; e gli animali muoiono dopo uno o due giorni, oppure sopravvivono all'esperimento.

[1]) Solo nelle esperienze 18, 20, 22 usai una soluzione di g-atomo 0,022 per L e nella esperienza 8 una soluzione a g-atomo 0,020 per L.

[2]) Solo in due esperienze adoperai soluzioni a g-atomo 0,0125 di manganese per litro.

Tabella III.

Fosfato di manganese iniettato nelle vene dei conigli.

Esperienze	Coniglio di kg	Soluzione di $Mn_3(PO_4)_2$ iniettata: g-atomi di Mn per L. $\times 10^4$	Soluzione di $Mn_3(PO_4)_2$ iniettata: in cc	Soluzione di $Mn_3(PO_4)_2$ iniettata: in minuti	Soluzione di $Mn_3(PO_4)_2$ iniettata: dell'età di minuti secondi	g-atomi di Mn $\times 10^4$ iniettati	g-atomi di Mn $\times 10^4$ per kg corporeo	g-atomi di Mn $\times 10^4$ per kg e minuto	Osservazioni
1	1,250	250	50,3	4,42 0,24	2, 0,80	1257	1006	211	morte dopo 19 ore dall'iniezione
2	1,250	250	38,9	2,50 0,76	2, 0,19	972	778	273	» 12 ore circa
3	1,150	125	68,3	3,15 0,40	1, 0,43	854	742	228	» 19 ore circa
4	1,150	125	63,4	3,2	4, 0,04	792	689	230	» poco i minuti dall'iniezione
5	1,300	250	29,2	1,34 0,54	1, 0,29	730	561	367	» 10 minuti
6	1,270	250	26,3	1,28 0,54	1, 0,70	657	517	351	» minuti 17 ½
7	1,100	250	19,8	1,12 0,60	1, 0,47	495	450	375	» 18 ore
8	1,500	250	25,2	1,59 0,51	1, 0,90	630	420	210	» 15 minuti
9	1,050	250	16,3	0,51 0,30	1, 0,57	407	400	461	sopravvive, cessa l'osservazione dopo 17 giorni
10	1,130	250	17	1,15 0,40	1, 0,77	425	379	301	sopravvive, cessa l'osservazione dopo 16 giorni
11	1,000	250	14	0,44 0,80	1, 0,28	350	350	466	morte dopo 36 ore circa
12	1,250	250	16,3	0,43 0,19	1, 0,32	407	326	453	sopravvive, cessa l'osservazione dopo 16 giorni
13	1,270	250	15,2	0,68 0,70	1, 0,52	380	300	306	morte dopo 18 ore circa
14	0,970	250	10,8	0,29	1, 0,71	270	278	556	sopravvive, cessa l'osservazione dopo 16 giorni
15	1,120	250	11,6	0,29	1,	290	259	555	» »
16	1,200	250	11,8	0,30 0,30	1, 0,04	290	242	484	» »

In fine con dosi minori di g-atomo 0,000390 per kg corporeo gli animali sopravvivono sicuramente.

La sintomatologia è uguale a quella che si ha col cloruro e col carbonato di manganese, quando gli animali muoiono solo qualche tempo dopo l'iniezione.

2. - *Fosfato di sodio e cloruro di manganese. Iniezioni contemporanee.*

Nelle esperienze precedenti il fosfato di manganese colloidale era preparato mescolando le due soluzioni reattive nel momento stesso dell'iniezione. In queste esperienze invece ho iniettato contemporaneamente nelle vene giugolari volumi uguali delle due soluzioni equivalenti di cloruro di manganese e di fosfato sodico, in modo che la

TABELLA IV.

Iniezioni endovenose contemporanee di Na_3PO_4 ed $MnCl_2$ nei conigli.

Esperienza	Coniglio di kg	Entrambe le soluzioni iniettate: a g-n-mol per L	in cc	in minuti	g-n-mol × 10⁶ di fosfato di sodio e di cloruro di manganese: iniettati	per kg di coniglio	per kg e minuto	Osservazioni
1	0,800	0,100	20,6	13,57	2060	2575	185	morte alla fine dell'iniezione
2	1,500	0,050	50,0	10	2500	1666	166	» dopo parecchie ore
3	1,170	0,100	17,9	2,27	1790	1530	624	» immediatamente
4	1,430	0,050	35,0	4,19	1750	1182	274	» dopo parecchie ore
5	1,300	0,050	25,0	2,33	1250	962	378	» immediatamente

mescolanza loro, la reazione chimica e la formazione del fosfato di manganese avvenisse nel sangue circolante. Anche in queste condizioni il fosfato di manganese che si forma nel plasma sanguigno è colloidale e non dà precipitati, grumi o coaguli, che possano compromettere per fatti meccanici l'osservazione farmacologica.

Il risultato di queste esperienze, che sono riassunte nella tabella IV, concorda bene con quelli ottenuti col fosfato di manganese colloidale, quando si tenga conto di quello che si è detto sopra, circa la mescolanza di vene liquide confluenti. Qui le due soluzioni entrano per le due vene giugulari, mescolate col sangue confluiscono al cuore e quivi per i vortici che si formano la mescolanza e la reazione chimica sarà completa; ma non è escluso che qualche filetto liquido

ancora separato possa dare sul cuore destro l'azione diretta ed immediata del cloruro di manganese. Così si comprende come in tre esperienze (1, 3, 5) si sia potuta avere morte immediata, ma sempre con dosi altissime di g-atomo di manganese (× 10⁶) 1287,5-765-481 per kg di coniglio.

3. - *Iniezioni preventive di fosfato sodico a quelle del cloruro di manganese.*

Mentre nelle esperienze precedenti iniettava nelle vene contemporaneamente le due soluzioni reattive di cloruro di manganese e di fosfato sodico, in queste inietto prima il fosfato e poi il manganese, che potrà così passare a fosfato in ragione del fosfato sodico che trova in circolo.

Queste esperienze sono riassunte nella tabella V.

Le prime due confermano pienamente quello che si è ottenuto col fosfato di manganese colloidale e colle iniezioni contemporanee di fosfato e di manganese. Le altre esperienze, fatte con una quantità costante di fosfato sodico, mostrano il rapporto che passa fra l'azione antidotica preventiva del fosfato sodico verso il cloruro di manganese ed il tempo che intercorre fra le iniezioni.

Subito dopo l'iniezione preventiva di fosfato comincia la sua eliminazione, quello che resta è tanto minore, quanto più lungo è l'intervallo fra le iniezioni. Corrispondentemente col crescere del tempo va diminuendo la quantità di manganese che può essere trasformata in fosfato, e conseguentemente la dose minima sicuramente ed immediatamente letale del cloruro di manganese si impicciolisce sempre più.

Le esperienze 3-6 della tabella V e la grafica della fig. 2 in cui sull'asse delle x è notato l'intervallo fra le due iniezioni e sull'asse delle y la dose di $MnCl_2$ in g-n-mol, mostrano come varî la dose minima, sicuramente ed immediatamente letale del manganese, in funzione del tempo trascorso dall'iniezione preventiva e costante di g-n-mol 0,001300 di fosfato sodico.

È logico ammettere che quando l'intervallo fra le due iniezioni sia lunghissimo, tutto il fosfato sia già eliminato e l'animale sia tornato in condizioni del tutto normali, come se non avesse ricevuto nulla. Per questo animale si prevede allora che la dose minima immediatamente e sicuramente letale sarà quella stessa già trovata sperimentalmente di g-atomo 0,000390 per kg e questo è il limite cui tende la funzione e la curva dopo un tempo infinito.

D'altro lato quando si suppone che l'intervento fra le due iniezioni sia ridotto a zero, allora è come se le iniezioni fossero fatte contemporaneamente ed in questo caso abbiamo già visto sperimentalmente che la tossicità immediata del cloruro di manganese diventa praticamente nulla. Esso è neutralizzato chimicamente e farmacologicamente dal fosfato, e poichè qui si iniettano g-n-mol 0,001300 di fosfato, l'animale teoricamente potrà sopportare senza danno g-n-mol 0,001300 di manganese, e per ucciderlo converrà iniettargli ancora del manganese, come se fosse un coniglio normale. Iniettarne cioè altri g-n-mol 0,000780, quali appunto occorrono a dare morte sicura ed immediata nel coniglio sano, del peso di un kg. Quindi teoricamente possiamo calcolare che dopo iniezione preventiva di g-n-mol 0,001300 di fosfato, quando l'intervallo delle iniezioni fosse ridotto a zero, la dose minima, immediatamente e sicuramente letale del cloruro di manganese diventerebbe di g-n-mol (0,001300 + + 0,000780 = 0,002080) 0,002080, ed a questo valore tende la funzione e la gratica, quando l'intervallo fra le iniezioni tende a zero.

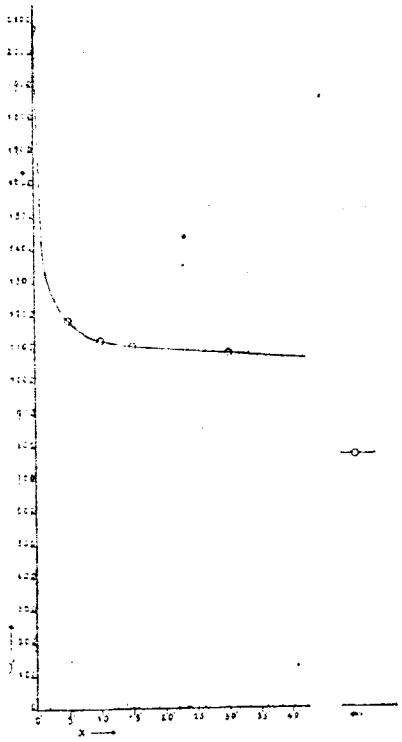

Fig. 2.

Da queste esperienze logicamente si deve dedurre che come il cloruro di manganese entrando in circolo raegisce col fosfato sodico che vi incontra, da noi stessi preventivamente introdotto, così reagisce anche col fosfato che vi incontra, fisiologicamente preesistente. Iniettiamo del cloruro, ma questo passa a fosfato di manganese. I fosfati alcalini normali agiranno così da antidoto naturalmente contro il manganese, che potrà riuscire letale solo quando abbia esaurita nell'animale questa riserva di difesa chimica-fisiologica. Contemporaneamente è logico ammettere che, impoverito l'organismo di fosfati alcalini naturali, che passano a fosfato colloidale di

TABELLA V.

Iniezioni successive di Na_3PO_4 ed $MnCl_2$ nelle vene dei conigli.

Esperienza	Coniglio di kg	I. Iniezione fosfato: a g-n mol per l.	I. Iniezione fosfato: in cc	I. Iniezione fosfato: in minuti	II. Iniezione manganese: a g-n mol per l.	II. Iniezione manganese: in cc	II. Iniezione manganese: in minuti	Intervallo fra le iniezioni in minuti	g-n-mol × 10⁴ per kg di coniglio: fosfato	g-n-mol × 10⁴ per kg di coniglio: manganese	g-n-mol × 10⁴ per kg di coniglio: differenza	g-n-mol × 10⁴ di manganese per kg di coniglio	Esito
1	1,200	0,050	50	3,51	0,050	30	2,52	1,10	2083	1250	- 833	625	morte dopo più di 12 ore
2	1,200	0,050	25	2,33	0,050	25	2,50	1,15	1042	1042	0	521	morte dopo più di 6 ore
[* 1,000	—	—	—	—	—	—	—	0	1300	2080	+ 780	1040	morte immediatamente]
3	0,970	0,050	25	2,40	0,050	23	2,5	5	1289	1186	— 103	593	»
4	0,800	0,100	10,4	1,3	0,100	9	1,20	10	1300	1125	— 175	562	»
5	0,820	0,100	10,7	1,35	0,100	9,1	1,30	15	1305	1110	195	555	»
6	1,200	0,100	15,6	0,37	0,100	18,1	1,28	30	1300	1092	- 208	546	»
[** 1,000	—	—	—	—	—	—	—	∞	1300	780	— 520	390	»

*) — Valori calcolati dalla somma della dose minima, sicuramente ed immediatamente letale (esp. 3, tabella I), per una quantità stechiometricamente uguale al fosfato, iniettato preventivamente.

**) — Valori calcolati dalla dose minima, sicuramente ed immediatamente letale (esp. 9, tabella I), supponendo che, trascorso un tempo lunghissimo dall'iniezione preventiva di fosfato, l'animale sia tornato del tutto normale.

manganese, da ciò stesso sorga un nuovo fattore di tossicità, che si unisce a quelli proprii del manganese.

Così la tossicità immediata del manganese dobbiamo intenderla come determinata da molti fattori chimici positivi e negativi, del manganese che entra estraneo e crea nuove forme chimiche, dei fosfati che vengono sottratti e rompono molteplici equilibri fisiologici.

V. Confronti e conclusioni.

Per lo scopo sintetico al quale mirano tutte queste ricerche sul manganese, nel riassumere i risultati ottenuti col cloruro, carbonato e fosfato, è opportuno confrontarli anche con quelli che ho ottenuti in lavori precedenti col permanganato di sodio [1] e col biossido di manganese [2].

La tossicità di tutti questi preparati, desunta dalle dosi letali e dal decorso dell'avvelenamento è riassunta comparativamente nella tabella VI. Con tutti questi preparati le dosi letali variano assai meno di quello che la diversità grandissima della struttura chimica e della solubilità loro farebbe sospettare.

Dosi. - La dose minima sicuramente letale è la stessa, 0,00023, col biossido di manganese e col permanganato che si converte in biossido nel momento stesso dell'iniezione endovenosa: è un po' maggiore, 0,00039, col carbonato e col cloruro: è leggermente più alta ancora, 0,00042, col fosfato nel quale verosimilmente si convertono il cloruro ed il carbonato durante l'iniezione endovenosa, incontrando i fosfati alcalini del sangue.

Il limite delle dosi sicuramente non letali è uguale coi primi tre, 0,00023; è leggermente più alto col carbonato, 0,00025; è un po' più alto ancora col fosfato, 0,00030.

Da ciò risulta che col permanganato di sodio e col biossido di manganese si trova una dose minima sicuramente letale, che segna un limite netto fra dosi letali e non letali; col cloruro, col carbonato e col fosfato di manganese fra le dosi sicuramente letali e quelle sicuramente non letali vi è un intervallo in cui il risultato sperimentale non è costante, ma variabile da animale ad animale. Questo intervallo diminuendo col diminuire della solubilità dei sali: dal cloruro al carbonato al fosfato.

Decorso dell'avvelenamento. - Col permanganato, col biossido

Tabella VI.

Tossicità comparata di composti di manganese.

Esito dell'animale			Con gatono di Mn per kg di coniglio			
			permanganato di sodio	biossido di manganese	cloruro di manganese	carbonato di manganese	fosfato di manganese
Esito sicuro, letale	immediatamente con dosi non minori di		—	—	0,000390	0,000389	—
	dopo qualche tempo con dosi non minori di		0,000230	0,000230		—	0,000420
Esito variabile	immediatamente letale		—	—	0,000390	0,000389	
	letale dopo qualche tempo		—	—	—	—	0,000420
	non letale				0,000250	0,000250	0,000300
Esito sicuro, non letale	non letale con dosi minori di		0,000230	0,000230	0,000250	0,000250	0,000300

e col fosfato di manganese, anche a dosi elevatissime, di regola non si ha mai morte immediata, perchè gli ultimi due sono colloidali ed il primo passa a forma colloidale di biossido al primo contatto col sangue durante l'iniezione endovenosa.

Col cloruro e col carbonato di manganese, che sono solubili, a dosi alte si ha sempre morte immediata.

Sintomatologia. - Il permanganato in un primo momento svolge l'azione sua ossidante diretta sul sangue, e mentre si riduce a biossido di manganese dà emolisi, emoglobinemia, emoglobinuria, anemia. In un secondo momento l'azione sua si identifica con quella del biossido di manganese colloidale. Questo pare che come tale non abbia un'azione apprezzabile: si riduce lentamente, passa a forma salina e l'azione paralizzante tardiva che dà poi sul cuore deve essere riferita all'jone manganoso. Il cloruro, il carbonato ed il fosfato hanno un'azione paralizzante sul cuore, che dipende dall'jone manganoso. Può comparire immediata coi sali solubili, cloruro e carbonato, coi quali si può arrivare rapidamente alla concentrazione ionica minima sufficiente a produrre la paralisi. Compare sempre tardi col fosfato colloidale, estremamente poco solubile, col quale solo lentamente si arriva alla concentrazione ionica minima sufficiente e produrre la paralisi.

L'azione farmacologica di tutti questi preparati è quindi in fondo molto simile per tutti; rispetto alle dosi, al decorso ed alla sintomatologia dell'avvelenamento. Ciò avviene perchè tutti nell'organismo animale tendono ad una forma salina unica di fosfato manganoso.

Fattori di tossicità. - I cambiamenti chimici che questi preparati di manganese subiscono nell'organismo sono esposti schematicamente nella fig. 3, che riassume fedelmente tutti i fatti osservati e mostra quali sono i fattori di attività farmacologica e la successione loro.

Sono fattori ossidanti energici, immediati, del permanganato con effetti molto vistosi per l'azione locale sul sangue. Sono fattori ossidanti più lenti e con effetti poco appariscenti del biossido di manganese. Sono fattori che dipendono dal catione $Mn^{\cdot\cdot}$, che si manifestano con effetti immediati e gravissimi, o lenti e tardivi a seconda della concentrazione che raggiunge. Sono immediati e gravissimi col cloruro, che è solubilissimo, e col carbonato, che è discretamente solubile; sono tardivi e lenti col permanganato, col biossido e col fosfato, coi quali la concentrazione ionica del manganese è sempre molto bassa.

A questi fattori diretti dell'attività farmacologica altri se ne debbono aggiungere, che sono indiretti e secondari. Tale è la distruzione di globuli rossi, prodotta dal permanganato, e la conseguente

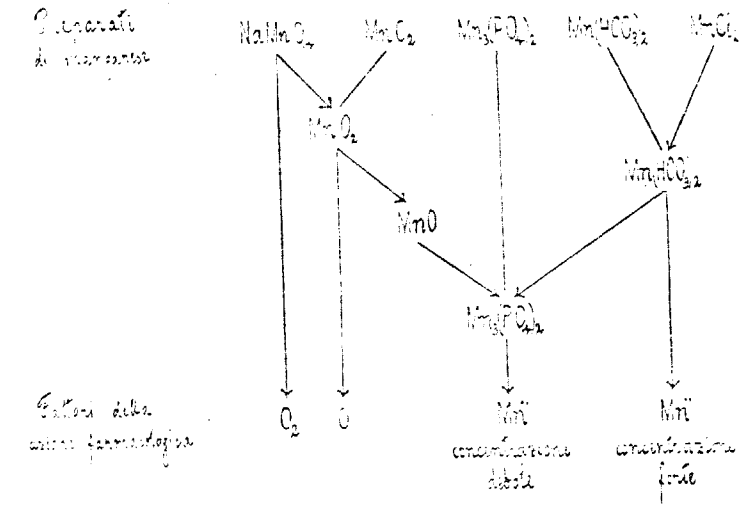

Fig. 3.

emoglobinemia, emoglobinuria ed anemia. Tale è la sottrazione di fosfati provocata in ultimo da tutti i preparati, ad eccezione del fosfato di manganese, il quale però si manifesta costantemente il meno tossico di tutti. Tale è l'alterazione dell'equilibrio ionico che consegue alla sottrazione dei fosfati.

Poteri di difesa dell'organismo. - Alcuni dei fatti sopradetti, che rispetto al manganese sono dei fattori di tossicità, rispetto all'organismo diventano dei mezzi di difesa, con cui riesce a neutralizzare il veleno.

L'azione riducente dei globuli rossi sul permanganato fa da antidoto all'azione ossidante sua. Il permanganato iniettato nelle vene si decompone sui globuli rossi, si ha così un'azione locale diretta ed intensa sul sangue, ma per ciò stesso diventa impossibile ottenere un'azione generale vera e propria da permanganato.

La tossicità immediata coi sali solubili di manganese è dovuta al catione, che con essi può raggiungere rapidamente un'alta concentrazione; ma i fosfati alcalini dell'organismo, reagendo con essi, li convertono in un fosfato poco solubile, che non ha una tossicità [illegible], [illegible] si avrà solo quando siano esauriti i mezzi di difesa.

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ESSENTIAL TRACE METALS IN MAN: MANGANESE

A STUDY IN HOMEOSTASIS*

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(*Received* 28 *May* 1965)

MANGANESE, a transitional metal with atomic number 25, has been considered essential for the normal physiological function of mammals and birds for 30 years. In spite of incontrovertible but indirect evidence of a requirement for dietary manganese, however, deficiency in man has not been recognized.

There is considerable interest in the physiological and biochemical role of manganese by nutritionists, enzymologists and pathologists. The subject has been excellently reviewed on two occasions by COTZIAS‡ [1, 2]. There are gaps, however, in some of the basic data and, at the risk of being repetitious, we have surveyed the field from biogeochemical and analytical viewpoints, as we have other trace elements, in order to answer certain questions pertinent to manganese metabolism in man. These are: (1) What are the environmental sources of manganese? (2) How good is the human homeostatic mechanism for the metal? (3) Does manganese deficiency exist in man even if slight? (4) Do diseases caused by accumulation of manganese occur in man? (5) What is the actual daily intake of manganese by normal human beings? (6) What living organisms have homeostatic mechanisms for manganese?

This report is the tenth of a series on trace elements in human beings [3–11] and represents the first on an essential one. Although there are much older data in the literature, we have attempted to present newer material not available elsewhere as well as to repeat many analyses done by others in order to arrive at our own viewpoint.

METHODS

Spectrographic analyses were performed on human tissues from subjects residing in the following cities of the United States: Baltimore, Birmingham, Chicago, Columbus (Ohio), Dallas, Denver, Memphis, Miami, New York, Richmond (Virginia), St. Louis,

*Supported by grants in aid from the National Heart Institute, United States Public Health Service (HE-05076-06), The United States Army (Contract D.A.-2595), CIBA Pharmaceutical Products, Inc. and Cooper-Tinsley Laboratories.

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‡COTZIAS points out that the name manganese is derived from the Greek *mangania*, meaning black magic, and *manganon*, the generic term for machines of both war and peace [2]. Manganese in the biosphere has many activities which appear magical, being "rich in phenomena and lacking in adequate guiding principles" [1]. As an instrument of war and peace, or of action, reaction and equilibrium, it not only catalyzes many strong reactions but its homeostatic control by living organisms is highly refined.

San Francisco, Seattle-Tacoma (Washington), Anchorage (Alaska) and Honolulu. Most cases involved 18–28 tissues [12]. Foreign specimens came from subjects residing in Taiwan, Tokyo, Kyoto, Chiba (Japan), Manila, Bangkok, Bombay, Delhi, Lucknow, Vellore (India), Beirut, Cairo, Addis Ababa, Lambarene (Gabon), Lagos (Nigeria), Welkom (Orange Free State), Usumbura (Burundi), Kampala (Uganda) and Bern (Switzerland). Most of these cases involved 10 organs [13]. Raw data were analyzed. The limits of detection for manganese by the method used [14] was 1.0 μg/g ash, or approximately 0.01 μg/g on a wet basis.

Biological material, soils and water were analyzed by the microanalytical method of SANDELL [15], based on formaldoxime. The limit of detection was 0.01 μg/g wet weight. Where 0.0 is shown in the tables, the amount was below the limit of detection.

Grains were grown in a long abandoned forest pasture near a mountain top and vegetables in a specially prepared garden which had not been fertilized; details have been reported [16]. Other foods were purchased from local chain stores. Laboratory animals were maintained in 'metal-free' quarters [17]. Wild animals were trapped or shot.

RESULTS

Manganese in human tissues according to age

Without exception, all human tissues contain manganese, according to emission spectroscopy. Concentrations are remarkably constant throughout most of life (Table 1). There is no tendency for decrease or accumulation to occur with aging.

TABLE 1. CONCENTRATION OF MANGANESE IN HUMAN TISSUES ACCORDING TO AGE (Mean values as p.p.m. ash)*

Age	Aorta	Brain	Diaphragm	Heart	Kidney	Liver	Lung	Pancreas	Spleen	Thyroid
Stillborn	37	46	66	74	79	101	54	—	75	—
	(2)	(1)	(6)	(7)	(13)	(19)	(10)		(4)	
1–45 days	39	11	15	20	51	82	35	—	25	—
	(2)	(1)	(1)	(2)	(11)	(12)	(2)		(2)	
45 days–10 yr	45	18	28	15	70	140	16	134	18	—
	(1)	(5)	(3)	(6)	(25)	(25)	(6)	(2)	(5)	
10–19 yr	11	18	17	19	88	151	36	112	12	—
	(6)	(10)	(7)	(11)	(15)	(14)	(13)	(12)	(13)	
20–29 yr	13	19	15	21	89	119	17	106	11	30
	(15)	(21)	(14)	(25)	(25)	(24)	(25)	(24)	(25)	(4)
30–39 yr	11	19	14	23	84	110	21	102	12	15
	(28)	(33)	(29)	(43)	(43)	(44)	(42)	(46)	(44)	(6)
40–49 yr	10	20	19	24	89	108	27	108	12	9
	(27)	(27)	(18)	(31)	(33)	(34)	(32)	(31)	(34)	(6)
50–59 yr	6	20	15	22	70	100	29	94	16	24
	(16)	(26)	(16)	(31)	(31)	(31)	(32)	(25)	(32)	(7)
60–69 yr	10	17	26	19	72	98	32	103	15	17
	(8)	(14)	(4)	(12)	(15)	(16)	(13)	(15)	(13)	(3)
70–79 yr	11	22	24	24	72	136	37	113	14	22
	(4)	(4)	(2)	(7)	(8)	(7)	(8)	(7)	(6)	(2)
80+ yr	4	18	21	18	88	86	17	69	6	11
	(2)	(2)	(1)	(2)	(2)	(2)	(2)	(2)	(2)	(2)
All cases, adult	11	20	17	23	91	130	24	110	11	19
S.E.M.	±1.2	±0.7	±1.2	±0.9	±4.0	±7.0	±2.0	±4.9	±0.8	±2.3
	(95)	(127)	(91)	(139)	(143)	(143)	(141)	(138)	(143)	(21)

*To nearest whole number. Number of cases in parentheses.

Infants up to 6 weeks of age contain relatively more manganese than do older children, especially in tissues normally low in this element, but a level is soon reached which remains relatively constant for 90 years. Ranges of values for each tissue are remarkably small, considering the method used, the calculation of each value in terms of ash weight, which reflects varying concentrations of major elements (K, Ca, Na, Mg, P) and the wide distribution of subjects from 15 cities of the United States.

Highest concentrations of manganese (means of 68–180 p.p.m. ash) occurred in two organs with several specialized metabolic activities, including the excretion of this metal, i.e., the pancreas and the liver, and in organs concerned with transport across membranes: the kidney and the large and small intestines (Tables 1 and 2). Lowest concentrations (<25 p.p.m.) occurred in organs and tissues with fewer metabolic

TABLE 2. MANGANESE IN OTHER HUMAN TISSUES, U.S. ADULTS

Tissue	No. cases	Mean±S.E.M.	Tissue	No. cases	Mean±S.E.M.
Adrenal	13	36±3.1	Trachea	54	14±1.0
Esophagus	66	17±1.5	Muscle	133	6±0.4
Stomach	129	47±2.5	Ovary	16	18±2.2
Duodenum	67	70±10.0	Uterus	31	12±1.3
Jejunum	101	68±3.9	Testes	72	14±0.6
Ileum	84	110±11.0	Prostate	50	19±2.9
Cecum	31	180±31.0	Bladder	109	18±1.7
Sigmoid colon	107	76±9.7	Omentum	75	48±9.8
Rectum	42	82±19.0	Skin	22	22±10.0
Larynx	35	8±1.1	Bone	91	2
			Fat	28	37±4.41

activities: aorta, heart, diaphragm, muscle, bladder, brain, esophagus, larynx, trachea, lung, spleen, skin, ovary, prostate, testes, uterus, thyroid and bone. Differences in concentrations between the two groups of tissues are significant, as is also the case for potassium, calcium, phosphorus, copper and zinc, but not for magnesium [12]. Intermediate concentrations (36–48 p.p.m.) were found in adrenal, stomach and omentum, the last also concerned with transport.

In Table 3 are shown concentrations of manganese in nine organs according to geographic origin of the subjects supplying the tissues. In general there was little deviation from place to place and no definite signs of manganese deficiency in any area. Concentrations in the tissues of 40 children (4–19 years of age) are remarkably similar to those of adults.*

In spite of the relative constancy of the levels of manganese specific for each organ and tissue, data were tested by computer to ascertain whether or not a linear relationship between the concentration of manganese and those of other elements, trace and bulk, existed. Choosing coefficients of correlation of 0.50 or more and of 0.30–0.50 and probabilities of chance occurrence (P) of 0.001 or less, a number of such relationships were shown to exist (Table 4). Of 28 tissues, levels of manganese were related to those of copper in 17, to magnesium and phosphorus in 14, to chromium and iron in 11 and to zinc in 8. Likewise manganese was related to the concentration of barium in 15 tissues and of lead in 9. Concentrations of manganese were related to those of

*Although data in the literature are generally similar to ours, they are not here compared because of some differences in analytical methods.

TABLE 3. CONCENTRATION OF MANGANESE IN HUMAN TISSUES ACCORDING TO GEOGRAPHIC ORIGIN (Median values as p.p.m. ash)

Area	No. cases*	Aorta	Brain	Heart	Kidney	Liver	Lung	Pancreas	Spleen	Testes
ADULT										
U.S. mainland	140	8†	18	20	82	100	17	98	9†	12
Honolulu	12	15	23	23	71	82	13	77	16	14
Switzerland	9	5	18	22	48	170	17	77	14	16
Africa	48	13	20	21	60	120	20	72	17	—
Middle East	46	17	21	22	70	110	38	91	15	18
Far East	76	19	20	23	68	110	24	86	18	18
Sub total and mean	351	13	20	22	67	115	22	84	15	16
CHILDREN < 20 yr										
U.S.	23	12	17	19	87	150	18	120	14	15
Africa	5	—	14	20	68	130	13	72	—	—
India	9	—	27	24	74	130	40	120	17	19
Far East	3	14	16	28	88	90	22	110	15	16
Sub total and mean	40	13	19	23	79	125	23	106	15	17
Mean		12.9	19.4	22.2	71.6	119.2	22.2	92.3	15.0	16.0
S.E.M.		±1.6	±1.2	±2.6	±3.9	±8.5	±3.1	±6.0	±0.9	±5.0

*Certain tissues were lacking in some of the cases, especially aorta, brain and testes (See Table 1).
†Concentrations in males aged 20–59 years differ ($P < 0.001$) from those from Africa, Middle and Far East [13].

both copper and iron in 12 tissues and of copper, iron and chromium in 7—muscle, the sigmoid, rectum, heart, esophagus, diaphragm and spleen. It is most unlikely that these relationships are due to chance. Concentrations in pancreas, thyroid, ovary, uterus, adrenal, duodenum, jejunum and cecum were little related to those of other trace metals, which may reflect the stability of manganese in these tissues.

Manganese in food

The content of manganese in an institutional diet is given in Table 5. For comparison are shown the values obtained by FELDMAN and JONES on 34 teenage diets from 10 United States cities, mean contents of which varied from 98 to 145 p.p.m. ash [18], and on balances of two adult persons [19], as well as the intakes of 9 college women as reported by NORTH, LEICHSENRING and NORRIS [20]. Older data are also given. Our institutional diet had less manganese than did most of the others.

In Table 6, the concentrations of manganese in a number of common foods are given. Sea foods and meats were generally low. Commercial vegetables had various amounts. Whole grains were especially high in manganese, as were their products, and nuts exceeded grains in content. Relatively enormous concentrations were found in tea, as is known [22], cloves, ginger, thyme and bay leaves. A cup of tea contained 1.3 mg whereas a cup of coffee provided only 0.15 mg. All oils but refined safflower contained manganese, as did commercial cholesterol. Pulpy fruit generally had little. On a caloric basis, grains, vegetables, cereals and nuts contained the largest amounts, sea foods, meats and dairy products the smallest. Some vegetable foods apparently had not concentrated manganese against the environmental gradient of soil.

Because superphosphate fertilizers of marine origin contain manganese, probably from sea water, a comparison was made of vegetables grown on heavily phosphated

TABLE 4. CORRELATIONS OF CONCENTRATIONS OF MANGANESE AND OTHER ELEMENTS IN HUMAN TISSUE OF HIGH SIGNIFICANCE ($P<0.001$)

Tissue	$r^*=0.50+$		$r^*=0.30$–0.50	
	Essential	Abnormal	Essential	Abnormal
Brain	Cu, Fe, Mg, P			Ba
Heart	Mg		Co, Cr, Cu, Fe, K, Zn	Ba, Pb, Ti
Aorta			K, Cu, Zn	
Kidney			Cr, Cu, Mg, Zn	Pb
Liver			Co, Cr, Mg, P	Ba
Pancreas	Mg		P	
Spleen	Cu, P	Ba	Cr, Fe, Mg, Zn	Pb
Muscle	Cu, P	Ba	Cr, Fe, Mg, Zn	Pb
Diaphragm	Cu, Fe	Ba, Pb	Cr, Mg, Ni, P, Zn	Ni, Sn, Ti
Lung	Cr	Ba, Ni, V	Cu	Al, Sn, Ti, B
Thyroid	P	B		
Ovary	None			
Testes	Mg		Cr, P, Zn	Pb
Uterus	Mg, P, Zn			
Bladder	Mg, P, Cu		Fe	Al, Au, Ba, Cd
Prostate	Fe, P		Cu	Pb
Skin	Fe	Al, Ba		
Larynx	Cu, Fe	Al	K	
Trachea	Cu, Fe, P			Ba, Ti
Adrenal	None			
Esophagus	Cu, Fe, Zn	Ba, Pb	Cr, Mg, P	Ni, Sr, Ti
Stomach			Cr, Cu, Mg	B, Cd
Duodenum	Cu		P	Cd
Jejunum			Cr	Pb
Ileum			Cu, Mo	Ba
Cecum		Ba, Sr		
Sigmoid colon	Cu, Mg	Ba, Pb, Sr	Ca, Cr, Fe	Ag, Al, B, Ni, Sn
Rectum	Ca, Cr, Cu, Fe	Ba, Sn	Mg	Ag, Al, Ni, Sr, Ti

SUMMARY (28 tissues)

Essential elements		Abnormal elements	
Cu	17	Ba	15
Mg	14	Pb	9
P	14	Al	6
Cr	11	Ti	6
Fe	11	Ni	5
Zn	8	Sr	4
K	3	Sn	4
Ca	2	B	3
Co	2	Cd	3
Mo	1	Ag	2
		Au	1
		V	1
		Bi	0

*r=correlation coefficient.
All correlations are positive except that for potassium in heart.

soil with those grown on unfertilized soil (Table 7). In 8 cases the amount increased, in 3 it decreased and in 11 it was relatively unchanged. Wheat and millet took up much manganese from superphosphated soil, whereas rye, barley, oats and corn did not.

Manganese in vegetation

There is a large amount of manganese in green leaves. In Table 8 are shown the

TABLE 5. MANGANESE IN SAMPLE DIETS

Item	Wet weight	% dry substance	μg/g wet	μg/g dry	Total μg
Institution, mental					
Breakfast	766.0	25.8	1.13	4.38	865.6
Lunch	860.8	18.4	1.43	7.77	1239.2
Dinner	898.7	19.1	0.33	1.72	296.7
Total or mean	2525.5	21.1	0.96	4.62	2401.5
Teenage, 10 U.S. cities (FELDMAN and JONES [18])		18.8	1.03	5.48	2575
Female, age 34	2372	19.5			6400
Male, age 35	2487	21.9			7500
(TIPTON and STEWART [19])					
College women, U.S. (NORTH *et al.* [20])	1230+				3700
Adults, England (KENT and MCCANCE [21])					
White flour					2200–2700
Whole wheat flour					8500–8800
Adults, Holland (BELZ [22])					2300
Adults, England (MONIER-WILLIAMS [23])					7000

concentrations found by HANNA and GRANT [25] in ornamental foliage in Rutgers, New Jersey, and by us in Vermont forest trees. From these data it is quite obvious that herbivorous animals and insects ingest much larger quantities of manganese than do human beings. HANNA and GRANT found several times as much manganese as iron in plants; of 17 trace elements it had the highest concentration and was present in amounts one-fourth to one-sixth as great as phosphorus or magnesium, therefore approaching the status of a 'bulk' element. All vegetation concentrated manganese to levels higher than those probably available in soil. LOUNAMAA [26], who conducted an exhaustive study of 15 trace elements in several hundred species of plants growing wild in Finland, found much more manganese than any other trace element, the next highest being zinc.

The amount of calcium in the soil influences the uptake of manganese by plants. In Table 9 are the concentrations in various types of plants growing wild on three main types of soil, as reported by LOUNAMAA [26]. The average concentration of manganese was less in plants growing on high-calcium soils than on low-calcium soils. Furthermore, on an ash basis all plants except lichens and mosses concentrated manganese against an environmental gradient.

Manganese in human blood and urine

Human blood has been found to contain variable quantities, depending upon the method of analysis (Table 10). Neutron activation analysis probably gives the most accurate results [27]. Part of the manganese is carried in plasma by transmanganin,

TABLE 6. MANGANESE IN FOODS
(wet weight)

Item	μg/g	μg/100 calories
Grains and cereals		
Wheat, whole, seed	11.32	340
Wheat, flour, all-purpose	5.20	150
Bread, white	1.78	67
Bread, whole wheat	1.43	38
Buckwheat, whole, seed	13.11	391
Rye, whole, seed	13.29	397
Oats, seed	6.46	160
Oatmeal	2.72	67
Barley, seed	17.80	494
Corn, whole	1.31	37
Corn, meal	2.05	59
Rice, American, brown	1.42	39
Rice, Madagascar	1.17	33
Rice, Japanese, unpolished	2.08	58
Rice, Japanese, polished	1.53	43
Millet, seed	2.04	57
Macaroni, dry	10.56	293
Grapenuts	30.76	854
Mean	7.00	199
Dairy products		
Milk, whole, bulk	0.19	29
Milk, direct from udder	0.14	21
Milk, dry, skimmed	0.0	0
Butter	0.96	12
Cheese, Swiss, domestic	1.32	33
Cheese, Gorgonzola, domestic	1.88	48
Eggs, whole	0.53	33
Egg whites	0.43	116
Egg yolks	0.88	25
Mean	0.70	35
Meat and poultry		
Beef, roasting	0.05	3
Beef, brisket	0.03	1
Beef liver	0.16	11
Beef kidney	0.0	0
Pork loin	0.0	0
Pork chops, lean	0.34	12
Pork liver	0.09	6
Pork kidney	0.75	63
Lamb chops, lean	0.34	18
Lamb kidney	0.30	30
Chicken breast	0.21	11
Mean	0.21	14
Fish and sea food		
Oysters, fresh, frozen	0.06	12
Clams, fresh, frozen	0.0	0
Scallops, fresh	0.11	11
Shrimp, fresh, frozen	0.02	2
Fish and sea food—*contd.*		
Cod steak	0.02	3
Halibut steak	0.12	9
Herring, smoked	0.04	2
Sardines, canned, Morocco	0.0	0
Anchovies, canned, Portugal	0.10	5
Mean	0.05	5
Fruit		
Orange, Florida	0.35	100
Tangerine, Florida	0.62	180
Peach	1.02	275
Pear, Anjou	0.33	83
Apple, MacIntosh	0.31	70
Raisins, packaged	4.68	190
Apricot, canned	0.20	33
Canteloupe	0.26	108
Mean	1.00	133
Nuts (edible part only)		
Pecans	35.09	585
Brazil	27.80	432
Almonds	24.69	412
Walnuts	7.52	137
Coconut, fresh (Virgin Islands)	0.38	10
Peanuts, salted	6.91	113
Mean	17.07	282
Vegetables		
Beans, green, canned	0.24	70
Peas, fresh	0.64	100
Peas, split green, dry	12.74	463
Carrots, canned	1.56	780
Beets, fresh	0.41	145
Beet greens, fresh	4.26	3873
Beets, canned	1.34	304
Turnip greens, fresh	4.71	4282
Spinach, canned	1.42	546
Spinach, fresh	7.77	2990
Cabbage, fresh	1.13	435
Broccoli, fresh	1.54	1000
Brussels sprouts, fresh	3.45	2156
Asparagus, fresh	0.32	178
Asparagus tips, canned	0.27	150
Cucumber, fresh	0.14	156
Rhubarb, fresh	4.57	7617
Tomatoes, canned	0.30	214
Tomato soup, canned	0.32	230
Mean	2.48	1352

TABLE 6. MANGANESE IN FOODS (*continued*)
(wet weight)

Item	µg/g	µg/100 calories	Item	µg/g	µg/100 calories
Soil of vegetables (fresh)	9.65	—	Coffee, infusion	0.85	—
			Tea, leaves	275.58	—
Condiments and beverages			Tea, infusion	6.9	—
Salt, table	0.0	—			
Pepper, black	47.48	—	Fats and oils		
Paprika	4.86	—	Cod liver oil, Norway	4.95	55
Cloves	262.86	—	Sunflower	1.47	16
Ginger, ground	87.33	—	Safflower	0.0	0
Thyme, ground	82.65	—	Cottonseed	2.52	28
Bay leaf, dried (laurel)	67.19	—	Corn	1.00	11
Garlic, fresh	0.95	—	Peanut, pressed	1.00	11
Garlic powder	0.45	—	Linseed, pressed	4.20	47
Chili powder	0.62	—	Lard, refined	0.98	10
Caraway seeds, whole	2.02	—	Cholesterol, U. S. P.	0.38	—
Mustard, dry	1.26	—			
Vinegar, cider	2.54	—	Mean	1.83	22
Coffee, ground	20.65	—			

a β_1 globulin which binds Mn (III) ions [28], and part in red cells either as a manganoporphyrin or chelated to heme [29].

A few analyses of urine are given in Table 9. Amounts were higher than those reported by PERRY and PERRY [34]. COTZIAS [2] quotes no excretion by this route, whereas NORTH *et al.* found manganese in 63 of 72 urine specimens [20].

Manganese in laboratory rats and other mammals

Our regimen provides 10 p.p.m. manganese as the citrate in the drinking water of rats [17]. The intake of water was measured at approximately 7 ml per 100 g body weight per day over a year's time [35], providing a daily intake of 70 µg manganese per 100 g. Food contained 12.7 µg/g, or approximately 63.5 µg/100 g per day. In spite of such large intakes, rat tissues contained little (Table 11). This intake of manganese is equivalent to 95 mg per day for a 70-kg man, whereas commercial rat food, which contains 6.0–20.7 µg/g, would provide a human equivalent of 21–72 mg manganese per day, or 7–24 times normal intakes. It is evident that the rat, like the human being, has an excellent homeostatic mechanism for manganese, having concentrations in his tissues in the ranges of the human and accumulating no appreciable quantities despite very high intakes. Similar mechanisms appear to exist for other mammals (Tables 6 and 11), especially herbivores which must consume large amounts, although a few animals had surprisingly high concentrations*.

Manganese in marine organisms

Man has approximately 20 mg or 0.286 p.p.m. manganese in his body, or about 3×10^{-5} per cent [36]. VINOGRADOV states: "In general, the amount of manganese is very small in fishes ($n \times 10^{-5}$ per cent of the living weight)" [37], the same order of

*Herbivorous animals by their homeostatic mechanisms reject excesses of manganese in food. Carnivores are therefore presented with low dietary intakes of this metal. The high concentration in the liver of the fox is unexplained.

TABLE 7. UPTAKE OF MANGANESE BY FRESH VEGETABLES AND GRAINS FROM SUPERPHOSPHATE IN SOIL (wet weight)

Items	Control		Superphosphate	
	μg/g	μg/100 calories	μg/g	μg/100 calories
Grains				
Wheat	9.78	294	24.62	739
Rye	0.87	26	6.93	207
Barley	17.80	494	3.98	108
Oats	6.46	161	3.88	97
Corn	1.31	37	1.11	32
Millet	2.04	58	7.96	227
Mean	6.38	178	8.07	235
Soil	32.11			
Superphosphate			1.00	
pH units	5.36		5.03	
Vegetables				
Potatoes, white	1.18	168	1.24	177
Carrots	0.12	52	0.12	52
Turnips	0.58	322	1.88	1044
Beets	0.67	152	0.41	93
Onions, white	0.0	0	0.0	0
Onions, red	0.45	187	1.10	479
Beans, Lima	0.0	0	2.33	88
Beans, string, bush	0.33	220	0.12	80
Beans, string, pole	0.99	660	1.18	787
Peas, green	5.47	855	1.01	158
Lettuce, Bibb	4.33	3936	7.41	6736
Swiss Chard	2.73	2730	5.70	5700
Spinach	4.43	1704	3.99	1535
Celery	3.22	3578	0.28	311
Kale	1.95	2437	10.05	12862
Cauliflower	1.82	1655	2.92	2654
Pepper, green	0.18	90	0.09	93
Squash, summer	1.40	2000	1.22	1717
Squash, winter	0.11	73	0.14	93
Squash, buttercup	0.12	80	0.13	87
Mean	1.37	1045	1.89	1735
Soil	40.56			
Superphosphate			1.00	
pH units	5.41		5.0	

magnitude as that in mammals. He quotes amounts in fish muscle similar to those obtained by us (Table 6). Fish concentrate manganese from sea water about 100-fold. Marine animals, having a liver, hepatopancreas or the equivalent, in general concentrate little more manganese from water than do fish, although there is variation within families (Table 12). Most molluscs, worms, arthropods and coelenterates have concentrations in or near this range. Fresh-water species, however, usually contain more than do marine; the concentration of manganese in fresh water is also considerably higher than in sea water. VINOGRADOV [37] proposed that the function of the hepatopancreas of molluscs may be to discharge manganese, thus preventing its

TABLE 8. MANGANESE IN VEGETATION, LEAVES (p.p.m.)

Item	Wet	Dry	Ash
HANNA and GRANT [25], suburban			
Acer rubrum (maple)		689	
Ilex opaca (holly)		594	
Kalmia latifolia (laurel)		178	
Pieris Japonica (quince)		508	
Pinus strobus (pine)		406	
Quercus palustris (oak)		566	
Rhododendron, Dresselhuys		681	
Rhododendron, *Roseum elegans*		495	
Tsuga canadensis (hemlock)		700	
Mean		535	
This report, forest			
Acer saccharum (maple)	4.06	11.9	222
Betula alba (birch)	10.23	30.0	458
Fagus grandifolia (beech)	1.02	2.9	43
Picea rubens (spruce)	94.40	295	10,600
Pinus strobus (pine)	46.70	117	3050
Pyrus Americana (ash)	27.777	85	1640
Quercus rubra (oak)	3.62	8.3	192
Tsuga canadensis (hemlock)	144.81	367	10,270
Ulmus Americana, heart wood (elm)	8.54	8.9	1550
Mean	37.91	102.9	3114

TABLE 9. MANGANESE IN VEGETATION GROWING ON DIFFERENT SOILS IN FINLAND*
(Mean values as p.p.m. ash)

Item	Calcareous soil (high calcium)	Silicic soil (low calcium)	Ultrabasic soil (no calcium)
Rock	2300	610	2200
Soil	1800	1500	1200
Lichens	—	410	1800
Mosses	770	3300	1200
Ferns			
Fronds	5500	2400	5600
Subterranean part	23,000	8900	29,000
Conifers			
Needles	7800	14,000	16,000
Twigs	6100	10,000	5200
Deciduous trees			
Leaves	5000	12,000	15,000
Twigs	7400	9000	11,000
Dwarf shrubs			
Leaves	6500	22,000	21,000
Stems	7300	26,000	30,000
Herbs			
Leaves	2800	7200	3700
Stems	2400	5800	3100
Flowers and fruit		6700	2400
Grasses			
Flowers		9200	6000
Leaves and stems		8100	5000
Subterranean part		8900	15,000
Mean, higher plants	7380	10,710	11,640

* After [illegible] [26]

TABLE 10. MANGANESE IN HUMAN BLOOD AND URINE

Item	Method*	Mean (μg/l.)	Range (μg/l.)	Mean, 24 hr (μg)	Author
Blood	Spect	29±2	25–34		BUTT *et al.* [30]
	Chem	30			FORE and MORETON [31]
Serum	X-ray	0.0	0–70		GOFMAN *et al.* [32]
Blood	NAA	2.4±0.8			BOWEN [33]
Blood	NAA		8.6–14.5		COTZIAS and PAPAVASILIOU [28]
Plasma	NAA	2.5	1.83–3.10		Ibid [28]
Red blood cells	NAA		0.46–1.21		Ibid [28]
Spinal fluid	NAA		0.83–1.50		Ibid [28]
Urine					
2 ♂ normal, 18–21 yr	Chem	200	150–250	280	This report
4 ♀ normal, 18–23 yr	Chem	395	150–590	552	This report
1 ♀, 74 yr	Chem	980	—	1370	This report
4 ♂ patients, 42–64 yr	Chem	155±5.7	150–170	220	This report†
18 ♀ patients, 27–84 yr	Chem	160±11.1	120–250	225	This report†
9 College women	Chem		0–340	200	NORTH *et al.* [20]
24 Adult Americans	Spect	27	10–70	39	PERRY and PERRY [34]
12 African patients	Spect	88	<30–560	123	Ibid [34]

*Spect=spectrographic method; Chem=chemical analyses; X-ray=X-ray fluorescence; NAA=neutron activation analysis.
†Schizophrenic patients in institution on intake of ±2400 μg per day.

TABLE 11. MANGANESE IN THE TISSUES OF LABORATORY RATS AND OTHER MAMMALS

Items	μg/g wet weight
Rats, Long-Evans strain (4)	
Liver	0.78
Kidney	1.00–1.06
Heart	0.36–0.88
Lung	0.54
Spleen	0.53–0.77
Mice, wild (17)	
Liver	1.81
Kidney	0.75
Heart	0.69
Lung	2.39
Deer (1)	
Kidney	1.44
Brain	0.66
Fat	1.55
Raccoon	
Aorta	2.31
Bat (whole)	1.44
Woodchucks (3)	
Liver	3.53
Kidney	3.80
Red fox (1)	
Liver	9.43
Dog	
Liver	4.29
Kidney	3.20

TABLE 12. CONCENTRATION OF MANGANESE BY LIVING ORGANISMS FROM THE ENVIRONMENT (SOIL, FOOD AND WATER)

Organism	Order of concentration p.p.m. living matter	Factor of concentration 10^n n
Plants		
Phytoplankton	—	3‡
Marine plants	100	5
Marine algae	1–10	2–3‡
Terrestrial plants	10–100	1–2
Animals, marine		
Zooplankton	—	2–3‡
Diatomaceae	0.1–100	2–5
Poriphera	10	4
Coelenterata	0.1–1.0	2–3
Bryozoa	1.0	3
Brachiopoda (shells)	—	(6)
Tunicata	10	4
Echinodermata*	1.0–10	3–4
Mollusca*	0.1–1.0†	2–3
Vermes*	0.001–1.0	0–3
Arthropoda*	0.1–10	2–4
Pisces*	0.1	2‡
Animals, terrestrial		
Mammals, including man	0.1	1
Sea water	0.001	–
Soil	1000	–
Food	1.0	–

Source: VINOGRADOV [37] and LOUNAMAA [26].
Note: The concentration in tissues was compared to that in sea water, soil or food.
*Animals with livers or the equivalent.
†Two species of fresh-water molluscs had 1000 p.p.m.
‡Also according to uptake of Mn^{54} as reported by FOLSOM *et al.* [39].

accumulation in other organs; his hypothesis is completely in agreement with the recent one of COTZIAS and PAPAVASILIOU [38], who demonstrated this route of excretion by mammals and discussed its extreme antiquity, developed before the kidney became necessary in the post-Cambrian era. Some marine plants and zooplankton accumulate much manganese, up to 400 p.p.m. wet weight, and fresh-water plants contain more. It is apparent that many marine animals feeding on plankton and vegetation consume large quantities and are able to reject or excrete any excess.

Manganese requirements

Estimated requirements of various species for manganese are shown in Table 13. It is apparent that man has one of the lowest. Absorption is inversely related to the level of calcium in the diet.

Unlike most other micronutrients, an intake of manganese sufficient for growth can cause deficiency in an organ or system [24]. Requirements for health are larger than requirements for growth. They may be much larger, as the table indicates. Such differences in adequate and optimal intakes have not been demonstrated for man.

Manganese depletion in man

Manganese was found regularly in every human organ and tissue in more than 7000

analyses on over 400 subjects from 15 United States cities and 20 foreign areas. Five adults from the United States, aged 50–79 years, had hepatic concentrations of 32–43 p.p.m. ash, which is below the 80 per cent range of the median (55–220 p.p.m.). Renal concentrations in all and pancreatic concentrations in four were below this range, but not those of other tissues. Traumatic accidents were the cause of death in 3 cases, and cardiovascular accidents in 2 cases. Three foreigners had hepatic concentrations of 7–18 p.p.m. ash and two had 35–43 p.p.m.; four had low levels in kidney and pancreas, but not in other tissues. Causes of death were trauma, lymphosarcoma, cancer of the

TABLE 13. MANGANESE REQUIREMENTS FOR VARIOUS SPECIES* (p.p.m.)

Item	Deficient diet	Marginal diet	Adequate diet
Mice	0.2–0.3		6–20+
Rats	0.2–0.3		6–20+
Rabbits	0.2–0.3	6+	20–80
Pigs	0.5	12	40
Cattle	1–7	10	30–600
Poultry	37	40	100–600
Man	?	?	3–5

*Largely from UNDERWOOD [24]. Requirements also depend upon level of calcium in diet.

stomach, cancer of the lung and lobar pneumonia. On these data, therefore, it is possible for some human beings (about 2.5 per cent) to have lower than normal concentrations of manganese in their target organs, although tissues normally low are relatively unaffected.

Manganese deficiency

Deficient intakes of manganese in experimental animals affect three systems—bone, reproduction and brain [1, 2, 24]. Abnormalities of the growth of bone occur in mice, rats, rabbits, pigs, cattle and poultry. In chicks with perosis (slipped tendon) caused by deficiency, activity of blood and bone alkaline phosphatase is depressed, a manifestation of decrease in content of the enzyme. Arginase is also reduced in livers of deficient mammals. Manganese is presumably a co-factor of both enzymes. There appears to be an interaction between manganese and choline, in that deficiency of the former is exaggerated by deficiency of the latter. Both substances are lipotropic.

To our knowledge, investigation of certain congenital abnormalities of bone in infants and children along these lines has not been made, an obvious lead from the experimental evidence [1]. Growing children may require more manganese than do adults [24] and diets low in this element may be fairly common.

In female rats, manganese deficiency is associated with deficiency in their young, stillbirths, early deaths and sterility in the mother, according to its degree. In birds, less eggs are produced and fewer embryos survive. In hogs and guinea pigs, litters are smaller and sows have abnormalities in estrus cycles. Cows exhibit delayed estrus and conception. Males of various species show sterility, absence of libido and seminal tubular degeneration, indicating a requirement of manganese for spermatogenesis.

Ataxia occurs in the offspring of manganese-deficient chicks, rats and guinea pigs. Rats show increased cerebral excitability and a tendency to convulsions [40, 41]. COMENS [42], in our laboratory, produced in rats what was evidently an acute and temporary manganese deficiency by injecting large doses of hydralazine, a drug useful

in hypertension. Resulting convulsions were prevented by injection of large doses of manganese concurrently. Potassium, calcium, cobalt, zinc and nickel had no such effect. This work was confirmed by HURLEY *et al.* [40]. Hydralazine, a chelating agent, binds manganese *in vitro* [43].

Manganese deficiency has not been demonstrated in man. In view of the large variations in dietary intake and the dependency of high levels on tea, whole grains, leafy vegetables and fruits, there is no logical reason why human beings should not be subject to deficiency of this essential trace metal under certain conditions. (1) Pregnant women on diets high in calcium, refined flour, sea foods, meats and dairy products might be expected to be depleted by the demands of the growing fetus for manganese just as much as for iron and chromium; tissues of the newborn have high concentrations of these metals [6]. (2) Growing children on diets subnormal as to manganese, with high intakes of milk, might be expected to show deficiencies, as they do for copper, iron and perhaps other elements. (3) Adult human beings on non-physiological reducing diets might develop deficient states; fortunately most crackpot regimens usually contain foods high in manganese.

From animal experiments, human disorders which require examination from the standpoint of manganese deficiency include; (1) conditions in pregnant women involving nervous instability and convulsions, such as toxemia of pregnancy and eclampsia—two cases of toxemia from Bangkok, however, had normal tissue levels; (2) as stated before, disorders of bony and cartilaginous growth in infants and children, such as congenital slipped epiphysis of the hip and Legg-Calvé-Perthes disease; and (3) certain types of sterility in both males and females. From what is now known, supplementation of the diets of pregnant women with manganese may be a logical, if unproven measure which can do no obvious harm.

COMENS [44] has advanced the hypothesis that disseminated lupus erythematosus might be the result of manganese deficiency, based on his observations on our patients with hydralazine disease [45], renal lesions produced by hydralazine in dogs [46] and the partial reversal of the L-E phenomenon in circulating blood of these dogs fed manganese citrate, as well as the prevention of perosis induced by hydralazine in chicks [42]. Although the experimental evidence for an interaction of hydralazine and manganese appears clear, we were unimpressed with the therapeutic results of feeding human beings doses amounting to half their body pools of manganese as the citrate daily for long periods; probably their plasma levels of the metal were elevated [47]. This approach, however, deserves further investigation. Disseminated lupus has not been reported among thousands of patients taking large doses of phenothiazines [48], which also bind manganese [1, 2].

Manganese excess

The fact that two inborn errors of metallic homeostasis involve intestinal transport of two trace elements, iron and copper, suggests that a similar defect might exist for manganese. Failure to reject dietary iron leads to accumulation and to hemochromatosis; the process may take many decades. Failure to reject dietary copper leads to accumulation and to hepatolenticular degeneration; the process often takes less than two decades. Considering the enormous intakes of manganese compared to the total body pool, it is conceivable that either partial deficiency of the mechanism of excretion of oral manganese by liver and pancreas, or congenital deficiency of plasma

transmanganin could occur, leading to accumulation and to disease—'hereditary manganism'. Poor hepatic and pancreatic secretion, however, is most unlikely in view of the biological antiquity of this mechanism and its dependency on bile [38]. High intakes of the oxide by another portal of entry, the lung, cause industrial manganism, a disorder mainly of the central nervous system. Similarity of manganism to Parkinsonism has led BORG and COTZIAS to suggest that the latter might be the result of accumulations [2]. Early manifestations of the disease mimic schizophrenia, which has not been studied from this viewpoint*.

In reviewing the human data case by case, we found several examples of excessive accumulation of manganese. Five-fold values above the means (> 500 p.p.m. ash) were found in 8 human livers and 1 pancreas. In 4 of those cases, other organs such as brain, aorta, heart and kidney contained concentrations of manganese above the 80 per cent range of normal. The subjects died of trauma, drowning, carcinoma of the pancreas and cor pulmonale. In the other 4 subjects, high hepatic levels were associated with elevations only in kidney and/or pancreas; the causes of death were chronic alcoholism, drowning and hepatoma (2 cases). In one additional case of carcinoma of the esophagus with metastases, high concentrations occurred in all organs but liver. Isolated accumulations of manganese at levels more than 5 times the mean for the organ occurred in 4 aortas, 4 brains and 2 spleens, without concurrent elevations in other organs. Therefore, 2.4 per cent of these subjects accumulated manganese in several organs and another 2.7 per cent accumulated the metal in one organ. Of 'normal' persons dying of trauma, about 1.7 per cent had accumulated manganese in several organs. There were no cases of Parkinsonism in this series, nor was a correlation with any single cause of death evident.

COTZIAS [2] has suggested that phenothiazines, which complex Mn^{3+}, produce Parkinsonism as a toxic effect by mobilizing manganese *in vivo*, somehow duplicating the disease of manganism. In that event, manganese from other tissues would be transported to the brain in excess amounts, diffusing gradually into cells.

DISCUSSION

Geochemistry

The average concentration of manganese in the universe is 69 atoms and in the solar atmosphere 200 atoms per 10,000 atoms of silicon [49], ranking 17th in abundance of all elements. The mean concentration on the earth's crust is about 1000 p.p.m. [50], ranking 4th in abundance of the trace elements, after aluminum, iron and titanium, and 15th of all elements. In sea water there is about 1 p.p.b. [49], 2 p.p.b. [51], or 4 p.p.b. [37], ranking 19th in abundance, evidence that most of the crustal manganese is in insoluble higher valence states (Mn^{3+}, Mn^{4+}). Marine sediments contain 1 per cent manganese [51], ten times that on land. River water generally carries much more manganese, 5–180 p.p.b. [52], than sea water, and fresh water can have more than 1000 p.p.b. [53], all of it probably divalent. Therefore the rains leach soluble manganese from the land and the rivers carry it to the sea where it is oxidized to an insoluble form and precipitated on the ocean floor. A geochemical and biogeochemical depletion of crustal manganese is thus evident; reduction of the tetra- and tri-valent

*No elevation of excretion of manganese in urine, however, was demonstrated in 22 chronic schizophrenic patients studied by us (Table 10).

manganese on land to a divalent state, solubility in fresh waters, oxidation to the tetravalent form in sea water, precipitation. Soil bacteria will both oxidize and reduce manganese [54], but known marine bacteria mainly oxidize it (*Leptothrix*, *Crenothrix*) [37].

In sea water, probably with plankton as a nucleus [37], manganese and iron slowly form nodules with onion-skin structures, 'scavenging' from sea water many other elements, including nickel (2 per cent), cobalt (0.2–2.0 per cent) and copper (2.3 per cent). According to MERO [55], the gross tonnage of these nodules is enormous, estimated for the Pacific Ocean at 1.5 trillion tons containing 16–50 per cent manganese. They also contain sizeable quantities of tellurium (10–125 p.p.m.) [56]. These nodules are forming at the rate of about 10 million tons per year (2.5 million tons of manganese) in the Pacific alone. If only 10 per cent could be mined economically, at the present rate of consumption of metals there would be enough manganese, cobalt, nickel, gallium, vanadium and other metals in the nodules to last 150,000 to 400,000 years. The rates of formation of 15 commonly used metals by these nodules per year are now 2–8 times their annual world consumptions [55].

Manganese in fresh water and soil

Treated public water supplies of the 100 largest cities of the United States, analyzed by DURFOR and BECKER [53] contained 0.0–2.5 p.p.m. manganese, with a median of 0.005 p.p.m.* We found 0.5 and 0.75 p.p.m. in tap water from our laboratory during a spring thaw. Analyses by the Public Health Service of tap water from 148 municipal supplies showed 0.002–1.0 p.p.m., with a median of 0.01 p.p.m.; 25 were 0.1 p.p.m. or more [57]. Manganese and iron are usually deliberately removed by aeration from municipal waters when concentrations are greater than 0.2 p.p.m. The intake by man from this source probably seldom exceeds 1.0 mg and is closer to 0.01 mg per day.

The mean concentration of manganese in soil is 850 p.p.m., most of it unavailable to plants [54]. Manganese levels shown in the tables are those for manganese soluble in hydrochloric acid and probably are fairly representative of the manganese available to growing vegetables as carbonates, chlorides, sulfates and organic acids.

Deep-rooted trees extract divalent manganese from the subsoil and concentrate it in their leaves which fall and enrich the top soil [54]. In dry regions with high oxidation potential, little vegetation and strong sunlight, manganese is oxidized to the tetravalent form which is completely insoluble in natural solutions and which forms 'desert varnish' [54]. Oxidation of manganese occurs only in relatively alkaline media, pH 6–8, and reduction occurs only in acid media, pH <6. Manganese, therefore, is poorly assimilated by plants from neutral or alkaline soils, giving rise to chlorosis of leaves, so common when soil is over-limed. Furthermore, at these pH values it 'scavenges' other trace metals (iron, cobalt), making them unavailable, as it does in sea water. Iron chlorosis of plants occurs in Kawai and other Hawaiian islands from this cause.

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*Coefficients of correlation of the concentrations of manganese and of other trace and bulk element in these waters were significant for nickel, lead and titanium ($P<0.005$) and for sulfate ($P<0.01$) but not for 19 others, including hardness, nor for total death rates or death rates from atherosclerotic heart disease.

Reduction of trivalent manganese in soils depends upon organic matter and its decay by bacteria in an acid soil. In alkaline soils, only traces of divalent manganese are found. Very acid soils with much moisture (muck) may lead to depletion of manganese because of its loss by run-off of water [54]. In such soils without leaching, manganese toxicity may become evident, obeying Bertrand's Law of optimum nutritive concentration [58].

The reactions of the manganese cycle in soil may be summarized as follows [54, 59]:

$$MnO \rightleftharpoons MnO \cdot nH_2O \underset{\text{Bacteria}}{\overset{\text{Bacteria}}{\rightleftharpoons}} Mn_2O_3 \cdot nH_2O \tag{1}$$

$$MnO \cdot nH_2O \xleftarrow{\text{Dismutation}} Mn_2O_3 \cdot nH_2O \xrightarrow{\text{Dismutation}} MnO_2 \cdot nH_2O \tag{2}$$

$$MnO \cdot nH_2O \underset{\text{Bacteria}}{\xleftarrow{\text{Chemical}}} MnO_2 \cdot nH_2O \longrightarrow MnO_2 \text{ (pyrolusite)} \tag{3}$$

$$MnO \cdot nH_2O \xrightarrow[\text{pH} > 6.5]{\text{Autoxidation}} MnO_2 \text{ (pyrolusite)} \tag{4}$$

Within the range of pH 3.2–8.0 in the reaction $Mn^{2+} \longrightarrow Mn^{4+}$, the redox potential is a linear function of pH. MnO_2 is partly unsatisfied in oxygen content ($O < 1.95$).

Using soil deficient in manganese, PASSIOMA and LEEPER [60] found that root growth of oats in terms of weight was much greater and root surface area was less in compacted than in loose soil. They offer the hypothesis that the root itself can reduce MnO_2 to a soluble form available for plants when it is in close contact. This idea is in agreement with the ability of roots to absorb other trace elements normally quite insoluble [10].

Deficiency has been described in grains, legumes, root vegetables, fruits, tea, sugar cane and trees. Because a slightly acid soil with plenty of organic matter provides available manganese, it is probable that much of the effect on plants of wood shavings, peat moss and other organic matter results from reducing manganic salts in soil to the manganous state. Manure and compost undoubtedly contain much manganese; digested sewage sludges are a rich source with 300 p.p.m. MnO [61].

In plants manganese functions in the formation of chlorophyll, controls the state of oxidation in several redox systems, and prevents the deposition of ferric iron in the leaves. It is a normal growth factor [62]. Manganese plays a direct role in photosynthesis independent of the level of chlorophyll [59], in keeping with its ability to be oxidized by direct sunlight. It is necessary for sporulation [63]. It catalyzes the destruction of indole acetic acid, an auxin, and activates nitrate reductase, the oxidation of dicarboxylic acids and decarboxylations in the citric acid cycle. Many bacterial and plant enzymes are activated by manganous ions, although specificity has been demonstrated unequivocally for only a few [59]. These necessary reactions account for the high concentrations in the green parts of plants and in the germinal portions of seeds.

WEINBERG [63] has pointed out that *Bacillus* requires manganese considerably in excess of that needed for normal vegetative growth in order to synthesize antibiotics, several enzymes, bacteriophage and protective antigens, and for the formation of

endospores and longevity of cultures; the requirement was satisfied by no other transitional metal of atomic numbers 24–30. This intriguing observation opens a wide field of investigation on other essential trace elements in amounts beyond minimal requirements for growth. WEINBERG [64] lists the following bacterial biosynthetic processes stimulated by manganese in excess of optimal growth requirements: formation of bacillin, bacitracin, mycobacillin, subtelin, polypeptides, cell dissociation, germination of spores, sporulation and mutagenesis. Therefore, amounts sufficient for growth are insufficient for optimal function. This concept, which applies equally to mammals, may be important in respect to possible human deficiencies.

Manganese as an essential trace element

As an intellectual exercise, we can briefly apply to manganese those criteria chosen for testing the biological essentiality of a trace element [58].

1. *Ubiquity.* Manganese is ubiquitous in biologically significant concentrations on the earth's crust and in sea water.

2. *Presence in living things.* All plants accumulate manganese, usually against an environmental gradient. All animals contain manganese. In so far as is known, all living organisms require manganese.

3. *Structure and function.* Manganese is a weak catalyst for oxidation-reduction systems, although it has nine oxidation states, more than any other transitional metal except rhenium. It is relatively inefficient for hydrogen binding and other hydrogen equilibration reactions, although it absorbs oxygen, acetylene, methane and carbon monoxide [65]. In the first transitional series it occupies an unique position by its cubic crystal structure. With organic compounds it has a strong tendency to form coordination compounds, especially with oxygen donors. It catalyzes the transfer of phosphoryl from one molecule to another and can act in transphosphorylation between ATP and orthophosphate. It promotes hydrolysis of amino acid esters and peptides* and stimulates decarboxylation, although this latter effect is weak when it is chelated to pyridoxal and amino acid complexes. It can form porphyrins.

In enzyme systems, by indirect evidence, manganous ion appears to be a co-factor of arginase and alkaline phosphatase. It is a less specific activator of a number of enzymes, most of them also dependent upon magnesium, but in some it is antagonistic to magnesium. However, prolidase and horse radish peroxidase require manganese as a co-factor [2]. Carnosinase, cystein desulfhydrase, thiaminase, deoxyribonuclease and glycyl-l-leucine dipeptidase are also activated, but specificity of manganese has not been proven [2, 59]. Therefore, whereas there is much evidence to implicate manganese in metabolic reactions, surprisingly few enzymes have been shown to be specifically dependent upon it.

4. *Presence in newborn and change with age.* In man manganese does not accumulate nor decline with age, it is stored in the newborn, is present in milk and obviously passes the placental barrier.

5. *Toxicity.* Divalent manganese has a low order of toxicity to living organisms, especially to vertebrate animals (Table 14). The concentrations reported toxic to

*Probably one example of its peptidase activity lies in the inactivation of angiotensin and pherentasin by manganous ion *in vitro* [66]. Pherentasin, a pressor substance found in human hypertensive arterial blood [67], is undoubtedly human angiotensin or a derivative.

TABLE 14. TOXICITY OF MANGANOUS ION IN WATER AND FOOD TO LIVING ORGANISMS*

Items	Nontoxic concentration (p.p.m.)	Toxic concentration (p.p.m.)
Plants grown in culture		
Yeast		500
Legumes		1–10
Various		3.5–62.5
Orange and mandarin seedlings		5
Tomatoes		5–10
Soybeans		10–25
Flax		25–100
Oats		150–500
Animals		
Daphnia magna, fresh-water	15	50†
Flatworms		700†
Fish, fresh water	15–20	2420–3450†
Eels, fresh water	2700†	
Birds	600	4800
Chicks	1000	4800
Rats	1000–2000	>2000
Rabbits		1250–6000
Pigs		500–2000
Cattle	50–600	
Lambs		5000

*From McKee and Wolf [68].
†Least toxic of trace metals tested, except for ferrous ion.
Note: Toxicity of permanganate is high, 2.3–4.1 p.p.m. as manganese for fish, 3–5 p.p.m. for eels, 0.28 p.p.m. for *Daphnia magna*.

plants grown in sand cultures are surprisingly low in view of normal manganese levels in soil, and suggest that only small amounts are available as soluble cation.

The large concentrations tolerated by vertebrates are obvious from Table 14 and are a manifestation of excellent homeostasis, less efficient in *Daphnia* but good in worms. Fish, molluscs and other marine animals, because of the large quantities of water passing through their gills and respiratory structures, are exposed constantly to manganese. As fresh water often contains 100–1000 times more than salt water, the capacity to reject or excrete the metal must be good. Copper, an essential trace element, is toxic to fish, for example, in concentrations less than 0.1 p.p.m. in soft water, and chromium (III), iron and zinc are toxic at less than 10 p.p.m. In fact, manganese is by far the least toxic to fish of all the trace elements tested, save iron [68]. Heptavalent manganese, which does not occur in nature, is very toxic, illustrating the general law that the natural valences of an element on the surface of the earth are the least toxic forms [11].

Industrial toxicity through an abnormal portal of entry, the lung, is well known. Manganism occurs in miners breathing manganic oxide dusts for 2 years or more. The first symptoms are those of psychosis resembling schizophrenia. Later, extrapyramidal disorders similar to paralysis agitans or Parkinsonism appear. The lungs serve as a depot from whence manganese is slowly and continuously absorbed; chelating agents have reversed the syndrome temporarily in a few cases, probably by removing some body manganese, only to have it recur as the lungs provide more. Tissue concentrations in the one case analyzed were normal; presumably the effects

of manganese on the brain are due to the presence of metal in extracellular fluids or to rapid turnover and not to deposition in nervous structures [2].

6. *Homeostasis.* The data here presented express signs of extraordinary homeostasis of manganese in the biosphere. Plants concentrate manganese from the soil in leaves, twigs and fruit, as they do boron, copper, zinc, molybdenum, silver, cadmium and lead [26]. Germinal structures are especially rich, to supply the growing seed. In spite of high soil concentrations, toxic accumulations seldom occur and deficiencies are usually the result of tampering with the soil.

Animals absorb enough manganese for their requirements and in almost all cases reject excesses*, which is the case for most essential trace elements. Because of the varied diets of animals, deficiency rarely exists except experimentally or from extreme conditions of soils, and that only in growing animals. The mechanisms for conserving manganese in the face of deficient intakes must be efficient.

Two practices in modern nutrition possibly could lead to relative deficiencies in human intake of manganese. Arable soils become depleted by removing organic matter and replacing it with inorganic fertilizers. Fortunately phosphates contain some manganese. However, we found no serious deficiency in plant foods. Manganese (and many other trace elements and vitamins) is removed from grains by refining procedures to make white flour and polished rice. The product is then naively 'enriched' with two or three of the organic but with none of the inorganic micronutrients removed†. In spite of this practice, retention of manganese by the human organism appears to be extremely efficient and the small body pool is not obviously depleted, although turnover may be depressed. At this writing it is doubtful that the disturbed lipid metabolism common to civilized man is caused by partial manganese deficiency, unless conditioned by other elements or dependent upon turnover‡.

An indirect illustration of the high degree of manganese homeostasis appears when total daily intakes of essential trace metals are compared with total bodily pools. Elements excreted via the kidneys are easily handled [58] and the ratios of intake to bodily content are often large. Of metals excreted via the intestine, manganese has the highest ratio (Table 15), large quantities being dealt with efficiently.

7. *Biological activities in mammals.* BERNHEIM and BERNHEIM [69] were the first to demonstrate a biological effect of manganese on isolated systems. Microgram quantities added to rat liver protein inhibited oxygen uptake and oxidation of phospholipids and cysteine. A manganese–vanadium antagonism was demonstrated, which was elegantly confirmed some 15 years later by CURRAN [70, 71], who showed that vanadium inhibited, while manganese and chromium stimulated the hepatic

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*One example of human homeostasis can be found in the widespread use of mildly alcoholic tonic formerly popular in the South, but no longer made. It contained 30 mg of manganese citrate per pint. In our laboratory, patients have been given 30 mg of this salt per day for many months without signs of toxicity.

†An illustration of the severe depletion of manganese from wheat is found in the analyses of CZERNIEJEWSKI *et al.* [89]. On a dry basis, 10 samples of wheat contained 46 p.p.m. on average, whereas 10 samples of white flour contained 6.5 p.p.m. manganese, and 10 samples of white bread, 5.9 p.p.m. Germ, bran and two other fractions had 121–165 p.p.m. A similar degree of depletion by milling of wheat was demonstrated for 5 bulk and 5 essential trace elements.

‡TIPTON *et al.* [13], however, point out that the mean concentration of manganese in the aortas (and spleens) of Americans is significantly less ($P < 0.001$) than those in the aortas of Africans and Near and Far Easterners. For a lipotropic element to be low in aortas subject to atherosclerosis may be important. The higher values were found predominantly where tea is the principal beverage.

TABLE 15. DAILY DIETARY INTAKES OF ESSENTIAL TRACE METALS COMPARED TO TOTAL BODY POOLS IN MAN, ESTIMATED [24, 36, 58]

Metal	Daily intake (mg)	Body pool (mg)	% Body pool ingested daily
Iron	12–15	4100	0.29–0.37
Zinc	10–15	2300	0.43–0.65
Copper	2–3	100	2–3
Manganese	2–5	20	10–25
Chromium	0.050–0.100	6	0.83–1.67
Molybdenum*	1–2	5	20–40
Cobalt	0.005–0.008	3	0.017–0.027
Sodium (for comparison)*	10,000–15,000	105,000	9.5–14.3

*Excreted by the kidney [58].
Note: As these metals do not ordinarily accumulate in man, total excretion equals intake.

synthesis of cholesterol and fatty acids in rats. In the synthesis of cholesterol from acetate, manganous ion is a necessary co-factor for the biocatalysis of mevalonic acid to squalene [72] by mevalonic kinase, and a phosphorylated derivative of mevalonic acid necessary for this reaction also requires manganese for its synthesis [73]. In the synthesis of fatty acids from acetate, manganous ion is probably chelated to coenzyme A. Therefore, lipid synthesis appears to be manganese-dependent [74].

COTZIAS [2] has accumulated a considerable body of indirect evidence that oxidative phosphorylation is dependent upon manganese: (1) Injected radiomanganese accumulates in mitochondria; (2) Bilirubin binds manganese and interferes with oxidative phosphorylation; (3) Manganese is the most effective metal for activating this system; (4) The system is depressed in manganese-deficient chicks. Manganese appears to be as basic a co-factor in metabolism as any other micronutrient.

COTZIAS *et al.* [75] have recently demonstrated a direct relationship between manganese levels and the darkness of hair, feathers and conjunctiva in man, the dog, the cow and birds, based on the idea that the number of free radicals in melanin is proportional to the intensity of color*. The idea that pigmentation was influenced by metal ions was first proposed by KIKKAWA *et al.* [76]; we have sporadically explored it with analyses of various trace elements [5–11]. As normal pigmentation is genetically controlled, this general hypothesis is of considerable interest in regard to the possible genetic transmission of a trace element for determining a genotype.

These considerations leave little doubt that manganese is an essential trace element for plants and animals. In fact they all point to the importance of this metal for many necessary biological reactions involved in growth, reproduction and health of all living organisms.

Manganese metabolism in mammals

COTZIAS and his co-workers have contributed most to our present knowledge of manganese absorption, excretion and turnover [1, 2]. Ingested divalent manganese as ion or in chelates probably remains unchanged in the acid stomach, but in the alkaline small intestine conditions are favorable for oxidation. The mechanism of

*Results of our analyses of human hair follow (p.p.m.): Blond, male, aged 2½ years, 0.95; brown, female, aged 22 years, 1.48; dark brown, male, aged 21 years, 1.68. Hair of a white English sheepdog contained 5.68 p.p.m.

intestinal transport is not known, but it apparently depends upon intestinal concentration and state of the body pool, as is the case for iron. Absorbed manganese is transported to the liver where it conjugates with bile and is excreted into the intestine; part is reabsorbed in a small enterohepatic circulation.

Manganese absorbed into the general circulation is transported by a β_1 globulin in plasma, transmanganin, in the trivalent form. *In vitro* binding to transmanganin is slow, but is increased by oxidizing agents ($Mn^{2+} \rightarrow Mn^{3+}$). The complex can be dissociated by reducing agents, which change Mn^{3+} into Mn^{2+}. The alkaline state of mammalian body fluids, however, is conducive to retention of manganese in the trivalent state.

Turnover of manganese is rapid [77, 78]. Injected radiomanganese quickly disappears from the blood and enters mitochondria in the liver and pancreas, entering into other organs more slowly, providing a method for estimation of the body's mitochondrial pool [2]. Challenge with stable manganese in any valence state promotes rapid excretion of radiomanganese. The largest fraction in the body is in a freely exchangeable, labile pool, various organs exhibiting markedly different rates of turnover. Skin, bone and erythrocytes are examples of tissues with low rates. Radiomanganese exchanges slowly with red cell manganese, which is held in a porphyrin complex [29].

When the hepatic route of excretion is blocked, the pancreas serves as a reserve organ of excretion of absorbed manganese, thus maintaining homeostasis. The mechanism of hepatopancreatic excretion is probably common to all animals with these organs, and evolutionarily antedates the renal route [38].

Partition of manganese in the bodies of mice is affected by glucocorticosteroids but not by mineralocorticoids. Livers retained less and carcasses more of injected radiomanganese, either in divalent or trivalent plasma-bound forms [79], when animals were treated with these steroids.

Injury to the myocardium of man releases large amounts of manganese into the peripheral blood. HEDGE *et al.* [80] have found serum concentrations of manganese almost universally elevated after acute coronary occlusion. The elevation was a more accurate index for diagnosis of myocardial infarction than were levels of glutamic oxalacetic transaminase, which can give false positive reactions. This observation is interesting in view of the relatively low concentrations of manganese in muscle of all types (Tables 1 and 2). The hypoglycemic effect of plant extracts as home remedies for diabetes mellitus has been traced to manganese. An unusual case responding dramatically to oral manganese chloride has been reported by RUBENSTEIN *et al.* [81], although other diabetics were unaffected. Vitamin E deficiency in the mouse is partially counteracted by manganese [82].

BRITTON and COTZIAS [83] have shown that turnover of manganese is dependent upon supply; the valence state of the supply is immaterial [79]. COTZIAS [47] has suggested that disorders due to manganese deficiency or excess may result from slow or rapid rates of turnover. This idea needs close examination. (1) Known manganese-dependent enzymes require divalent metal. (2) The alkaline body media favors oxidation of manganese to the trivalent form; reduction may be difficult in that media. (3) The evolutionary need for development of a strong manganese-reducing system in mammals may have been offset by the abundance of divalent manganese in the environment. In that event, low dietary intakes of Mn^{2+} would cause lessening of a

normal Mn^{2+} : Mn^{3+} ratio in tissues and high intakes would increase this ratio without change in tissue concentrations. If deficiency or excess of Mn^{2+} could be demonstrated in man, it would open a promising field of investigation, shake some of our established concepts on metallic micronutrients and place manganese homeostasis [83–85] in a new light.

Manganese balance in man

The average daily balance of manganese in normal human beings can be estimated.

Intake (mg)		Output (mg)	
In food	3.0 (2.0–7.0)	In urine	0.150
In water	0.005 (0.0–1.0)	In feces	2.857
In air	0.002 (0.0–0.039)	Retained	0.0

Variations of intake depend upon the type of diet, the water supply and the amount of particulate matter in air. Dietary intakes as low as 0.5 mg per day in 2300 calories can be readily obtained from meat and sea foods, dairy products, bread, fats and sugar as the main source of calories. Without green vegetables, tea or coffee, a low carbohydrate, high protein, high fat reducing diet of 1500 calories could contain as little as 0.28 mg manganese. Intakes as high as 13–20 mg per day are provided by a 2300-calorie vegetarian diet of whole grains, fruit, nuts, tea and fresh vegetables, well spiced. The amount from air is obviously absorbed into the body, for there is no demonstrable accumulation in lungs with age. These large differences in intake are probably reflected in equally large differences in the rates of turnover of manganese in various human bodies.*

SUMMARY AND CONCLUSIONS

A survey of manganese in the environment of man has involved analyses of food, foliage and tissues. Manganese is omnipresent in living organisms. Trees concentrate it in leaves and twigs against an environmental gradient; some vegetable foods do not. Manganese is available to plants when soil pH is in the range of 4–6; in more alkaline soils it becomes insoluble by oxidation, but can be reduced by bacteria in decaying organic matter. Deficiencies in plants are known.

In vegetable foods, manganese is especially concentrated in the germinal portions of grains and fruits and in nuts, as well as in leafy vegetables. Tea and some spices are rich in manganese. Refining of flour removes much of the manganese. Root vegetables contain little. Diets low in manganese may be fairly common.

Animals, both marine and terrestrial, have a remarkable ability to reject manganese taken in from the environment. No form of animal life accumulates manganese. Hence, animal foods are generally low in this element. An efficient homeostatic mechanism for manganese appears to operate in all vertebrate and most invertebrate animals.

*Because of these great differences in intake and the large amounts in tea, one might expect that the tissues of human subjects from areas where tea is the principal beverage would differ from those in areas where coffee is largely drunk. No such differences are apparent in Table 3. The data of STITCH on British subjects [86], of HARDING-BARLOW on South Africans [87] and of BUTT *et al.* [88] on Californians were also compared to those here reported, although spectrographic analyses were made by somewhat different techniques. Manganese levels in liver and pancreas were somewhat higher in South Africans and the British than in our subjects, but those in other tissues were generally similar. BUTT's levels in pancreas were somewhat lower than ours.

All human tissues analyzed from around the world contained manganese. Organ specificity was demonstrated for liver, kidney, intestine and pancreas. Muscle had low concentrations. Variations in any single tissue in differing groups of subjects were remarkably small. No significant change in manganese concentration occurred in any organ with age, except that tissues normally low in this element were higher in the newborn. The human lung did not accumulate manganese with age, in spite of appreciable concentrations in the air of industrial cities.

The usual daily intake of manganese by man approximates 2–5 mg or 10–25 per cent of a total bodily pool of 20 mg, a ratio higher than that of any other cationic trace metal. A small increment appears in the urine; the remainder is excreted by the liver and pancreas or is unabsorbed.

Overt manganese deficiency has not been recognized in man. As measured by concentrations in liver, depletion is fairly uncommon. Excess accumulation of manganese occurs in a few human beings. Whether or not such excesses cause disease is not known. The theoretical existence of an inborn error of metabolism leading to accumulation of manganese in some organ is possible but has yet to be proven.

The weight of evidence that manganese is essential for man is overwhelming. The metal is probably concerned in oxidative phosphorylation and lipid metabolism.

In so far as is known, manganese is an essential trace element for all living organisms.

Acknowledgements—We are indebted to SANDRA FILGATE for performing the analyses.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 241, No. 15, Issue of August 10, pp. 3480-3487, 1966
Printed in U.S.A.

Pyruvate Carboxylase

VI. THE PRESENCE OF TIGHTLY BOUND MANGANESE*

(Received for publication, February 10, 1966)

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SUMMARY

The presence of a paramagnetic metal ion in pyruvate carboxylase has been shown by the effect of this enzyme on the longitudinal relaxation rate of the protons of water as measured by pulsed nuclear magnetic resonance. The metal ion shows an enhanced effect on the proton relaxation rate (ϵ_b = 4:2) when bound to the enzyme as compared to that of the free metal ion in the presence of denatured enzyme. This observation suggested that the metal ion is manganese, and the presence of manganese in the purified enzyme is confirmed by chemical, neutron activation, emission spectral, and atomic absorption spectral analyses. Quantitative analysis by various methods has given the metal content as 2.5 to 4.3 moles of manganese per mole of enzyme or 0.6 to 1.1 moles of manganese per mole of biotin. ^{54}Mn is incorporated into pyruvate carboxylase *in vivo*. When the enzyme is isolated from chicken liver mitochondria, a good correlation is obtained between radioactivity and enzymic activity at all stages of purification subsequent to the initial mitochondrial extract. Correlation between total manganese content and enzymic activity has also been shown at various stages of purification. Studies on the ^{54}Mn-labeled enzyme have shown that release of ^{54}Mn and reduction in the enhancement of the proton relaxation rate occur only after irreversible denaturation of the enzyme. Neither the ^{54}Mn content nor the proton relaxation rate is affected by exposure of the enzyme to cold or ethylenediaminetetraacetate. No exchange of the bound metal ion with added manganese can be shown. Although the protein-bound manganese possesses magnetic properties consistent with divalent manganese ion, higher valency states cannot be excluded.

Pyruvate carboxylase (pyruvate-CO_2 ligase (ADP), EC 6.4.1.1), purified from chicken liver mitochondria, catalyzes an adenosine triphosphate-dependent CO_2 fixation on pyruvate (Reaction 1) (1, 2).

$$\text{Pyruvate} + \text{ATP} + HCO_3^- \xrightleftharpoons{\text{acetyl-CoA, } Mg^{2+}} \text{oxalacetate} + \text{ADP} + P_i \quad (1)$$

Manganese can replace magnesium in Reaction 1 with no significant change in the maximal rate (2), and, hence, the role of the added divalent cation in the above reaction can be studied by the proton relaxation rate method described by Cohn and Leigh (3) and Cohn (4). During the course of these studies, the pyruvate carboxylase preparation itself was observed to cause a marked increase of the proton relaxation rate. The characteristics of the effect suggested that the paramagnetic metal species responsible was manganese. Further studies designed to elucidate the identity of the metal, its stoichiometry, and the general nature of its binding to the protein are described below.

Evidence indicating that the bound manganese has a functional role in the pyruvate carboxylase reaction is presented in the succeeding article (5).

A preliminary account of some of these findings has already appeared (6).

EXPERIMENTAL PROCEDURE

Methods—Pyruvate carboxylase was prepared as described previously (7) and was assayed spectrophotometrically in the direction of CO_2 fixation (1), with specific activities expressed as micromoles per min per mg of protein at 25°. Protein was estimated spectrophotometrically (8) or by a micromodification of the biuret procedure (7). The PRR[1] of water was measured as described by Mildvan and Cohn (9). The observed enhancement (ϵ^*) of the effect of manganese on the PRR of water which is due to binding of this metal ion by the protein is defined as

$$\epsilon^* = \frac{1/T_1^* - 1/T_1^0}{1/T_1 - 1/T_1^0} \quad (2)$$

where $1/T_1^*$ is the observed PRR of the solution of pyruvate carboxylase and $1/T_1$ is the PRR of a solution containing an equivalent concentration of divalent manganese as $MnCl_2$. The correction value, $1/T_1^0$, represents the PRR of water, buffer, and salts. The protein component of pyruvate carboxylase is as-

* This work was supported in part by Contract AT (11-1) 1242 of the Atomic Energy Commission, by United States Public Health Service Grant GM 08320, and by National Science Foundation Grant G-23384.

‡ This work was done during the tenure of an advanced postdoctoral fellowship from the American Heart Association.

[1] The abbreviations used are: PRR, proton relaxation rate; EPR, electron paramagnetic resonance.

sumed to have negligible effect on the PRR of water at the concentrations used (5 to 12 mg per ml). This assumption is supported by observations on bovine serum albumin (9) and pyruvate kinase (10). Since all the manganese is bound to protein in the present system, ϵ^* equals ϵ_b, the enhancement of the bound manganese.

Electron paramagnetic resonance spectra were measured as described previously (9). ^{54}Mn and ^{59}Fe were estimated with the use of a Nuclear-Chicago gas flow counter.

Emission spectral analyses of pyruvate carboxylase were carried out in a Jarrell-Ash emission spectrograph with the use of a graphite arc. The quantitative estimation of the metal content of these preparations was performed by densitometric comparison with standards. Manganese was detected and estimated chemically by the formaldoxime method as described by Sandell (11). A linear relationship between absorbance at 450 mμ and manganese content was shown for the range 0 to 5 μg of manganese, and the absorbance was decreased approximately 15% by standing for 2 hours at 65°. Pyruvate carboxylase samples were prepared for chemical analysis of their manganese content by wet ashing with H_2SO_4-HNO_3 (11). Similar treatment of standard manganese solutions in the presence and absence of bovine serum albumin showed that color development was not significantly affected by the presence of protein.

Analysis of the manganese content of the various fractions obtained during purification of pyruvate carboxylase was performed by atomic absorption spectroscopy with the Aztec instrument manufactured by Techtron, Ltd., Melbourne, Australia. A linear relationship between manganese concentration and optical density at 2793 A was observed up to 10^{-4} M Mn^{2+}. The linear region of 95 to 100% transmission was expanded by the use of a Leeds and Northrup Speedomax 1-mv recorder, which resulted in a noise level equivalent to less than 1 μM Mn^{2+}. Proteins in solution were denatured and extracted with 7% $HClO_4$ for 15 min and centrifuged. The supernatant solution, which contained 3 to 6 μM manganese, was used for analysis. The same results were obtained without denaturation or removal of protein, indicating that the extraction was essentially complete.

Iron analysis by atomic absorption at 2480 A was only one-fifth as sensitive as manganese analysis. The noise level was equivalent to 2 μM iron. Analyses were carried out with the use of protein-free extracts in 7% $HClO_4$ or 1.2 M HCl at a level of 6 to 8 μM iron.

Materials—$^{54}MnCl_2$ (carrier-free) was obtained from New England Nuclear; $^{59}FeCl_3$ from Oak Ridge National Laboratory; 5,5′-dithiobis(2-nitrobenzoic acid) from Aldrich; and EGTA[2] from Eastman. All other reagents were obtained or prepared as described previously (7).

RESULTS

Identification of Metal Ion—In contrast to many other proteins, pyruvate carboxylase caused a marked increase in the PRR of water. For example (Table I), bovine serum albumin and pyruvate kinase had no significant effect on the PRR of the buffer (0.35 to 0.42 sec^{-1}), but pyruvate carboxylase caused a marked increase in the relaxation rate (1.92 sec^{-1}). The PRR of a solution of the latter enzyme after denaturation with perchloric acid (0.68 sec^{-1}) was much reduced as compared with the native enzyme, but was greater than the PRR of pure buffer.

[2] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid.

TABLE I

Presence of paramagnetic metal in pyruvate carboxylase

For the measurements on pyruvate carboxylase, the system contained, in 0.1 ml, 5 μmoles of Tris-Cl (pH 7.8), 8 μmoles of Tris-Cl (pH 7.2), 32 μmoles of KCl, and 0.94 mg of pyruvate carboxylase (specific activity, 12.6). The PRR was measured as described by Cohn (4). The values for the effects of bovine serum albumin and pyruvate kinase on the PRR of water are taken from the data of Mildvan and Cohn (9) and Mildvan and Cohn (10), respectively.

System	PRR
	sec^{-1}
Buffer	0.35–0.42
Bovine serum albumin (24 mg/ml)	0.40–0.45
Pyruvate kinase (9.2 mg/ml)	0.40–0.45
Pyruvate carboxylase (9.04 mg/ml)	1.92
Pyruvate carboxylase (9.04 mg/ml) + 14% perchloric acid	0.68
ϵ_b (enhancement)[a]	4.2

[a] The enhancement (ϵ_b) of pyruvate carboxylase was calculated according to Equation 2.

The increment in the PRR of the denatured enzyme as compared with the PRR of the buffer is due to the metal ion which has been released from the protein.

The nature of the PRR effects suggested that Mn(II) is the metal ion responsible and that the metal-containing site is accessible to water. The identification of the paramagnetic ion as Mn(II) is based on the following considerations. On the assumption that a reasonable stoichiometric relationship holds between the metal content and the biotin content of the enzyme (4 moles of biotin per mole of enzyme) (7), the PRR value for the metal ion after release from the protein by denaturation (cf. Table I) is consistent only with elements with 5 unpaired electrons, *i.e.* high spin complexes of Mn(II) and Fe(III). The observed enhancement value when the metal ion is bound to the protein (ϵ_b = 4.2; cf. Table I) rules out those metal ions with a short electron spin relaxation time such as Fe(III), Fe(II), Co(II), and Ni(II), but does not exclude Cu(II), Cr(III), or Mn(II) (12).

Examination of the EPR spectrum of pyruvate carboxylase in 0.05 M Tris-Cl (pH 7.0), containing 0.2 M KCl at 77° K and 300° K, revealed only a small free radical signal at g = 2 in one preparation and no detectable signal in another preparation. However, after denaturation of the enzyme with perchloric acid and removal of the protein by centrifugation, an EPR spectrum typical of manganese was obtained. These considerations suggest that the bound paramagnetic metal ion in pyruvate carboxylase is manganese.[3]

Examination of pyruvate carboxylase preparations by emission spectroscopy (Table II) shows the presence of significant quantities of manganese. In the first experiment, the enzyme was not treated to remove adventitious metal ions, and the manganese content of the preparation may be somewhat high. Significant amounts of iron and several other elements were also noted, although copper and chromium were found only in trace amounts. In a later experiment, the enzyme was pretreated with 10^{-2} M

[3] In preliminary experiments, pyruvate carboxylase in 1 M sucrose and 0.1 to 0.2 M $(NH_4)_2SO_4$ gave a slightly altered manganese spectrum at 30° K and at 77° K, which may be due to the release of bound manganese from the enzyme under these conditions.

TABLE II

Emission spectral analysis of pyruvate carboxylase

For analysis of the untreated enzyme, a sample containing 16.4 mg of protein with a specific activity of 16.8 was equilibrated with 0.05 M potassium phosphate (pH 7.2), containing 0.06 M $(NH_4)_2SO_4$, by passage through a Sephadex G-50 column equilibrated with the same buffer mixture. Buffer obtained from the equilibrated column was used as a blank. The stoichiometric calculations are based on an estimated molecular weight of 655,000 (7). For the treated enzyme, a sample containing 18 mg of protein with a specific activity of 28 was pretreated with 0.01 M EDTA and then passed through a Sephadex G-50 column equilibrated with 0.2 M KCl-0.05 M Tris-Cl (pH 7.2). Both KCl and Tris had previously been recrystallized from EDTA. A sample of the KCl-Tris Cl analogous to that containing the protein was used as the blank.

Element	Enzyme (untreated)		Enzyme (treated with EDTA)	
	Net content[a]	Ratio	Net content[a]	Ratio
	ppm	*moles/mole enzyme*	*ppm*	*moles/mole enzyme*
Mn	500 (0.5)	6.0	300 (0)	3.6
Fe	250 (50)	2.9	100 (200)	1.2
Cu	21 (4)	0.2	10 (80)	0.1
Cr	15 (10)	0.2	0 (0)	0
Ni	10 (5)	0.1	0 (0)	0
Mg	450 (50)	12.1	0 (600)	0
Ca	200 (200)	7	0 (800)	0
Al	300 (40)	3.3	100 (100)	0.9
Mo	200 (50)	1.4	0 (80)	0
Si	300 (200)	8.7	800 (500)	23
Ba	20 (5)	0.1	0 (0)	0
Pb	15 (5)	0.1	0 (0)	0

[a] Content after subtraction of the blank. The values of the latter are given in parentheses.

EDTA, which does not remove the paramagnetic component of pyruvate carboxylase (see Table V), and the manganese content was determined as 3.6 moles per mole of protein. In this determination, all other elements tested were absent except iron, aluminum, and silicon. The presence of aluminum and silicon and perhaps of iron may have been due to contamination of the sample during the ignition procedure by clay remaining in the furnace from previous samples, and this circumstance probably also contributed to the high blank for certain elements in the second sample. Unfortunately, these conjectures cannot be established with certainty.

The emission spectral analyses are consistent with the hypothesis that manganese is the metal ion present in pyruvate carboxylase, but do not eliminate iron as a possible constituent of this enzyme. To obtain evidence of the nature of the metal by other means, we attempted to label pyruvate carboxylase by administration of $^{54}MnCl_2$ and $^{59}FeCl_3$ to chickens. The enzyme was subsequently isolated from the livers and examined for radioactivity. $^{54}MnCl_2$ (250 to 300 μC) was administered by intravenous or intraperitoneal injection to 8-week-old chickens which were killed by decapitation after 4 to 5 days on a normal diet. Lyophilized mitochondrial powder was prepared as described previously (7). Considerable radioactivity was present in the mitochondrial powder and in the initial crude extract prepared from this powder, and, in addition, the purified enzyme contained significant radioactivity. Examination of the ^{54}Mn distribution during purification (Table III) showed an essentially constant ratio of ^{54}Mn to enzymic activity at all stages subsequent to the 25 to 33% $(NH_4)_2SO_4$ precipitate (Stage 2). When 500 μC of $^{59}FeCl_3$ were used in a similar experiment, no radioactivity was found in the purified enzyme, although considerable incorporation was observed both in the mitochondrial powder and in the initial crude extract.

Correlation of ^{54}Mn and CO_2 fixation in the purified enzyme has also been examined when ^{54}Mn-pyruvate carboxylase (Stage 5) was fractionated by gel filtration on Sephadex G-200 (Fig. 1) and by sucrose density gradient centrifugation according to the procedure of Martin and Ames (13) (Fig. 2). On Sephadex G-200 (Fig. 1), there was good correlation between pyruvate carboxylase activity and radioactivity in the early and middle fractions (Fractions 1 to 10), but a small excess of radioactivity appeared beyond the main peak (Fractions 12 to 14). Preparations of the enzyme similar to those used in the gel filtration experiment have been shown to contain two components, the sedimentation coefficients of which when extrapolated to infinite dilution are 14.8 S and 6.75 S (7). Clear resolution of the 14.8 S and 6.75 S components is obtained on centrifugation through a sucrose density gradient in contrast to the incomplete separation given by gel filtration on Sephadex G-200. Enzymic activity was shown to be confined to the 14.8 S component, but the 6.75 S component also contained biotin at a reduced ratio to protein as compared with the 14.8 S protein (7). Examination of the 6.75 S component (Fig. 2, fraction centering on tubes 9 and 10) indicated that radioactivity was present, but at a reduced ratio to protein as compared with the 14.8 S component (Fractions 1 to 3). The relationship between the ^{54}Mn to protein ratios of the 14.8 S and 6.75 S components is similar to that previously observed for the biotin to protein ratios of these two fractions (7).

While these experiments show unequivocally that ^{54}Mn is incorporated into pyruvate carboxylase *in vivo*, the low incorporation (about 0.001% of the added dose) necessitated a rigorous identification of the radioactive component as ^{54}Mn. The γ emission spectrum of ^{54}Mn is characteristic of this radioisotope, and the spectrum obtained from the radioactive enzyme with the use of a γ emission spectrometer (Fig. 3) was identical with the spectrum of a sample of carrier-free $^{54}MnCl_2$ and clearly different from that given by carrier-free $^{59}FeCl_3$.

New protein synthesis under conditions *in vivo* is presumably required for manganese incorporation since incubation of isolated mitochondria with $^{54}MnCl_2$ and subsequent purification of pyruvate carboxylase showed no incorporation of ^{54}Mn by exchange.

Total Manganese Content during Purification of Pyruvate Carboxylase—A more definitive correlation of manganese with pyruvate carboxylase was sought by atomic absorption spectral analysis of the total manganese content as compared with the specific activity of various fractions obtained during purification of the enzyme (Table IV). The manganese content increased in approximately the same proportion as the specific activity after the first $(NH_4)_2SO_4$ precipitation, with some tendency for the ratio of manganese to activity to fall. The reasonably good correlation observed offers support to the thesis that pyruvate carboxylase contains manganese as a constitutive element. The manganese content of the final fraction as estimated by this method is 3.0 ± 0.5 moles per mole of enzyme, which is slightly lower than the values obtained by other methods (*cf.* Table VI). The final sample also contained significant quantities of iron (0.8

TABLE III

Balance study of incorporation of ^{54}Mn into fractions from lyophilized chicken liver mitochondria

Carrier-free $^{54}MnCl_2$ (250 μC) was administered to two 8-week-old White Rock chickens by intraperitoneal injection. After 4 days, lyophilized liver mitochondria were prepared as described by Utter and Keech (1). Twenty-seven grams of nonradioactive lyophilized mitochondria were added and pyruvate carboxylase was prepared by the procedure of Scrutton and Utter (7). The stage numbers correspond to the description of this purification method with percentage of $(NH_4)_2SO_4$ referring to percentage of saturation at 25°. The fractions were assayed for CO_2 fixation, protein, and ^{54}Mn as described under "Experimental Procedure." Protein-bound ^{54}Mn was estimated in the crude extract and in the 33% $(NH_4)_2SO_4$ supernatant fraction after gel filtration of a 1-ml aliquot on a Sephadex G-50 column (25 × 1 cm) equilibrated with 0.05 M potassium phosphate (pH 7.2) containing 0.06 M $(NH_4)_2SO_4$ and 10^{-2} M EDTA.

Fraction	Pyruvate carboxylase		^{54}Mn		^{54}Mn to PC ratio
	units	*units/mg*	*total cpm*	*cpm/mg*	*cpm/unit*
Stage 1					
Crude extract			82,600	12.5	
Crude extract (after Sephadex G-50 with EDTA)	3,000	0.81	31,400	8.06	10.0
0–25% $(NH_4)_2SO_4$ precipitate	378	1.8	1,345	6.45	3.6
Stage 2					
25–33% $(NH_4)_2SO_4$ precipitate	2,230	3.1	7,400	10.3	3.3
33% $(NH_4)_2SO_4$ supernatant fraction			64,400	11.6	
33% $(NH_4)_2SO_4$ (after Sephadex G-50 with EDTA)	510	0.1	19,100	3.4	34.0
Stage 3					
$Ca_3(PO_4)_2$ gel supernatant	1,910	2.9	7,070	10.7	3.7
Stage 4					
$(NH_4)_2SO_4$ extracts					
30%	10	0.5	40	2.0	4.0
28%	34	1.8	123	6.4	3.6
22% (I)	1,180	19.8	4,130	67.2	3.5
22% (II)	520	22.0	1,650	69.6	3.2
20%	241	10.6	820	36.2	3.4
Stage 5					
70% $(NH_4)_2SO_4$ precipitate	1,320	15.0	5,410	61.4	4.1

mole per mole of enzyme) as determined by the atomic absorption spectral analysis.

Properties of Bound Manganese—The availability of ^{54}Mn-pyruvate carboxylase has permitted examination of the nature of the binding of the metal to the enzyme. The radioactive protein has been subjected to various treatments which might replace or remove the metal. The effects of these manipulations on the ^{54}Mn content of the enzyme can also be correlated with changes produced in PRR and enzymic activity. The results of a number of such experiments are shown in Table V. Denaturing agents, such as trichloracetic acid and sodium dodecyl sulfate, caused both complete release of radioactivity and complete loss of the enhancement observed with the native enzyme. These results provide experimental justification for the measurement of the manganese content by PRR analysis (see Table VI).

Preincubation of the enzyme for 5 hours with EDTA or with EGTA, an analogue of EDTA with a higher affinity for manganese (14), did not affect the PRR or the radioactivity. The catalytic activity was also unaffected under these conditions. These observations suggest either that manganese is very firmly bound to pyruvate carboxylase or that access to the manganese-containing site in pyruvate carboxylase is restricted.

Pyruvate carboxylase is rapidly and almost completely inactivated by exposure to cold (7), and cold inactivation is accompanied by dissociation of the active enzyme (14.8 S) to yield a 6.75 S-subunit which lacks enzymic activity. However, cold inactivation affected neither the radioactivity nor the enhancement of the bound manganese, and this finding is consistent with results from sucrose density gradient centrifugation of ^{54}Mn-pyruvate carboxylase which afforded evidence for the presence of manganese in the 6.75 S component. The results are in contrast with the release of ^{54}Mn and reduction in enhancement observed in the presence of 1% sodium dodecyl sulfate. This agent has previously been shown to cause dissociation of pyruvate carboxylase to yield a subunit (s_{20} = 2.3 S) (7). The results presented in Table V, therefore, support the suggestion (7) that the subunit produced by cold inactivation differs markedly from that produced by dissociation of the enzyme in the presence of 1% sodium dodecyl sulfate.

The observations with —SH inhibitors are equivocal. While 5,5′-dithiobis(2-nitrobenzoic acid) (15) affected neither property, *p*-chloromercuribenzoate caused almost complete loss of radioactivity but only an incomplete reduction of ϵ^*. The failure of mercuribenzoate to cause complete reduction in enhancement may be due to binding of the released manganese to other sites on the protein in the PRR experiment.[4] However, other factors cannot be excluded since mercuribenzoate also caused rapid precipitation of pyruvate carboxylase on certain occasions.

All attempts to remove manganese reversibly from this enzyme have been entirely unsuccessful. As discussed in the following article (5), some substrates and inhibitors, *e.g.* pyruvate and oxalate, produced large decreases in the enhancement (ϵ^*) of the bound manganese resulting from the formation of freely dissociable enzyme-substrate or enzyme-inhibitor complexes. However, the presence of such substances did not cause loss of ^{54}Mn from the radioactive enzyme. Experiments not shown here

[4] A. S. Mildvan and M. C. Scrutton, unpublished observations.

afforded evidence that bound manganese in purified pyruvate carboxylase did not exchange with added manganese in the presence or absence of glutathione. By contrast, in some other metalloproteins, *e.g.* carboxypeptidase (16), exchange with added metal ion occurs readily under these conditions.

Manganese-Pyruvate Carboxylase Stoichiometry—The results

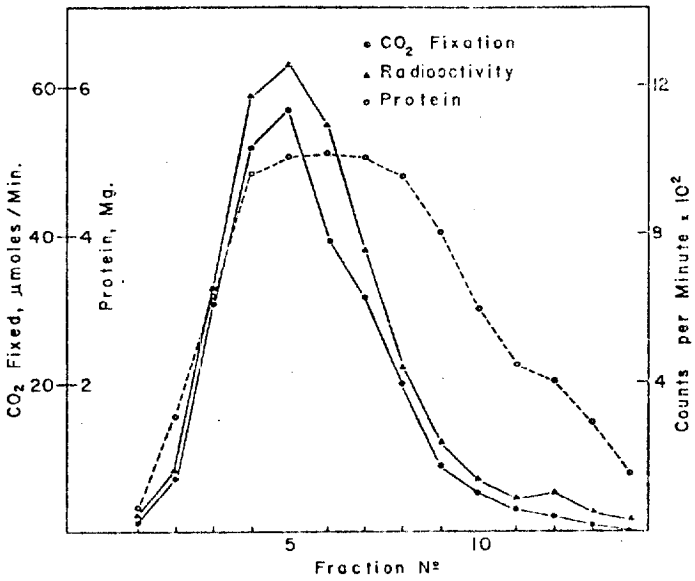

FIG. 1. Correlation of radioactivity and CO_2 fixation by gel filtration on Sephadex G-200. A sample of pyruvate carboxylase (Stage 5) (47.5 mg; specific activity, 7.2), purified from chickens treated by injection with $^{54}MnCl_2$, was placed on a column (12 × 2.5 cm) of Sephadex G-200 equilibrated with 0.1 M Tris-sulfate (pH 7.2) containing 0.06 M $(NH_4)_2SO_4$ and 0.01 M EDTA. Three-milliliter fractions were collected and analyzed for CO_2 fixation, radioactivity, and protein as described under "Experimental Procedure."

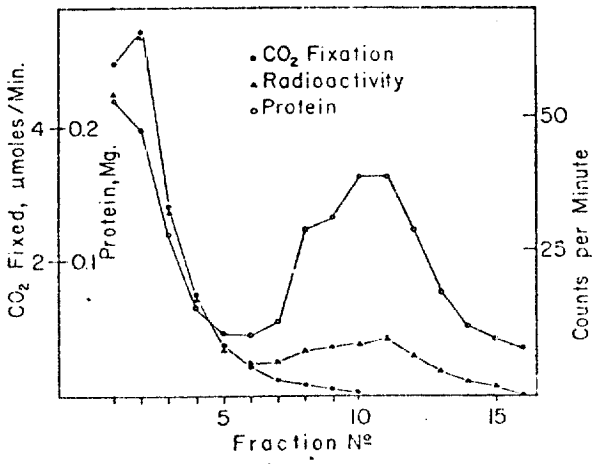

FIG. 2. Correlation of radioactivity and CO_2 fixation by centrifugation through a sucrose density gradient. Pyruvate carboxylase (Stage 5) (1.47 mg; specific activity, 12.1), purified from chickens treated by injection with $^{54}MnCl_2$ and dissolved in 0.2 ml of 0.05 M potassium phosphate (pH 7.2) containing 0.06 M $(NH_4)_2SO_4$ and 0.01 M EDTA, was layered onto a 5 to 20% sucrose density gradient containing the same buffer as described by Martin and Ames (13). After centrifugation for 8 hours at 141,000 × *g*, the gradients were fractionated as described by Martin and Ames (13) but with approximately 0.2-ml fractions. Analysis for protein, radioactivity, and CO_2 fixation was as described for Fig. 1.

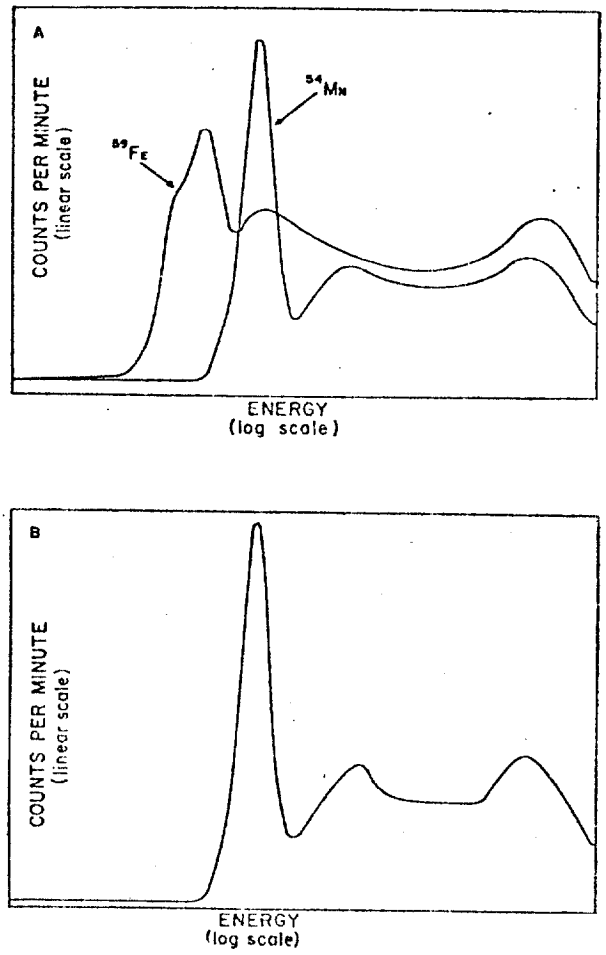

FIG. 3. The γ emission spectra of radioactive pyruvate carboxylase, $^{54}MnCl_2$, and $^{59}FeCl_3$. The spectra were obtained with a single channel pulse height analyzer (Nuclear Instruments). *A*, spectra of authentic $^{54}MnCl_2$ and $^{59}FeCl_3$ standards; *B*, spectrum of radioactive pyruvate carboxylase purified from chickens treated by injection with $^{54}MnCl_2$.

TABLE IV

Correlation of manganese content and enzymic activity during purification of pyruvate carboxylase

The purification procedure was that described by Scrutton and Utter (7). Manganese content was estimated by atomic absorption spectral analysis as described under "Experimental Procedure." The fractions subsequent to the first $(NH_4)_2SO_4$ precipitate were treated with 0.01 M EDTA.

Fraction	Enzymic activity		Manganese	Ratio
	total units	*units/mg*	*mμ-moles/mg*	*Mn/unit*
Crude extract	2100	0.63	0.70	1.1
25-33% $(NH_4)_2SO_4$ precipitate	3450	7.9	1.78	0.22
$Ca_3(PO_4)_2$ gel supernatant fraction	2630	8.6	1.90	0.22
$(NH_4)_2SO_4$ extracts				
22% (I)	1330	27.7	4.11	0.15
22% (II)	386	29.6	3.93	0.135
70% $(NH_4)_2SO_4$ precipitate	1570	29.6	4.56	0.155

obtained for the manganese content by a number of different analytical techniques are shown in Table VI, with the values ranging from 2.5 to 4.3 moles of manganese per mole of pyruvate carboxylase. The results, which involve five different analytical techniques as applied to a number of different preparations of the enzyme, suggest that the stoichiometry may be 4 moles of manganese per mole of enzyme, as appears to be the case with biotin (7). It should be mentioned that neutron activation and atomic absorption analysis of one sample which had undergone slow inactivation for a period of several weeks gave values of approximately 1.7 to 1.8 moles of manganese per mole. This result suggests that under some circumstances manganese can be lost from the enzyme by slow aging and inactivation.

For the formaldoxime analysis, two samples of the enzyme were ashed in HNO_3-H_2SO_4 and the manganese content was determined in quintuplicate on aliquots from the digest. Iron may interfere with this method but may be distinguished from manganese by the fact that the iron formaldoxime complex is largely destroyed on standing at 65° for 2 hours, while the manganese formaldoxime complex is little affected by these conditions. The decrease in absorbance at 450 mμ after 2 hours at 65° (15%) was not significantly greater in the samples obtained by ashing pyruvate carboxylase than the decrease found for the manganese standard.

The values for PRR analysis are based on denaturation of samples from the two different enzyme preparations with each denaturing agent. The manganese content was calculated by comparison of the value for the PRR obtained after denaturation with a standard curve relating the PRR to the concentration of $MnCl_2$ in the same concentration of denaturing agent. The experiments with ^{54}Mn-pyruvate carboxylase described above established that the bound manganese was totally released under the three conditions of denaturation used (Table V).

TABLE V

Effect of denaturing and chelating agents, cold, and thiol inhibitors on enzyme-bound radioactivity and enhancement of bound manganese

The relative catalytic activities (native enzyme = 100) are not necessarily derived from the same experiment as the radioactivity measurements, but are given on the basis of the known effects of these conditions on the enzymic activity from other studies.

Treatment	Relative catalytic activity	ϵ^{*a}	^{54}Mn[b]
			cpm/mg protein
None	100	4.18	246
2% trichloracetic acid	0	0.83	19
7% perchloric acid	0	0.79	
1% sodium dodecyl sulfate	0	1.23	3.3
5.7×10^{-2} M EDTA	100	4.20	234
5.4×10^{-2} M EGTA	100	4.10	241
2° (10 hours)	<5	4.36	222
1.1×10^{-3} M mercuribenzoate	0	1.67	38
1.9×10^{-4} M 5,5′-dithiobis(2-nitrobenzoic acid)	0	4.16	225

[a] The observed enhancement values (ϵ^*) are calculated as described under "Experimental Procedure."

[b] For the measurement of radioactivity, the protein was separated from the supernatant fraction either by centrifugation, *e.g.* for trichloracetic acid, or by gel filtration on a Sephadex G-50 column (25 × 1 cm). ^{54}Mn and protein were estimated as described under "Experimental Procedure."

TABLE VI

Manganese content of pyruvate carboxylase

In all cases, the pyruvate carboxylase was pretreated with 10^{-2} M EDTA and passed through a Sephadex G-50 column to remove extraneous metals.

Method	Manganese		No. of preparations examined
	mμmoles/mg	*moles/mole enzyme*	
Emission spectrum	5.45	3.6	1
Atomic absorption spectrometry	4.73 ± 0.19	3.1 ± 0.2	3
Neutron activation	3.76	2.5 ± 0.1	1
Formaldoxime	6.54	4.3	1
PRR after denaturation with			
Trichloracetic acid	4.38	2.9	1
Perchloric acid	5.77 ± 0.69	3.8 ± 0.4	2
Dodecyl sulfate	5.99	3.9	1
Weighted average[a]		3.4 ± 0.3	

[a] The weighting factor was the number of preparations examined as given in the last column.

DISCUSSION

To provide unequivocal proof that a given metal is a constitutive component of a protein has generally proved to be a task of considerable difficulty (17). However, several diverse lines of evidence indicate that manganese has such a relationship with pyruvate carboxylase purified from chicken liver. First, many different analytical techniques have shown similar amounts of manganese in the highly purified enzyme with the amounts of manganese related to the enzymic activity; second, the characteristics of the PRR effects of pyruvate carboxylase are in good agreement with those predicted for a protein containing bound manganese; and finally, incorporation *in vivo* of ^{54}Mn into pyruvate carboxylase has been shown. The sum of these observations strongly supports the thesis that pyruvate carboxylase is a manganese-metalloenzyme in which manganese is present in a stoichiometry similar to that of biotin, *i.e.* 4 moles per mole of enzyme. The data showing the absence of other paramagnetic metals from pyruvate carboxylase are convincing except in the case of iron, a significant amount of which (0.8 to 0.9 mole per mole of enzyme) has been detected by two analytical techniques. However, the effect of pyruvate carboxylase on the PRR of water is not consistent with that predicted for protein-bound iron (12), and ^{59}Fe is not incorporated into pyruvate carboxylase *in vivo*.

Proteins have been reported to contain a wide variety of nondissociable metals such as zinc, iron, calcium, molybdenum, and copper, but, as far as we are aware, this is the first case in which a highly purified enzyme has been shown to contain nondissociable manganese (*cf.* Footnote 5). There are many examples of enzymes which require the addition of a divalent metal ion such as Mn(II) for activity, *e.g.* kinases, enolase, and some dehydrogenases, but in these cases the manganese is readily dissociable. Pyruvate carboxylase also belongs to this class of enzymes since the addition of a divalent metal ion is required for enzymic activity. Therefore, this enzyme appears both to require an

added dissociable divalent metal ion and to contain a bound metal ion. As shown in the following article, the functional roles of these two types of metal are different.

Although the enhancement of the bound manganese ($\epsilon_b = 4.2$) was calculated with reference to Mn(II), none of the experiments reported here provide definitive evidence on the valence state of manganese when it is bound to pyruvate carboxylase. If the manganese were trivalent in this complex, the enhancement value of 4.2 would be too small by a factor of 1.5. The enhanced effect on the PRR of water and the failure to observe an EPR spectrum with native pyruvate carboxylase in Tris-Cl + KCl at either 77° or 300° K are compatible with the known properties of divalent manganese when bound to a protein, but too little information is available on the properties of the higher valence states of manganese, *e.g.* Mn(III) and Mn(IV), to permit their exclusion. On the basis of chemical evidence, Cotzias and Bertinchamps (20) have suggested that manganese is trivalent in a complex with β-globulin. However, the finding that manganese can replace magnesium in the pyruvate kinase complex (10) with retention of catalytic activity suggests that the metal ion remains divalent when bound to this enzyme. Similar findings have been made with other kinases.

While pyruvate carboxylase appears to be the first manganese metalloenzyme to be described,[5] the results summarized in Table III suggest that other proteins become radioactive when ^{54}Mn is administered to chickens. If the radioactivity remaining in the protein fraction after gel filtration on Sephadex G-50 equilibrated with 10^{-2} M EDTA represents protein-bound ^{54}Mn, 28% of the protein-bound radioactivity of the Tris-acetate extract is accounted for as pyruvate carboxylase. The latter appears to be the only radioactive component in the 0 to 25% and 25 to 33% $(NH_4)_2SO_4$ precipitates. Considerable radioactivity is present in the 33% $(NH_4)_2SO_4$ supernatant fraction, which contains very little pyruvate carboxylase (1). Other manganese proteins may, therefore, be present in this fraction, although no systematic examination has been undertaken to attempt their identification. However, purification of phosphoenolpyruvate carboxykinase from the 33% $(NH_4)_2SO_4$ supernatant fraction showed that this enzyme did not become radioactive under conditions when pyruvate carboxylase was labeled.[6] Table II shows also that only 38% of the radioactivity in the Tris-acetate extract of the mitochondria is protein-bound as determined by the loss of ^{54}Mn after gel filtration on Sephadex G-50. A value of 20% is given for the nondialyzable manganese of liver homogenates by Fore and Morton (21).

The apparently low incorporation of ^{54}Mn *in vivo* appeared disturbing in relation to the significance of the presence of manganese in pyruvate carboxylase. However, only about 10 to 20% of the total pyruvate carboxylase content of intact liver is recovered as the purified enzyme, mainly because of losses in the preparation of the mitochondria and the extraction of the mitochondrial powder (1, 7). The true incorporation is therefore 0.005 to 0.01% of the added dose, *i.e.* a dilution factor of approximately 10^4. Since 300 μC of $^{54}MnCl_2$ contain approximately 6×10^{-3} μmole of manganese, the corrected dilution factor would be obtained with a body pool of 60 μmoles of manganese. Calculation of the approximate size of the body manganese pool in chickens based on published tissue analyses (22) gives a value of 10 to 20 μmoles. The apparent dilution would be larger than expected if pyruvate carboxylase underwent incomplete turnover during exposure to ^{54}Mn or if radioactivity was lost through rapid equilibration of the manganese pools of the serum and bile (23).

No systematic investigation of the rate of turnover of the nondialyzable manganese fraction appears to have been made, and all figures given are for total manganese turnover, which has been found to be rapid with the use of tracer techniques (24). The finding of bound manganese in pyruvate carboxylase should permit investigation of the rate of turnover both of this enzyme and of nondialyzable manganese in liver. Preliminary results suggest that the incorporation of ^{54}Mn into pyruvate carboxylase is maximal after approximately 5 days and decreases very slowly thereafter, indicating that the turnover rate of protein-bound manganese may be much slower than that of the total manganese pool. More extensive studies are required to clarify and confirm these points. Such investigations should lead to a clearer understanding of the pathways of manganese metabolism *in vivo*, which are largely obscure (24). Labeling with ^{54}Mn may also prove useful in examination for changes in the level of pyruvate carboxylase under various physiological conditions, and manganese deficiency states may be used to provide an independent test of the metabolic role of pyruvate carboxylase.

Acknowledgments—This work would not have been possible without the advice and encouragement of Dr. M. Cohn. We are also very grateful to Mr. H. Carter of the Standard Oil Company of Ohio for the emission spectral analyses; to Mr. T. Houser for the γ emission spectrum; to Dr. E. J. Harris and Dr. B. C. Pressman for their advice in the use of the atomic absorption spectrometer; to Dr. W. F. Bethard and Dr. R. Schmitt of General Atomic for the neutron activation analyses; and to Dr. R. Sands and Dr. G. Palmer for the low temperature electron paramagnetic resonance study.

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Studies on the Phosphorylating Action of the Intestinal Mucosa.

III. On the Influence of Several Chemicals on the Dephosphorylating (Hydrolysing) and Phosphorylating Actions of the Intestinal Mucosa of the Rabbit.

By

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In the previous paper,[1] the author has reported that the mucosa enzyme which gave rise to hydrolysis of glycerophosphate and that which catalysed phosphorylation, might be both identical, since the both activities behaved similarly towards different inactivating factors, namely pH, heat and dialysis. From these comparative points of view, further investigations were carried out, and this report concerns with the influence of several chemicals which are known as activators or inhibitors of phosphatase on the both actions of the mucosa enzyme.

Experimentals.

The methods and materials used in these experiments were similar to those in the previous experiments.[1] The glycerol extract of acetone powder prepared from the mucosa of the small intestine of the rabbit, was also here utilized. The ratio of g weight of the acetone powder to ml volume of glycerol was 1:20 or 1:30, as was shown in each table.

The reaction mixtures were adjusted to pH 9.0 and incubated under toluene at 37°.

In the hydrolysis test, 2.0 ml of each reaction mixture was added to 1.0 ml of 10 per cent trichloroacetic acid at a certain interval of incubation and filtered after standing more than one hour. The amount of inorganic phosphorus in 1.0 ml of this deprotenized filtrate was estimated by the method of ALLEN,[2] using a photoelectric colorimeter.

In the case of synthesis test, 0.5 ml of each reaction mixture was added to 2.0 ml of 10 per cent trichloroacetic acid; inorganic phosphorus in 0.5 ml of the filtrate was estimated.

The control test without substrate was conducted under the same conditions as the main tests. These values were reduced from those of the main tests.

The dephosphorylating and phosphorylating activities are indicated as the increase and decrease of these values, calculated for 1.0 ml of digest.

I) Influences of $MgSO_4$, $CoCl_2$ and $MnCl_2$ on the dephosphorylating (hydrolysing) and phosphorylating activities of the intestinal mucosa of the rabbit.

Hydrolysis test: 1.0 ml enzyme extract + 5.0 ml 0.32 g/dl Ca α-glycerophosphate solution + 2.0 ml 0.1 M glycine-NaOH buffer or 0.1 M veronal Na-HCl buffer solution (pH 9.3) + 2.0 ml $MgSO_4$ solution of various M-concentrations or water alone as control.

Synthesis test: 3.0 ml enzyme extract + 1.0 ml 0.35 M Na_2HPO_4 solution in 0.1 M glycine-NaOH buffer or 0.1 M veronal Na-HCl buffer solution (pH 9.3) + 1.0 ml $MgSO_4$ solution of various M concentrations or water alone as control.

Table I Influence of $MgSO_4$ on the dephosphorylating (hydrolysing) and phosphorylating actions of the intestinal mucosa (glycerol extract of acetone powder (1:20))

Buffer	Glycine-NaOH buffer				Veronal Na-HCl buffer			
Final M conc. of $MgSO_4$ / Hours	0 (control)	M/200	M/100	M/50	0 (control)	M/200	M/100	M/50
Hydrolysis: Increase of inorganic P. (mg in 1.0 ml digest)								
2	0.035	0.035	0.035	0.035	0.044	**0.047**	**0.047**	**0.047**
5	0.080	0.081	**0.086**	**0.086**	0.093	**0.107**	**0.108**	**0.105**
24	0.185	0.200	**0.200**	**0.200**	0.200	**0.212**	**0.212**	**0.212**
Synthesis: Decrease of inorganic P. (mg in 1.0 ml digest)								
24	0.11	0.10	0.10	0.11	0.10	0.11	0.11	0.07
72	0.22	0.23	0.23	0.21	**0.25**	0.21	0.24	0.12
120	**0.37**	0.35	0.34	0.30	**0.37**	0.29	0.30	0.27

Hydrolysis test: 1.0 ml Enzyme extract + 5.0 ml 0.32 g/dl Ca α-glycerophosphate solution + 2.0 ml 0.1 M glycine-NaOH buffer soultion (pH 9.3) + 2.0 ml $CoCl_2$ solution of different concentrations of water

alone as control.

Synthesis test: 2.0 ml Enzyme extract + 1.0 ml 0.35 M Na_2HPO_4 solution in 0.1 M glycine-NaOH buffer solution (pH 9.3) + 1.0 ml $CoCl_2$.

Table II Influence of $CoCl_2$ on the dephosphorylating (hydrolysing) and phosphorylating actions of the intestinal mucosa (glycerol extract (1:30))

Hours \ Final M conc. of $CoCl_2$	0 (control)	M/200	M/100	M/50
Hydrolysis: Increase of inorganic P. (mg in 1.0 ml digest)				
2	0.003	0.017	0.024	0.029
5	0.020	0.057	0.055	0.068
24	0.072	0.102	0.155	0.134
Synthesis: Decrease of inorganic P. (mg in 1.0 ml digest)				
24	0.08	0.07	0.07	0.02
72	0.11	0.10	0.09	0.07
120	0.20	0.18	0.16	0.15

Hydrolysis test: 1.0 ml Enzyme extract + 5.0 ml 0.32 g/dl Ca α-glycerophosphate solution + 2.0 ml 0.1 M veronal Na-HCl buffer solution (pH 9.3) + 2.0 ml $MnCl_2$ solution of different concentrations or water alone as control.

Synthesis test: 3.0 ml Enzyme extract + 1.0 ml 0.35 M Na_2HPO_4 solution in 0.1 M veronal Na-HCl buffer solution (pH 9.3) + 1.0 ml $MnCl_2$.

Table III Influence of $MnCl_2$ on the dephosphorylating and phosphorylating actions of the intestinal mucosa (glycerol extract (1:30))

Hours \ Final M conc. of $MnCl_2$	0 (control)	M/200	M/100	M/50
Hydrolysis: Increase of inorganic P. (mg in 1.0 ml digest)				
2	0.027	0.021	0.012	0.012
5	0.053	0.039	0.029	0.023
24	0.117	0.090	0.062	0.051
Synthesis: Decrease of inorganic P. (mg in 1.0 ml digest)				
24	0.09	0.07	0.06	0
72	0.20	0.20	0.21	0.11
120	0.29	0.25	0.33	0.13

III. Influence of NaF, KCN, monoiodoacetic acid, cysteine, and glycine on the dephosphorylating and phosphorylating actions of the intestinal mucosa (glycerol extract).

Hydrolysis test: 1.0 ml Enzyme extract + 5.0 ml 0.32 g/dl Ca α-glycerophosphate solution + 2.0 ml 0.1 M glycine-NaOH buffer solution (pH 9.3) + 2.0 ml of additive substance of different concentrations or water as control.

Synthesis test: 2.0 ml Enzyme extract + 1.0 ml glycerol + 1.0 ml 0.35 M Na_2HPO_4 solution in 0.1 M glycine-NaOH buffer solution (pH 9.3) + 1.0 ml of additive substance of various concentrations or water as control.

Table IV Influence of NaF on the dephosphorylating and phosphorylating actions of the intestinal mucosa

Hours \ Final M conc. of NaF	0 (control)	M/200	M/100	M/50
Hydrolysis: Increase of inorganic P. (mg in 1.0 ml digest)				
2	0.017	0.015	0.018	0.018
5	0.032	0.030	0.035	0.033
24	0.089	0.087	0.085	0.089
Synthesis: Decrease of inorganic P. (mg in 1.0 ml digest)				
24	0.04	0.05	0.05	0.04
72	0.09	0.07	0.08	0.07
120	0.12	0.09	0.12	0.11

Table V Influence of KCN on the dephosphorylating and phosphorylating actions of the intestinal mucosa

Hours \ Final M conc. of KCN	0 (control)	M/200	M/100	M/50
Hydrolysis: Increase of inorganic P. (mg in 1.0 ml digest)				
2	0.033	0.011	0.005	0.003
5	0.070	0.023	0.014	0.009
24	0.147	0.069	0.054	0.038
Synthesis: Decrease of inorganic P. (mg in 1.0 ml digest)				
24	0.11	0.09	0.12	0.09
72	0.26	0.18	0.14	0.10
120	0.33	0.25	0.20	0.12

Table VI. Influence of monoiodoacetic acid on the dephosphorylating and phosphorylating actions of the intestinal mucosa

Final M conc. of MIA / Hours	0 (control)	M 200	M 100	M 50
Hydrolysis: Increase of inorganic P. (mg in 1.0 ml digest)				
2	0.033	0.034	0.021	0.021
5	0.063	0.062	0.059	0.051
24	0.165	0.152	0.160	0.160
Synthesis: Decrease of inorganic P. (mg in 1.0 ml digest)				
24	0.04	0.05	0.04	0.03
72	0.07	0.08	0.07	0.05
12	0.12	0.11	0.10	0.07

Table VII. Influence of cysteine on the dephosphorylating and phosphorylating actions of the intestinal mucosa

Final M Conc. of cysteine / Hours	0 (control)	M 200	M 100	M 50
Hydrolysis: Increase of inorganic P. (mg in 1.0 ml digest)				
2	0.039	0.014	0.011	0.011
5	0.060	0.032	0.027	0.018
24	0.165	0.142	0.143	0.086
Synthesis: Decrease of inorganic P. (mg in 1.0 ml digest)				
24	0.04	0.02	0.01	0
72	0.07	0.05	0.02	0.01
12	0.12	0.06	0.04	0.01

Hydrolysis test: 1.0 ml Enzyme extract + 5.0 ml 0.32 g/dl Ca α-glycerophosphate solution + 2.0 ml 0.1 M veronal Na-HCl buffer solution (pH 9.3) + 2.0 ml of glycine solution of various concentrations or water only as control.

Synthesis test: 3.0 ml Enzyme extract + 1.0 ml 0.35 M Na_2HPO_4 solution in 0.1 M veronal Na-HCl buffer solution (pH 9.3) + 1.0 ml glycine solution.

Table VIII. Influence of glycine on the dephosphorylating and phosphorylating actions of the intestinal mucosa (glycerol extract (1:300)

Final M conc. of glycine / Hours	[illegible]	M 200	M 100	M 50
Hydrolysis: Increase of inorganic P. (mg in 1.0 ml digest)				
[illegible]	0.037	[illegible]	[illegible]	0.031

5	0.053	[illegible]	0.039	0.041
24	0.117	0.102	0.098	0.098
Synthesis: Decrease of inorganic P. (mg in 1.0 ml digest)				
24	0.09	0.07	0.08	0.07
72	0.17	0.16	0.20	0.18
12	0.25	[illegible]	0.30	0.28

Results and Discussions.

It is well known that what an enzyme changes is not the state of equilibrium of the reaction, but the velocity. Therefore, in an enzymatic reversible reaction, if the reaction of one direction is activated or inhibited by the addition of one substance, the reverse reaction must be also influenced in the same manner, when this substance relates itself to the catalytic action of the enzyme. If one substance would activate one direction of a reaction and inhibit on the opposite, it must be taken into consideration that this substance does not concern with the catalytic action of the enzyme itself, but affects the reaction system in some other way.

Regarding the effect of magnesium on the phosphorylating activity, Table I denotes that $MgSO_4$ stimulated the hydrolysing phosphatase activity of the intestinal mucosa of the rabbit but inhibited the phosphorylating activity in its 0.02 M concentration. Therefore, it follows that $MgSO_4$ is considered not to participate in the catalytic action itself of the phosphatase.

As to the mechanism of activation of phosphatase by $MgSO_4$, ERDTMAN[1] described that the enzyme will be rather strongly inhibited by phosphate than the enzyme-magnesium complex, that is, the affinity of magnesium to phosphatase is greater than to phosphate, and the affinity of phosphate to phosphatase-magnesium complex is little or zero. Magnesium would act as a kind of buffer to phosphate. And then, he pointed out that it is a worthy basis, if we can show that magnesium inhibits or retards the enzymatic synthesis which is performed in high concentrations of phosphate and glycerol. But he could not succeed in enzymatic synthesis.

BOLLE and TIROAT[2] also reported that magnesium salts are antagonistic to the inhibitory effect of phosphate, and that this action may be due to the formation of a phosphate of Mg or an indirect influence of Mg^{++} ions on active groups in the enzyme to which phosphate is fixed.

These experiments proved that the supposition of ERDTMAN is just

right; and they also would supply another basis to the evidence that magnesium is an antagonist to the inhibitory effect of phosphate and not a true activator for the catalytic action.

Table II shows that $CoCl_2$ acted on phosphatase of the intestinal mucosa of the rabbit in the same manner as $MgSO_4$, accelerating the hydrolysis and inhibiting the synthesis of glycerophosphate.

As for the influence of Mn, CLOETTA[5] reported that the phosphatase activity of the intestine was activated by $MnSO_4$ in a concentration of 10^{-3}M concentration but inhibited in 10^{-2}M concentration. It is obvious from Table III that $MnCl_2$ inhibited the dephosphorylating action of the intestinal mucosa in concentrations of M/50 to M/200, and also inhibited the phosphorylating action in its M/50 concentration.

Regarding the influence of NaF on the dephosphorylating and phosphorylating activities of the intestinal mucosa, ROCHE and associates[6] reported that sodium fluoride did not affect the phosphomonoesterase of the intestine. Table IV shows that the phosphatase activity of the intestinal mucosa is not affected by NaF. As to the synthetic activity, KAY[7] demonstrated that the phosphorylating actions of duodenum and kidney were inhibited by NaF in concentration of 0.08 to 0.16 M. From the results of Table IV, the retardation effect of NaF in its 0.02 M concentration on the phosphatase activity could not be ascertained.

The inhibition of purified alkaline intestinal phosphatase by cyanide was reported by SCHMIDT and THANHAUSER.[8] It is demostrated by Table V that KCN strongly inhibited the dephosphorylating (hydrolysing) and phosphorylating action of the intestinal mucosa.

As for the influence of monoiodoacetic acid, SCHÄFFNER and BAUER[9] observed that yeast phosphatase was slightly inhibited by monoiodoacetic acid but kidney phosphatase was not affected. From Table VI it seems that monoiodoacetic acid might have only a slight inhibitory influence on the dephosphorylating and phosphorylating activities of the mucosa in its high concentration.

Regarding the influence of cysteine, WALDSCHMIDT-LEITZ and SCHÄFFNER[10] demonstrated that the activity of alkaline kidney phosphatase was inhibited by cysteine and also by H_2S, but phosphorylating action remained unaffected. From these observations they came to the conclusion that whether the hydrolysis or synthesis of phosphoric ester overcomes the other, was depend upon the relation of quantity of SH and SS-compounds which must exist in the unpurified enzyme. The inhibitory effect of cystein on phosphatase had been questioned by LEHMAN[11] and KÖSTER and BERSIN.[12]

Now it is the present view that cysteine inhibits the alkaline phosphatase.[13]

It is easily seen from Table VII that the phosphorylating activity of the intestinal mucosa of the rabbit was also remarkably inactivated by cysteine.

According to BODANSKY,[14] α-amino acids accelerated the activity of the intestinal phosphatase in a very low concentration, and retarded it in a high concentration. And in direct extracts of tissue without dialysis, the concentration of α-amino acids, because of the high autolytic capacity of intestine, are greater than optimal and retarding in effect. From Table VIII it follows that glycine had an inhibitory effect on the phosphatase activity of the intestinal mucosa in its high concentration of 0.02 M. However, it has scarcely affected the phosphorylating activity (Table VIII). These facts are also obvious from the results of Table I, in which glycine-NaOH buffer and veronal Na-HCl buffer were used.

Summary.

The influence of some substances on the dephosphorylating (hydrolysing) and phosphorylating activities of the intestinal mucosa was studied at pH 9.0, using glycerol extract of the acetone powder of the rabbit mucosa as an enzyme source.

1) $MgSO_4$ slightly activated hydrolytic activity of the enzyme, while inhibited the phosphorylating activity in high concentrations. From these observations, mechanism of activation of phosphatase by magnesium was discussed.

2) $CoCl_2$ stimulated hydrolytic action, while it seemed to preclude synthetic action in high concentrations similarly to the case of $MgSO_4$.

3) The dephosphorylating as well as phosphorylating action was inhibited by $MnCl_2$ in its high concentrations.

4) NaF had no effect on the both actions.

5) Monoiodoacetic acid seemed to depress slightly the both actions of the intestinal mucosa only in its high concentrations.

6) Cysteine and KCN remarkably injured the both actions.

7) The hydrolysing action of the mucosa phosphatase was slightly depressed by glycine in its high concentrations, while it remained without effect on the phosphorylating action of the mucosa.

In closing, the author wishes to express his deepest gratitude to Prof. Dr. *S. Utzino* for his incessant guidance and helpful suggestions during the course of this research. This work was carried out by the grants from the Ministry of Education for the Scientific Research, for which the author intends here to express his appreciation.

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Vrach. Delo. 7: 121-123, 1965

USE OF MANGANESE WITH VITAMINS C AND B_1 IN THE COMPREHENSIVE TREATMENT OF INFECTIOUS HEPATITIS

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We used a 0.1 to 0.05% manganous chloride solution to treat infectious hepatitis: 1 teaspoonful twice a day at the rate of 10 to 20 mg daily for 2 weeks. The dose of vitamin C for adults was 0.2 g 3 times daily. Vitamin B_1 was administered in the form of an 0.02% thiamine bromide solution 3 times daily. The doses for children were proportionately smaller.

In addition to manganese and vitamins C and B_1, we prescribed dietotherapy (Pevzner's No. 5 diet), 2% magnesium sulfate solution (200 to 300 mg daily), subcutaneous drop injection of 40% and 5% glucose (500 to 1000 ml every other day), pancreatin (0.5 to 1 mg [?] 3 times a day), duodenal and cecal sounding.

We divided the 94 patients under observation into 2 groups. The first, 49 persons, were treated with manganese and vitamins C and B_1. The second, 45 persons (control) were treated only with the vitamins. The 2 groups were approximately the same in age composition and severity of the disease. The patients of the first group were distributed by age as follows: 33 from 6 to 15, 9 from 16 to 30, and 7 from 31 to 60. The second (control): 30 from 6 to 15, 6 from 16 to 30, and 8 from 31 to 60. Thus, more than half the patients were children whose manganese requirement, according to A. O. Voynar (1960), is 3 times that of adults.

The patients were divided into 2 groups according to the severity of the disease, mild and moderate. In the first group, the course was mild in 24 patients and moderately severe in 21. We followed A. F. Bilibin's instructions for determining the severity of the disease. In analyzing the result we used, in addition to the physical findings, the following indices: general improvement, number of bed-days, time for blood bilirubin and cholesterol to become normal

pyruvic acid in urine, and change in daily diuresis.

The first group of patients with a moderately severe course were in the hospital 3 days less than the second group. Among those with a mild course, there was no appreciable difference between the groups with respect to the number of bed-days.

The bilirubin level was determined by Bokal'chuk's method. It ranged in the first group of patients with a moderately severe course from 78 to 409 (average 212 mg%) and returned to normal after 32.3 days. But in the second group of patients with a moderately severe course in whom the bilirubin level ranged from 25 to 409 (average 152.7 mg%), it did not return to normal until after 37.5 days, i.e., 5.2 days later than in the first group. There was no appreciable difference in this respect between the patients with a mild course.

Blood cholesterol was determined by Bloor's test. The normal serum cholesterol level, according to O. V. Travina, ranges from 140 to 200 mg%. It varied considerably in our patients, from 104 to 500 mg%. Hypocholesterolemia was noted in the first group of patients with a moderately severe course, whereas the cholesterol level was normal in the second group. The cholesterol level return to normal in the first group after treatment. The first group of patients with a mild course suffered from hypercholesterolemia, but the cholesterol level returned to normal after treatment even though we did not give these individuals any lipotropic agents. The cholesterol level was somewhat high in the second group after treatment. In the first group of patients with a mild course, the liver shrank 3.8 days sooner than in the second group. There was no appreciable difference between the patients with a moderately severe course.

We paid a great deal of attention during treatment to the pyruvic acid level in the urine and to the disturbance of carbohydrate metabolism. Bisulfute-binding substances (chiefly pyruvic acid) were determined in the urine by the method of Banarji and Harris. The daily normal pyruvic acid level (according to Gulevich) was taken as 100 to 200 mg. We found it to be high in most patients at the onset of the disease, peaking at the height and dropping at the time of recovery; it returned to normal in only a few cases. It will be noted that the level in the first group was high in 21 patients with a moderately severe course and in 15 patients of the second group.

Among those suffering from a moderately severe course, the pyruvic acid level was normal in only 4 patients (2 in each group). Among those with a mild course, it was high in 8 patients of the first group and in 5 patients of the second.

Among those with a mild course (first group), the pyruvic acid level was high in 17 patients and in 13 of the second group. When the patients were discharged, the level was normal in 8 patients of the first group with a moderately severe course and in 6 of the second group. It averaged 260.8 mg in the first group and 359 mg in the second, i.e., it was 100 mg less in the first group of patients than in the second group.

The pyruvic acid level dropped in 16 persons of the first group with a moderately severe course and in 5 of the second.

The pyruvic acid level dropped in 9 patients of the first group with a mild course and in only 2 of the second group. It averaged 196.5 mg in the first group, i.e., it became completely normal, and 256 mg in the second group. It is interesting to note that it amounted to 220.3 mg in the first group of patients at the height of the disease but 359 mg a day in the second group.

It is reasonable to conclude, therefore, that the use of manganese in the treatment of infectious hepatitis results in a more rapid normalization or lowering of the pyruvic acid level in the urine and, consequently, in a more rapid normalization of carbohydrate metabolism.

Daily diuresis decreases in infectious hepatitis patients at the height of the disease but increases as recovery proceeds. For example, it amounted to 1269 ml at the height of the disease in the first group of patients with a moderately severe course, i.e., it increased by 317 ml, but in the second group it increased by only 80 ml. Among those with a mild course, it increased by 413 ml and in the second group by only 263 ml. It is evident from the foregoing data that daily diuresis increased more rapidly in the first group of patients. Despite the marked increase in daily diuresis of these patients, the drop in pyruvic acid level at the time of discharge was quite substantial in a rather large number of patients, an indication of fairly rapid normalization of carbohydrate metabolism.

Thus, although the levels of blood bilirubin, and pyruvic acid in urine were quite high in the first group of patients at the start of treatment and they suffered from hypocholesterolemia (evidence of marked disturbances of pigment, carbohydrate, and lipid metabolisms), normalization of these indices set in more quickly and was more complete than in the second group. Moreover, the general condition of the first group improved sooner. Their appetite returned and pain subsided more quickly.

Our findings show that the combined use of manganese with vitamins C and B_1 is beneficial in the comprehensive treatment of infectious hepatitis.

УДК 616.36-002.14-085+615.857.06+615.739.14

ПРИМЕНЕНИЕ МАРГАНЦА ВМЕСТЕ С ВИТАМИНАМИ С И B_1 В КОМПЛЕКСНОМ ЛЕЧЕНИИ БОЛЕЗНИ БОТКИНА

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Для лечения больных болезнью Боткина мы применяли 0,1—0,05% раствор хлористого марганца, который давали по одной десертной ложке два раза в день из расчета 10—20 мг в сутки на протяжении двух недель.

Доза витамина С при лечении взрослых составляла 0,2 г три раза в день. Витамин B_1 применялся в виде раствора 0,02% бромистого тиамина три раза в день. Дозы для детей соответственно уменьшались.

Кроме марганца и витаминов С и B_1, для лечения применяли: диетотерапия (стол № 5 по Певзнеру), 2% раствор сернокислой магнезии по 200—300 мг в сутки, внутривенные введения 40% и 5% растворов глюкозы для подкожного капельного введения от 500 до 1000 мл в сутки через день, панкреатин по 0,5—1 три раза в день, дуоденальное и слепое зондирование.

Под нашим наблюдением находилось 94 больных, которых мы разделили на две группы. Первая группа состояла из 49 человек, которых лечили марганцем с витаминами С и B_1, вторая группа (контрольная) из 45 человек, которых лечили только витаминами С и B_1. По возрастным показателям и тяжести заболевания обе группы примерно одинаковы. По возрастному составу больные первой группы распределялись следующим образом: в возрасте от 6 до 15 лет — 33 человека, от 16 до 30 лет — девять, от 31 до 60 лет — семь. Вторая группа (контрольная): в возрасте от 6 до 15 лет — 30 больных, от 16 до 30 лет — шесть, от 31 до 60 лет — восемь.

Итак, больше половины больных составляют дети, у которых, как указывает А. О. Войнар (1960), потребность в марганце в три раза выше, чем у взрослых. По тяжести заболевания больные были разделены на две группы: с легким и средней тяжести течением.

Так, в первой группе легкое течение наблюдалось у 24 больных и средней тяжести — у 21. При определении тяжести заболевания мы руководствовались указаниями А. Ф. Билибина. Анализируя результаты, кроме данных клинического обследования, мы использовали следующие показатели: улучшение общего состояния, количество койко-дней, сроки нормализации уровня билирубина и холестерина в крови, пировиноградной кислоты в моче и изменение суточного диуреза.

Больные первой группы со средней тяжестью заболевания находились в стационаре на три дня меньше, чем больные второй группы.

При легком течении болезни заметной разницы по группам в количестве койко-дней не было.

Уровень билирубина сыворотки крови определялся по методу Бокальчука. У больных первой группы при средней тяжести болезни он колебался в пределах от 78 до 409 (в среднем 212 мг%) и нормализация его наступила к 32,3 дня. У больных же второй группы со средней тяжестью болезни, где уровень билирубина колебался в пределах от 25 до 409 (в среднем 152,7 мг%), нормализация его наступила только на 37 с половиной день болезни, то есть на 5,2 дня позже, чем у больных первой группы. У больных с легким течением заметной разницы не было.

Холестерин крови определялся по методу Блюра. Нормальный уровень холестерина сыворотки крови по О. В. Травиной колеблется в пределах от 140—200 мг%. При исследовании он колебался в значительных пределах от 104 до 500 мг%. У больных первой группы со средней тяжестью заболевания наблюдалась гипохолестеринемия, в то время, как у больных второй группы уровень холестерина был нормальным. После лечения у больных первой группы уровень холестерина нормализовался. При легком течении в первой группе наблюдалась гиперхолестеринемия, а после лечения наступила нормализация уровня холестерина, несмотря на то, что мы не давали больным липотропных веществ. Во второй группе после лечения уровень холестерина был несколько повышенным. У больных первой группы с легким течением болезни сокращение размеров печени наступило на 3,8 дня быстрее, чем у второй группы, а при средней тяжести — заметной разницы не было.

При лечении значительное внимание уделялось определению уровня пировиноградной кислоты в моче — показателю нарушения углеводного обмена. Бисульфитсвязывающие вещества (в основном

пировиноградная кислота) в моче определялись по методу Бенерджи и Гарриса. За суточный нормальный уровень пировиноградной кислоты (по Гулевичу) принимали 100—200 мг. В результате выяснилось, что уровень пировиноградной кислоты у подавляющего большинства больных в начале заболевания повышен, а в разгар его достигает самого высокого уровня и к выздоровлению снижается, приходит в норму только в некоторых случаях. Следует также отметить, что высокий уровень пировиноградной кислоты в первой группе был у 21 больного средней тяжести, в то время как во второй группе — у 15.

При средней тяжести болезни нормальный уровень пировиноградной кислоты был только у четырех больных (по два человека в каждой группе), а при легком течении в первой группе — у восьми, а во второй группе — у пяти человек.

При легком течении болезни (первая группа) уровень пировиноградной кислоты был повышенным у 17 человек, а во второй группе — у 13. К моменту выписки нормализация ее уровня в первой группе со средней тяжестью заболевания наступила у восьми человек, во второй группе — у шести. В средних показателях ее уровень у больных первой группы был 260,8, а во второй группе — 359 мг, то есть уровень пировиноградной кислоты у больных первой группы был на 100 мг меньше, чем у больных второй группы.

Уровень пировиноградной кислоты в первой группе при средней тяжести заболевания снизился у 16 человек, во второй группе — у 5.

При легком течении в первой группе уровень пировиноградной кислоты снизился у десяти человек, а во второй группе — только у двух. В средних показателях ее уровень был в первой группе — 196,5 мг, то есть полностью нормализовался, у больных второй группы равнялся 256 мг. Интересно, что у больных первой группы в разгар заболевания показатель пировиноградной кислоты составляет 220,3 мг, в то время, как у больных второй группы — 359 мг в сутки.

Таким образом, можно сделать вывод, что применение марганца при лечении болезни Боткина ведет к более быстрой нормализации или снижению уровня пировиноградной кислоты в моче, а следовательно, и к более быстрой нормализации углеводного обмена.

Суточный диурез у больных болезнью Боткина уменьшается в разгар заболевания и увеличивается по мере выздоровления. Так, у больных первой группы со средней тяжестью суточный диурез в разгар заболевания составлял 1269 мл, то есть увеличился на 317 мл, а у больных второй группы — только на 80 мл. При легком течении он увеличился на 413 мл, а во второй группе — только на 263 мл. Из приведенных данных видно, что у больных первой группы наблюдается более быстрое увеличение суточного диуреза. Несмотря на значительное увеличение суточного диуреза у больных первой группы, снижение уровня пировиноградной кислоты к моменту выписки было более значительным и у большего количества больных, что свидетельствует о более быстрой нормализации углеводного обмена.

Таким образом, хотя в начале лечения у больных первой группы уровень билирубина в крови, пировиноградной кислоты в моче был более высоким и наблюдалась гипохолестеринемия — все это свидетельствует о более значительных нарушениях пигментного, углеводного и липоидного обменов — нормализация их наступала быстрее и была более полной.

Кроме того, у больных первой группы быстрее наступало улучшение общего состояния, появлялся аппетит, проходили боли. Заметной разницы в сроках исчезновения желтухи не было.

Полученные данные свидетельствуют о пользе совместного применения марганца с витаминами С и B_1 в комплексной терапии болезни Боткина.

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 76, 78–86 (1958)

The Mechanism of Chemical Mutagenesis. II. Interactions of Selected Compounds with Manganous Chloride[1]

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Received October 9, 1957

Introduction

Kinetic studies on the action of chemical mutagens on bacteria were reported by Demerec and Hanson (1), and in the first paper of this series (2). A complementary approach is based on analysis of interactions between a chosen mutagen, manganous chloride, and other compounds active in the mutational process. The first phase, which is presented here, is a survey of some selected substances, most of which are known antineoplastic agents. The rationale governing the selection is the earlier observation that many of these agents are endowed with mutagenic properties.

The choice of manganous chloride as the standard mutagen was prompted by availability of methods and experimental results obtained by Demerec and Hanson (1) in their extensive studies with this compound. The lack of pronounced bactericidal activity further recommended $MnCl_2$ as a convenient mutagen.

From the study of Demerec and Hanson (1), it appeared that the mutagenic action of manganous ions might be highly sensitive to extraneous agents; pretreatment of the bacteria with buffers of varying pH or with different salt solutions had a pronounced effect, and the coaction of two mutagens, $MnCl_2$ and ultraviolet light, was less than additive. Other examples of antimutagenic effects in different systems

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include: the photoreactivation phenomenon whereby the lethal and mutagenic activities of ultraviolet light are reversed (3); the conditional reversal of the mutagenic effect of formaldehyde by undefined constituents of yeast fed to *Drosophila* (4); and the suppression of mutation, either spontaneous or theophylline-induced, at the T_5 locus of *Escherichia coli* by guanosine, adenosine, and inosine (5).

Materials and Methods

The mutational system employed in the present studies was the reversal from streptomycin dependence to independence and from cysteine requirement to prototrophy in strain Sd-4-73 of *E. coli*, obtained from Dr. M. Demerec and previously used in the extensive studies of his group (1, 6–8). The method of inducing mutations was essentially similar to that described by Demerec and Hanson (1). Cultures were grown overnight at 35°C. in aerated nutrient broth containing 20 μg. streptomycin/ml. Approximately 10^{10} cells, washed and pretreated with 0.3 *M* NaCl, were subsequently exposed for 2 hr. to 2 ml. of 0.04% manganous chloride alone or in combination with another compound. The mutagen was removed either by centrifugation and washing with 0.5% saline or by dilution below its biologically active concentration. The percentage of survival and number of mutants were assayed for the cultures treated with $MnCl_2$, the test compound, and the combination of the two, as well as for the controls. The assay media were as follows: nutrient agar containing 100 μg. streptomycin/ml. for scoring the total number of colonies; nutrient agar for revertants from streptomycin dependence (*sd-4*) to independence; and minimal agar containing 10 μg. cysteine/ml. for cysteine-independent mutants (*cys-2* reversions).

The compounds tested are listed in Tables I and II. The indicated concentrations were close to the toxicity level, as far as the solubility of the compound allowed. Sterile distilled water was used as the solvent. Acetone was employed for preparation of concentrated stock solutions of compounds poorly soluble in water. Bacterial suspensions were treated at pH 6.5–7.0. Acidification of $MnCl_2$ solution resulted in the loss of mutagenic activity. This observation confirmed earlier results of Bryson *et al.* (9).

Results

The experimental results are summarized in Tables I, II, and III. Considerable variability in the mutagenicity of $MnCl_2$ and other compounds when assayed on different cultures made it imperative to assay the individual and joint action of mutagens on the same culture. Therefore, every horizontal row in the tables represents data referable to a particular bacterial suspension. This variability in mutagenic activity, particularly with $MnCl_2$, is a well-known phenomenon [cf. (1)]. Individual determinations of induced mutagenicity obtained with a particular, freshly prepared bacterial suspension have been shown to be con-

TABLE I

Effect of a Variety of Agents on the Number of Mutants Induced by Manganous Chloride in E. coli *Sd-4-73 at the Streptomycin-Dependence and Cysteine-Requirement Loci*

Test compound					Manganous chloride (0.04%)			Test compound plus 0.04% manganous chloride		
Name	Concn.	Survivors	Number of induced mutants per 10^8 survivors		Survivors	Number of induced mutants per 10^8 survivors		Survivors	Number of induced mutants per 10^8 survivors	
			sd-4	*cys-2*		*sd-4*	*cys-2*		*sd-4*	*cys-2*
	μg./ml.	%			%			%		
Actinomycin					SEE Table II					
Aminopterin	10	100	0	0	87.6	8200	80	93.8	9200	80
Amethopterin	5	100	0	0	41.4	3900	550	50.4	2100	620
8-Azaguanine	100	100	0	0	7.6	2200	180	100	2200	210
Azaserine					SEE Table III					
Benzimidazole	100	15.3	0	0	20.2	7400	290	33.8	2200	110
Carzinophilin	0.05	22.9	45	0	41.4	3900	550	<0.05	—	—
	0.01	47.2	310	0	100	210	31	26.7	2600	0
6-Chloropurine	10	60.2	1.6	0	78.1	800	70	70.1	500	50
Cycloserine	50	—	—	—	100	1100	120	32.3	1000	610
2,6-Diaminopurine	100	63.0	0	0	76.2	2200	80	63.1	1800	110
2,4-Diamino-5-*p*-chlorophenol-6-ethylpyrimidine (Daraprim)	10	92.3	0	0	94.2	1700	200	78.8	2200	260
2,4-Diamino-5-(3′,4′-dichlorophenyl)6-ethylpyrimidine	10	56.1	5	0	78.1	800	80	45.2	1600	150
6-Diazo-5-oxynorleucine (DON)	1000	68.0	0	0	55.2	2200	230	38.3	800	70
Kinetin	5	187.0	0	0	41.4	3900	550	35.1	3000	1200
6-Mercaptopurine	100	47.8	0	0	76.2	2200	80	50.6	5500	450
1,4-Dimethane sulfonoxybutane (Myleran)	10	100	0	0	94.1	1700	200	90.3	1600	170
Nutrient broth (Difco)	800	100	0	0	78.1	4100	250	76.2	2500	190
Puromycin	10	97.1	0	0	60.1	1200	380	42.4	1900	400
Triethylene melamine	5	100	970	2	41.4	3900	550	73.8	1400	190
Urethan	1000	93.8	0	0	87.6	1200	80	69.4	2700	220

TABLE II

Effect of Actinomycin D on the Mutagenic Activity of $MnCl_2$

Expt.	Actinomycin[a]				$MnCl_2$ (400 μg./ml.)			Actinomycin and $MnCl_2$			Per cent decrease in $MnCl_2$ mutagenicity	
	Concn.	Survival	Induced mutants per 10^8 survivors		Survival	Induced mutants per 10^8 survivors		Survival	Induced mutants per 10^8 survivors			
			sd-4	*cys-2*		*sd-4*	*cys-2*		*sd-4*	*cys-2*	*sd-4*	*cys-2*
	μg./ml.	%			%			%				
1	50	39	0	0	70	2400	600	85	12	60	99.5	99
2	1	—	—	—	55	4200	250	50	21	60	99.5	76
3	1	—	—	—	98	3200	410	52	4300	61	[b]	85
	1	—	—	—				27	4700	95	[b]	77
4	1	30	0	0	70	870	120	85	71	1	92	91
	0.1	—	—	—				100	600	7	31	94
5	1	80	0	0	98	1100	120	78	26	0	87	100
	0.05	—	—	—				68	810	100	[b]	[b]
6	1	63.8	0	0				35.4	5000	550	45	[b]
	10	3.1	0	0	22.4	9100	660	0.2	820	780	91	[b]
	100	0.15	0	0				0.07	6700	330	27	50
7	10	100	5	0	78	840	80	2.4	250	150	70	[b]
8	5	51	0	0	60	1200	380	28	2400	670	[b]	[b]
9	2	—	—	—	95.7	2000	50	62.7	880	150	66	[b]
	3	—	—	—				61.7	52	<17	97	>60
10	5	—	—	—	100	1300	190	100	1100	370	[b]	[b]
11	1	—	—	—	40	1200	330	46.5	71	8	94	75
12	1	—	—	—	65	1000	60	97	92	3	91	57

[a] Stock solution of actinomycin D in acetone (10 mg./ml.) was employed throughout these experiments. All subsequent dilutions were made in demineralized water. At the resulting concentrations, acetone was not found to interfere with any of the tests.

[b] Actinomycin had no antimutagenic effect in these experiments.

sistent within limits of ±30% (2). Therefore, a change in the mutagenic action of $MnCl_2$ by greater than a factor of 2 might be considered on the border line of significance.

Measured by this standard, with a few exceptions the compounds tested did not have any pronounced effects on the mutagenic action of $MnCl_2$. The negative data (Table I) illustrate also the relatively high reproducibility of the mutagenic action of $MnCl_2$ when assayed on a single culture in the presence or absence of the inactive supplement.

Antimutagenic activity was exhibited by actinomycin D and possibly by benzimidazole and 6-diazo-5-oxynorleucine (DON). The results obtained with actinomycin are presented in Table II. In many experiments, an actinomycin D concentration as low as 1 μg./ml. almost completely suppressed the mutagenic action of manganous ions. This antimutagenic effect, however, was highly variable, and in some series of experiments actinomycin did not exhibit any such activity, seemingly under the same experimental conditions. Benzimidazole and DON suppressed the mutagenicity of $MnCl_2$ by approximately a factor of 3. Urethan and 6-mercaptopurine, which by themselves did not show any mutagenic activity, slightly enhanced the mutagenic action of manganous ions.

Mutagenicity was exhibited by several substances tested. Of these, azaserine was strongly synergistic with manganous ions both in the mutagenic and bactericidal properties. Table III illustrates this pronounced effect. Actinomycin D, which was introduced in Expt. 4, again seemed to suppress the mutagenicity of $MnCl_2$ without affecting the action of azaserine. In other experiments, it was confirmed that actinomycin had no effect on the mutagenic activity of azaserine or triethylene melamine (TEM).

Carzinophilin, another mutagenic drug, showed synergism with manganous chloride at the lower concentration employed. The mixture was fivefold more efficient in producing reversions at the streptomycin dependence locus than the calculated additive effect. The highly synergistic bactericidal effect of higher levels of carzinophilin (Table I) with $MnCl_2$ made the determination of joint mutagenic effect impossible. 2,4-Diamino-5-(3′,4′-dichlorophenyl)-6-ethylpyrimidine had a slightly synergistic effect on the mutagenicity of $MnCl_2$, while the joint action of manganous ions and TEM or manganous ions and 6-chloropurine was le[illegible]n additive.

Kin[illegible] a plant-growth factor, did not exhibit any bactericidal,

TABLE III

Combined Mutagenic Effect of Azaserine and $MnCl_2$

Expt.	Mutagens	Concn.	Survivors	Number of mutants per 10^8 survivors		Factor of synergism[a]	
				sd-4	*cys-2*	*sd-4*	*cys-2*
		μg./ml.	%				
1	Azaserine	5	35.3	1460	130		
	$MnCl_2$	400	87.6	8200	85		
	Azaserine + $MnCl_2$	5 + 400	1.4	520000	650	54	30
2	Azaserine	5	45.5	2600	40		
	$MnCl_2$	400	100	800	21		
	Azaserine + $MnCl_2$	5 + 400	11.5	7700	165	2.3	27
3	Azaserine	5	89.3	1700	260		
	$MnCl_2$	400	80.2	1100	180		
	Azaserine + $MnCl_2$	5 + 400	16.7	10400	1800	3.7	8.7
4	Azaserine	10	34.1	1400	14		
	$MnCl_2$	400	39.1	1000	16		
	Azaserine + $MnCl_2$	10 + 400	1.9	9700	120	4.0	4.0
	Azaserine + $MnCl_2$ + actinomycin	10 + 400 + 1	52.1	1500	12	0.6	0.4
5	Azaserine	1	42.4	1200	15		
	$MnCl_2$	400	100	3000	37		
	Azaserine + $MnCl_2$	1 + 400	67.2	170000	220	40.5	12

[a] The ratio of the experimentally assessed mutagenicity of the combination to the calculated sum of the mutagenic effects of the two separate components.

mutagenic, anti- or promutagenic activity, but had an interesting side effect: it stimulated many cells in a resting population of *E. coli* to undergo an additional division, thereby almost doubling the cell number (187% "survival").

Discussion

The majority of drugs employed in the present study were known to have antineoplastic activity. Earlier studies (7, 10) had shown that some of them were endowed with mutagenic activity. It was interesting, therefore, to see whether those which were not mutagenic might participate in the mutagenic process either as promutagens or antim[illegible]s.

Moreover, the observed interaction between mutagens, either synergistic, additive, or antagonistic, would also be of interest. Consequently, the results were analyzed in terms of these two broad patterns: (*a*) interaction between $MnCl_2$ and substances inactive as mutagens; and (*b*) interaction between $MnCl_2$ and compounds which by themselves proved to be mutagens.

(*a*) Within the first group, no compound showed pronounced antimutagenicity, with the possible exception of actinomycin D, which inconsistently annulled the activity of $MnCl_2$. A direct chemical reaction between $MnCl_2$ and actinomycin D outside the cell, with resulting total inactivation of manganous ions, does not seem probable, because the molecular ratio of less than one molecule of actinomycin to 5000 manganous ions would imply that each molecule of actinomycin must inactivate close to 5000 manganous ions. DON and benzimidazole slightly depressed $MnCl_2$ activity. Since the concentrations of these drugs were rather high, a direct inactivation of Mn ions cannot be excluded. Promutagenic activity, the second type of interaction, was exhibited by 6-mercaptopurine and urethan.

(*b*) The second group of substances, which were shown to be mutagenic by themselves, included azaserine, carzinophilin, 6-chloropurine, 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-ethylpyrimidine, and TEM. Their interactions with $MnCl_2$ comprised a broad spectrum, from slightly antagonistic (6-chloropurine, TEM), through weakly synergistic [2,4-diamino-5-(3′,4′-dichlorophenyl)-6-ethylpyrimidine], to moderately or strongly synergistic (carzinophilin, azaserine). The site of mutagenic interactions may be either inside or outside the bacterial cell. While the low concentrations of the accessory mutagens do not permit an explanation of their antagonism toward $MnCl_2$ on the basis of direct inactivation, the formation of a highly active compound between synergistically acting mutagens cannot be easily excluded. Present experimental data do not permit determination of the site of such an interaction.

A general conclusion which may be drawn from the data is the relative indifference of the manganous chloride mutagenic system to interference by the majority of drugs tested. This property is rather remarkable in view of the otherwise observed variability in $MnCl_2$-induced mutation rates in different cultures; the reproducibility in samples derived from the s[illegible]ulture is quite satisfactory. The experimental data point to a co[illegible]on between mutagenicity and the antineoplastic property.

Of the 17 substances which were known to be active on experimental tumors, 5 were mutagenic and an additional 4 either antagonized or promoted the mutagenic action of $MnCl_2$. Thus, over half of the cancerostatic agents tested were directly or indirectly active in the process of mutation. This correlation, however, may be spurious because the assortment tested was small and not necessarily representative.

The strong synergistic interaction of $MnCl_2$ and azaserine in mutation is of considerable interest. No synergism of this magnitude has been reported for other systems, where increases of 2–5-fold were maximal [(11–14); summarized by Muller (15)]. The data indicate that the actions of these chemically unrelated mutagens in combination are not direct and not independent of each other. Further studies on this system may lead to an understanding of the mechanism of chemical mutagenesis, and indicate a relation to other types of synergisms as encountered in multiple chemotherapy of bacterial and neoplastic diseases (16–18).

Acknowledgments

We are indebted to Dr. J. M. Ruegsegger of Lederle Laboratories for aminopterin, amethopterin, puromycin, and TEM; to Dr. G. H. Hitchings of the Wellcome Research Laboratories for the samples of 8-azaguanine, 6-chloropurine, 2,6-diaminopurine, Daraprim, 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-ethylpyrimidine, 6-mercaptopurine, and Myleran; to Dr. J. Ehrlich of Parke, Davis & Co. for azaserine and DON; to Merck & Co. for actinomycin D; to Dr. S. Wakaki of Kyowa Fermentation Industry, Tokyo, for carzinophilin (1 unit equal to 0.01 μg. of pure, crystalline material); to Eli Lilly Co. for cycloserine; and to E. R. Squibb & Sons, Inc. for kinetin.

One of us (V.N.I.) wishes to express his gratitude to the U. S. Government for providing the travel grant and postdoctoral fellowship. The skillful technical assistance of Miss Clara E. Biro is gratefully acknowledged.

Summary

Interactions between $MnCl_2$ and other mutagens, assessed by measuring changes in mutation rates from streptomycin-dependence (*sd-4*) to independence, and from cysteine-requirement (*cys-2*) to prototrophy in *E. coli*, Sd-4-73, varied from highly synergistic to less than additive (antagonistic). The combined action of $MnCl_2$ and azaserine exceeded the theoretical additive effect by a factor of 50. A few nonmutagenic substances influenced the $MnCl_2$ activity as promutagens or antimutagens. Most notable among them was actinomycin D, which, al[illegible] in-

consistently, was capable of almost completely suppressing the action of the mutagen.

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Biochem. J. (1965) 95, 94

Effect of Manganese on Calcification of Bone

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(*Received* 30 *June* 1964)

1. Young mice were maintained on a basal diet composed of meat, which is poor in both manganese and calcium. 2. The addition of small amounts (2·5–5·0mg./kg. of meat) of manganese improved weight gain and calcification of bone and decreased incorporation of injected radiocalcium into bone. 3. Prolonged treatment with larger amounts (10·0–25·0mg./kg. of meat) of manganese depressed growth, induced defective calcification of bone and increased incorporation of radiocalcium into bone.

It is well known that dietary lack of manganese induces skeletal abnormalities, including generalized rarefaction of bone, that are preventable by appropriate manganese supplementation (Underwood, 1962). These findings were reported in animals that had subsisted on diets containing sufficient or abundant amounts of calcium. Recently, manganese has been found to increase bone ash of mice subsisting on a diet composed of ox muscle only, which is poor in calcium as well as in manganese (Han, Schwartz & Guggenheim, 1961). Young mice maintained for 6 weeks on unsupplemented meat developed a severe bone disorder. The ash content of the bone was markedly decreased, and osteoporosis could be demonstrated both radiologically (Han *et al.* 1961) and histologically (Ullmansky, 1964). The addition of 2·5mg. of manganese/kg. of meat increased bone ash from 46·9g./100g. of dry fat-free bone to 55·1g./100g., in spite of the fact that the diet was very poor in calcium. The effect of larger amounts of manganese is reported in the present paper. Mice were kept for various times on ox meat to which different amounts of manganese (2·5–25·0mg./kg.) were added. Their femurs were then analysed for bone ash, calcium, phosphorus and manganese. The incorporation of radioactive calcium in bone was also determined.

METHODS

Sixteen groups of 24 male Swiss mice, 3 weeks old (10–13g.), were used. Their diet consisted of raw lean ox muscle, which was offered either unsupplemented ('negative' control), or supplemented with 2·5, 5·0, 10·0 or 25·0mg. of manganese (as $MnSO_4$)/kg. or with 3·0g. of calcium (as $CaCO_3$)/kg. ('positive' control). Femurs of mice fed on meat and given a similar supplement of calcium (3·3g./kg.) were previously found to contain a normal percentage of ash (Han *et al.* 1961). The calcium content of the meat was examined in 12 samples and found to be 95 (S.E.M. ± 5·9) mg./kg.

Four groups ('negative' and 'positive' controls and mice supplemented with 10·0 and 25·0mg. of manganese/kg. of meat) were killed after 2 weeks. 'Negative' and 'positive' controls and four groups supplemented with the four different concentrations of manganese were killed after 4 and 6 weeks respectively. The femurs were cleaned from adherent tissue and weighed. They were then broken, extracted for 6hr. with ethanol in a Soxhlet apparatus, dried to constant weight and ashed at 600°. The calcium (Baron & Bell, 1956), phosphorus (Fiske & Subbarrow, 1925) and manganese (Gaies & Ellis, 1947) contents of the ash were determined. Determination of phosphorus and manganese were performed in 18 mice only of each group. Measured portions from the ash of three pairs of femurs were pooled. The values in the Tables thus indicate means ± S.E.M. of six samples each comprising three pairs of femurs.

In six animals of each group the incorporation of radioactive calcium was studied. These mice received a dose of $^{45}CaCl_2$ subcutaneously at the end of the experimental period (0·1μc in 1·0mg. of calcium/10g. body wt.). On the following day the animals were killed and their femurs ashed at 600°. The ash was dissolved in 1N-HCl and a measured portion was taken for determination of radioactivity. The results are expressed as μg. of the dose administered per g. of dry fat-free bone or per 100mg. of bone calcium.

RESULTS

Table 1 presents results obtained after an experimental period of 2 weeks. The mice that were fed on a diet supplemented with calcium increased their weight more than the mice fed on the unsupplemented diet. The addition of manganese to the meat also produced an increase in weight, but this was not as great as that with calcium. The calcium supplement significantly ($P < 0·01$) increased bone ash and the concentrations of calcium and phosphorus in both ash and in dry fat-free bone, but decreased the incorporation of radioactive calcium in bone ($P < 0·01$). A similar, though smaller, effect on bone was observed when meat was supplemented with 25mg. of manganese/kg., whereas

Table 1. *Composition of bones and uptake of radioactive calcium by femurs of mice maintained for 2 weeks on a meat diet*

Experimental details are given in the text. The results are given as means ± S.E.M.

Supplement ...	None	10·0 mg. of Mn/kg. of meat	25·0 mg. of Mn/kg. of meat	3600 mg. of Ca/kg. of meat	No. of pairs of femurs in each sample	No. of samples in each mean
Weight increase (g.)	4·6 ± 1·7	5·5 ± 0·8	6·5 ± 1·0	7·8 ± 0·9	—	24
Weight of femurs (mg.)	68 ± 5·5	77 ± 5·3	85 ± 3·5	98 ± 5·6	1	24
Ash:						
(mg.)	13·7 ± 1·3	15·5 ± 1·1	20·2 ± 1·2	28·4 ± 2·2	1	24
(% of dry fat-free bone)	42·1 ± 0·63	42·2 ± 1·55	48·8 ± 0·49	53·6 ± 0·71	1	24
Ca:						
(% of ash)	30·4 ± 0·21	29·5 ± 0·15	32·1 ± 0·14	35·1 ± 0·10	1	24
(% of dry fat-free bone)	12·7 ± 0·18	12·4 ± 0·20	15·6 ± 0·22	18·9 ± 0·23	1	25
Ca incorporated:						
(μg./g. of dry fat-free bone)	78 ± 0·55	80 ± 0·53	77 ± 0·66	56 ± 0·21	1	6
(μg./100 mg. of bone Ca)	62 ± 0·22	64 ± 0·32	53 ± 0·48	30 ± 0·53	1	6
P:						
(% of ash)	15·7 ± 0·17	14·8 ± 0·10	16·3 ± 0·12	17·7 ± 0·15	3	6
(% of dry fat-free bone)	6·6 ± 0·12	6·3 ± 0·15	8·0 ± 0·13	9·6 ± 0·17	3	6
Mn:						
(mg./100 g. of ash)	490 ± 14	788 ± 12	811 ± 13	411 ± 12	3	6
(mg./100 g. of dry fat-free bone)	203 ± 27	332 ± 22	389 ± 24	219 ± 22	3	6

Table 2. *Composition of bones and uptake of radioactive calcium by femurs of mice maintained for 4 weeks on a meat diet*

Experimental details are given in the text. The results are given as means ± S.E.M.

Supplement ...	None	2·5 mg. of Mn/kg. of meat	5·0 mg. of Mn/kg. of meat	10·0 mg. of Mn/kg. of meat	25·0 mg. of Mn/kg. of meat	3600 mg. of Ca/kg. of meat	No. of pairs of femurs in each sample	No. of samples in each mean
Weight increase (g.)	4·4 ± 1·3	7·0 ± 1·2	6·0 ± 0·9	3·9 ± 0·8	4·2 ± 0·8	9·3 ± 1·5	—	24
Weight of femurs (mg.)	97 ± 4·9	107 ± 9·0	97 ± 9·7	95 ± 7·3	111 ± 8·2	129 ± 9·5	1	24
Ash:								
(mg.)	19·0 ± 3·6	23·9 ± 1·8	21·1 ± 1·7	20·9 ± 3·2	23·0 ± 5·0	43·5 ± 3·6	1	24
(% of dry fat-free bone)	40·8 ± 0·48	48·1 ± 0·45	47·0 ± 0·57	45·4 ± 0·86	43·1 ± 0·93	59·6 ± 1·10	1	24
Ca:								
(% of ash)	28·9 ± 0·15	32·3 ± 0·22	32·3 ± 0·17	32·5 ± 0·16	31·3 ± 0·16	37·4 ± 0·18	1	24
(% of dry fat-free bone)	11·6 ± 0·18	15·4 ± 0·20	15·3 ± 0·10	14·7 ± 0·12	13·6 ± 0·15	22·6 ± 0·19	1	24
Ca incorporated:								
(μg./g. of dry fat-free bone)	73 ± 0·43	67 ± 0·41	70 ± 0·53	61 ± 0·38	67 ± 0·44	44 ± 0·59	1	6
(μg./100 mg. of bone Ca)	62 ± 0·53	42 ± 0·39	46 ± 0·40	40 ± 0·52	47 ± 0·35	20 ± 0·38	1	6
P:								
(% of ash)	15·0 ± 0·18	16·7 ± 0·12	16·6 ± 0·15	15·9 ± 0·13	15·7 ± 0·14	18·8 ± 0·19	3	6
(% of dry fat-free bone)	6·0 ± 0·13	8·5 ± 0·15	7·8 ± 0·17	7·1 ± 0·18	7·0 ± 0·11	11·5 ± 0·16	3	6
Mn:								
(mg./100 g. of ash)	474 ± 13	861 ± 19	901 ± 18	906 ± 24	956 ± 18	401 ± 20	3	6
(mg./100 g. of dry fat-free bone)	192 ± 15	450 ± 12	410 ± 20	416 ± 14	398 ± 12	243 ± 11	3	6

supplementation with only 10mg. of manganese/kg. had no effect. Both concentrations of manganese (10 and 25mg./kg.) increased the manganese content of ash and bone ($P < 0.01$). It follows that the addition of 25mg. of manganese/kg. of meat considerably improves the calcification of bone even within 2 weeks.

Although no further weight increase was observed in mice maintained on the meat diet for 4 weeks, the mice whose diet was supplemented with calcium continued growing (Table 2). The addition of small amounts of manganese to the meat (2·5–5·0mg./kg.) brought about some increase in weight, but larger amounts (10·0–25·0mg./kg.) had no effect. The increased bone calcification resulting from the addition of calcium to the meat, which was apparent after 2 weeks, became more evident after 4 weeks. Small additions of manganese also improved calcification, though to a smaller degree. They increased bone ash and the calcium and phosphorus contents of ash and of dry fat-free bone, and decreased the incorporation of radioactive calcium in bones and in bone calcium. Larger amounts of manganese, i.e. 10·0 and particularly 25·0mg./kg. of meat, brought about smaller increases in bone ash and in the concentrations of calcium and phosphorus in bone and in ash. The calcium and phosphorus contents of the bones of mice that were fed on meat supplemented with 25mg. of manganese/kg. were significantly ($P < 0.01$) lower than those of mice that received the 2·5 or 5·0mg./kg. supplement, and differed little from those found in animals kept on the unsupplemented meat diet. This is in contrast with the results obtained with the high concentration of manganese after 2 weeks. The bone manganese content was similar in the four groups that received manganese, irrespective of the concentration of manganese supplementation. The lowest concentration is apparently adequate for maximal storage of manganese in bone.

Prolonging the experimental period to 6 weeks (Table 3) widened the differences in weight increase and weight of femurs between mice raised on the unsupplemented meat diet and those kept on the same diet with calcium added. Small amounts of manganese (2·5–5·0mg./kg. of meat) slightly increased the amount (P 0·2–0·05) and percentage (P 0·02–0·05) of bone ash, and decreased the incorporation of radioactive calcium in dry fat-free bone ($P < 0.01$) and in bone calcium ($P < 0.01$). Higher concentrations of manganese (10·0–25·0mg./kg. of meat) depressed growth and decreased the bone ash

Table 3. *Composition of bones and uptake of radioactive calcium by femurs of mice maintained for 6 weeks on a meat diet*

Experimental details are given in the text. The results are given as means ± S.E.M.

Supplement ...	None	2·5mg. of Mn/kg. of meat	5·0mg. of Mn/kg. of meat	10·0mg. of Mn/kg. of meat	25·0mg. of Mn/kg. of meat	3600mg. of Ca/kg. of meat	No. of pairs of femurs in each sample	No. of samples in each mean
Weight increase (g.)	5·4 ± 1·4	6·9 ± 1·1	7·1 ± 1·0	6·9 ± 0·6	4·2 ± 0·8	13·5 ± 1·0	—	24
Weight of femurs (mg.)	98 ± 9·2	113 ± 5·5	109 ± 5·2	104 ± 6·8	111 ± 5·7	141 ± 4·4	1	24
Ash:								
(mg.)	19·8 ± 1·7	24·2 ± 1·4	22·7 ± 1·8	19·6 ± 1·3	20·6 ± 0·7	44·0 ± 1·6	1	24
(% of dry fat-free bone)	44·3 ± 0·41	46·1 ± 0·63	45·6 ± 0·58	39·9 ± 0·50	37·1 ± 0·65	59·3 ± 0·73	1	24
Ca:								
(% of ash)	32·9 ± 0·29	32·3 ± 0·37	31·4 ± 0·20	30·7 ± 0·21	28·8 ± 0·28	41·2 ± 0·20	1	24
(% of dry fat-free bone)	14·5 ± 0·19	15·0 ± 0·36	14·0 ± 0·24	12·1 ± 0·20	10·7 ± 0·13	24·5 ± 0·20	1	24
Ca incorporated:								
(μg./g. of dry fat-free bone)	72 ± 0·20	67 ± 0·21	67 ± 0·33	64 ± 0·29	70 ± 0·20	39 ± 0·72	1	6
(μg./100mg. of bone Ca)	50 ± 0·29	45 ± 0·11	47 ± 0·28	52 ± 0·38	63 ± 0·13	17 ± 0·55	1	6
P:								
(% of ash)	17·6 ± 0·42	17·5 ± 0·28	16·8 ± 0·12	16·2 ± 0·12	15·0 ± 0·09	20·9 ± 0·21	3	6
(% of dry fat-free bone)	7·6 ± 0·30	8·0 ± 0·25	7·8 ± 0·13	6·2 ± 0·11	5·8 ± 0·16	12·2 ± 0·43	3	6
Mn:								
(mg./100g. of ash)	530 ± 10	990 ± 12	915 ± 13	980 ± 14	1087 ± 24	475 ± 4	3	6
(mg./100g. of dry fat-free bone)	236 ± 19	456 ± 14	432 ± 22	381 ± 33	398 ± 28	275 ± 12	3	6

and calcium and phosphorus contents of bone. At the highest concentration of manganese supplementation, the incorporation of radioactive calcium in bone calcium was increased. It therefore appears that relatively large doses of manganese are toxic when administered over a prolonged period and induce defective calcification of bone. The manganese content of bone was similar to that found after 4 weeks.

DISCUSSION

Our results confirm and extend previous observations (Han *et al.* 1961) that young mice kept on a meat diet develop an osteoporosis-like condition that can be prevented by the addition of calcium to the meat. In the absence of any supplement bone ash and calcium and phosphorus concentrations of bone decrease and the incorporation of radioactive calcium by bone becomes higher than when calcium is added. These changes suggest decreased calcification (Carlsson, 1951; Menezel, Schraer, Pakis, Posner & Likins, 1963). The possibility that the addition of unlabelled calcium to meat would dilute the concentration of radioactive calcium in the serum cannot be excluded, although the serum calcium concentration was not found to be affected by the meat diet (Han *et al.* 1961). The addition, over a period of 4 weeks, of small amounts of manganese (2·5–5·0mg./kg.) to the meat diet improves calcification of bone. The possibility that these supplements of manganese affected food intake and thus calcification cannot be excluded. However, the nature of the diet used, namely raw minced meat, made an exact measurement of food consumption unreliable. After 6 weeks no clear effect on the bone composition is discernible, although the incorporation of radiocalcium is increased. The same supplementation concentration, i.e. 2·5mg./kg., was found by Han *et al.* (1961) to increase bone ash even after a 6-week observation period.

The deleterious effect exerted by a meat diet on the skeleton of kittens (Scott, Greaves & Scott, 1961) and of rats (Moore & Sharman, 1960) has been reported. Growth of rats ceased after a few weeks, and the bones were undersized, had thin walls and a low ash content and were often fractured. The disorder could be prevented by adding adequate amounts of calcium to meat (Moore *et al.* 1962). Our results indicate that not only calcium is involved. Muscle meat is a poor source of manganese (Underwood, 1962). Radiographic, histological and biochemical investigations have established the important role of manganese in osteogenesis (Underwood, 1962). Dietary lack of manganese is reported to inhibit growth and to cause poor calcification of bone in mice, rats and rabbits (Shils & McCollum, 1943; Wachtel, Elvehjem & Hart, 1943; Smith & Ellis, 1947). Skeletal maturation of the young is retarded (Frost, Asling & Nelson, 1959), and the density, breaking strength and ash content of bones is decreased. Microscopically, extensive deviations from normal were found that were interpreted as suppressed osteogenesis rather than as accelerated bone resorption (Smith, Medlicott & Ellis, 1944).

As mentioned above, these findings were reported in animals that had subsisted on manganese-deficient diets containing adequate amounts of calcium. The fact that meat is deficient in manganese as well as in calcium renders it particularly suitable for studying the effect of manganese on bone calcification and calcium metabolism without interference by calcium. In mice maintained on meat small amounts of manganese play an important role in calcification, whereas larger doses lead apparently to defective calcification of newly formed bone.

The larger doses are, however, much smaller than those reported to be toxic for guinea pigs (500mg./kg of food), rats (over 2000mg./kg.) and chicks (4800mg./kg.) (Underwood, 1962). It is possible that the increased sensitivity of our animals results from the low calcium content of their diet, and that no adverse effects on bone would have been observed if the diet had provided adequate amounts of calcium.

This study was supported by U.S. Public Health Service Research Grant A-4385 from the National Institutes of Health, Bethesda, Md., U.S.A.

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pages 177-207

Frankfurter Zeitschrift fur Pathologie [Frankfort Journal of Pathology]
74: 572-578, (1965)

From the Institute for Forensic Medicine of the University of Lund (Sweden)
(Director: Prof. Dr. G. E. VOIGT)

On the protective action of zinc in the presence of liver damage induced by manganese sulfate or carbon tetrachloride *

By G. E. VOIGT and T. SALDEEN with three text illustrations

(Received on 17 Feb. 1965)

Zinc is well known as an important constituent of various enzymes or is capable of activating the effectiveness of certain enzyme systems.

Thus this metal is one of the most important trace elements in the organism, and it is not surprising that in the past few years there has been increasing interest in the zinc content of different organs, of the serum, and of the urine under normal and pathological conditions, especially in cases of cirrhosis of the liver. As LUNDEGARDH and H. BERGSTRAND; VALLEE, WACKER, BARTHOLOMAY and HOCH have shown, the zinc content of the cirrhotic liver can be reduced as compared with the norm.

The idea naturally suggested itself to investigate whether large amounts of zinc introduced into the organism exert an influence on degenerative and/or inflammatory changes of the liver, like they may be experimentally induced, e.g., through manganese sulfate or carbon tetrachloride. It seemed especially justifiable to undertake investigations along this line, since SADASIVAN has demonstrated a lipotropic action of zinc in the liver.

* Excerpts presented at the meeting of the "Nordic Society of Forensic Medicine", at 5 Oct. 1964.

Manganese Sulfate

As investigations by KOBERT; HANDOVSKY, SCHULTZ and STAEMMLER, among others, have shown, after repeated injections, manganese induces icteric necroses and possibly* a cirrhosis of the liver. Degenerative changes in the brain and kidney, and vascular changes in the spleen, have also been described (MENON).

After preliminary orientational tests, the Syrian Golden hamster showed itself to be the most suitable experimental animal for the experiments to be conducted; it is significantly more sensitive to manganese than the rat, guinea pig, or mouse. For the experiments, golden hamsters from an institute-bred stock were employed; their weight was between 60 g and 130 g and their age between 3 to 9 months. The appropriate dosage was found to be 4.5 mg manganese sulfate in aqueous solution per 100 g body weight. This was injected subcutaneously on the back of the animal every day up to 10 days. Manganese sulfate causes local necroses, which forbid a continuation of the injections beyond the period stated. Since there are no reports in the literature on the effect of repeated administrations of manganese in the hamster, a group of 60 animals received the cited dosage of manganese sulfate subcutaneously for 10 days, and in the course of a month, beginning with the first day after commencement of the experiment, two animals were sacrificed every day. Results of the microscopic examinations were as follows:

As the first sign of liver damage, beginning in the vicinity of the periportal spaces, there occurs as early as one day after commencement of the experiment large droplet fatty deposition in the parenchyma cells in the periphery of the hepatic lobules. Very shortly thereafter, clear

* also "eventually".

indications appear of a necrosis of these hepatic cells, with pyknotic nuclei and later, a karyolysis. The cytoplasm becomes eosinophilic. In the early stages, this necrosis is limited to smaller areas, but soon afterwards spreads from the surrounding area of the periportal spaces along the periphery of the hepatic lobules, and thence toward the latters' center. The necrotic cells are surrounded by abundantly polymorphonuclear leukocytes, which are already occasionally clustered in the periportal spaces before extensive necroses set in. The cells of Kupffer, located primarily in the periphery of the lobules, are already swollen only a few days after the commencement of the investigation, and contain fat. From the seventh day on, iron can be demonstrated in them. On the sixth or seventh day after beginning the experiment, the icteric necroses are clearly developed. Thereafter, a regeneration of the liver parenchyma cells gradually sets in. Only in individual animals that have survived more than 15 days, are a periportal connective tissue increased and indications of cystic duct proliferation to be observed, whereas in those animals sacrificed one month after commencement of the experiment, there are present simply an increased number of the so called dark liver parenchyma cells in the peripheny of the lobules. Occasionally, small round cell foci are also observable; these are mostly at a distance from the periportal spaces, in the periphery of the lobules. The cells of Kupffer are rich in iron.

Parallel with the icteric necroses, there regularly appear in the kidneys, too, necroses of the epithelial cells in the distal portion of the Tubuli cont. I, Tubuli cont. II*. and the initial collecting tubules.

* =convoluted renal tubules I and II.

In cases of longer survival periods, a regeneration of the destroyed epithelia may be observed; the latter are replaced by squamous epithelial cells. In the myocardium, small necroses with agglomerations of polymorphonuclear leukocytes are seen almost regularly.

Zinc Sulfate

In a dosage of 4.5 mg per 100 g body weight, zinc sulfate was administered daily for ten days subcutaneously on the thighs. Zinc sulfate occasioned local necroses to a still greater degree than manganese sulfate.

Subsequent to the administration of zinc, no morphological changes at all could be observed in the microscopic examination of the liver.

Lethality following administation of manganese sulfate and zinc sulfate

Two hundred and two golden hamsters were divided up into three groups. Of these, Group 1 (92 animals), received up to 10 days, daily, 4.5 mg manganese sulfate per 100 g body weight; Group 2 (40 animals), up to 10 days, daily, 4.5 mg zinc sulfate; and Group 3 (70 animals), up to 10 days, daily, 4.5 mg manganese sulfate and simultaneously also 4.5 mg zinc sulfate. The period of survival was logged. Surviving animals were decapitated one month following commencement of the experiment. The results are shown in Fig. 1.

In Group 1, the mortalities could be ascribed primarily to the hepatic changes, but also to the myocardial necroses and to the tubule necroses in the kidneys. The mortalities in Group 2 could very well be connected with the local necroses in the area surrounding the point of injection. In these animals, liver, kidney, and heart changes are lacking. In Group 3, it is to be assumed the animals succumbed as a result of the

kidney changes and the extensive local necroses in the neighborhood of the points of injection. No myocardial necroses were found in the microscopic examination, while changes in the liver were either more weakly developed or altogether absent.

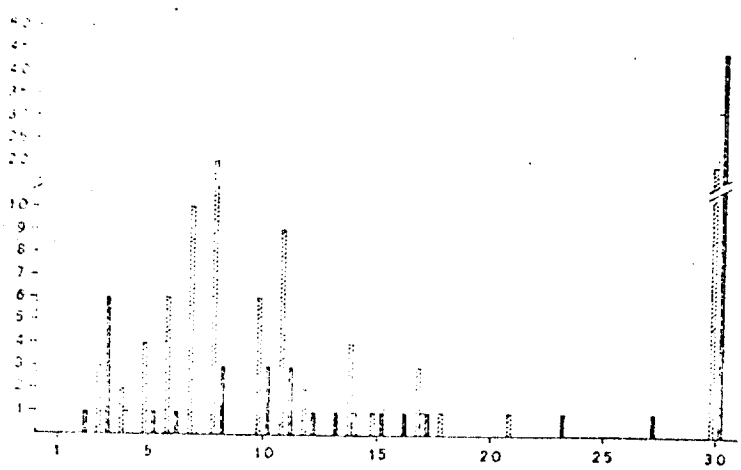

Fig. 1. Survival period of golden hamsters calculated from the first day after treatment with manganese sulfate (▨); zinc sulfate (▭); or manganese sulfate and zinc sulfate (▬). Surviving animals were sacrificed on the thirtieth day.

As may be seen from Fig. 1, lethality is highest on the sixth to seventh day following repeated injections of manganese sulfate; this agrees with the observations on the severity of the changes in the liver.

The most striking finding was that the hepatic necroses in Group 3 were significantly less pronounced than in Group 1. In order to examine more closely the effect of zinc as opposed to the manganese induced liver changes, the morphological changes of the liver following injection of manganese sulfate, resp., manganese sulfate and zinc sulfate, were investigated on the six and seventh day after commencement of the experiment.

Comparative investigations of the liver changes on the sixth and seventh day after commencing the experiment

Thirty animals were administered daily 4.5 mg manganese sulfate, and 45 animals received 4.5 mg manganese sulfate and 4.5 mg zinc sulfate, per 100 g body weight. Animals that were in a poor general condition were sacrificed already on the sixth day, the remainder on the seventh day.

Fatty depositions in the liver parenchyma cells and liver necroses were registered on a scale of 0 to 3 in accordance with the following schema: 0 = no changes; 1 = fatty deposition and necroses in the periphery of the lobes of the liver; 2 = fatty deposition and necrosis also in the intermediary zone of the acini*; and 3 = diffuse fatty deposition and necrosis of all the acini*. The changes were evaluated by two different investigators. The mean values of the independent evaluations are apparent from the table below. The differences between the liver changes were statistically significant ($p<0.01$).

Table: Effect of zinc upon the manganese sulfate- resp. carbon tetrachloride-induced liver changes. Mean values of evaluation in the microscopic examination (see text).

	$MnSO_4$	$MnSO_4$ and $ZnSO_4$	CCl_4	CCl_4 and $ZnCCl_4$
Number of experimental animals	30	45	23	16
Fatty deposition	1.6	0.2	2.8	1.9
Necrosis	2.1	0.4		

* Hepatic lobules.

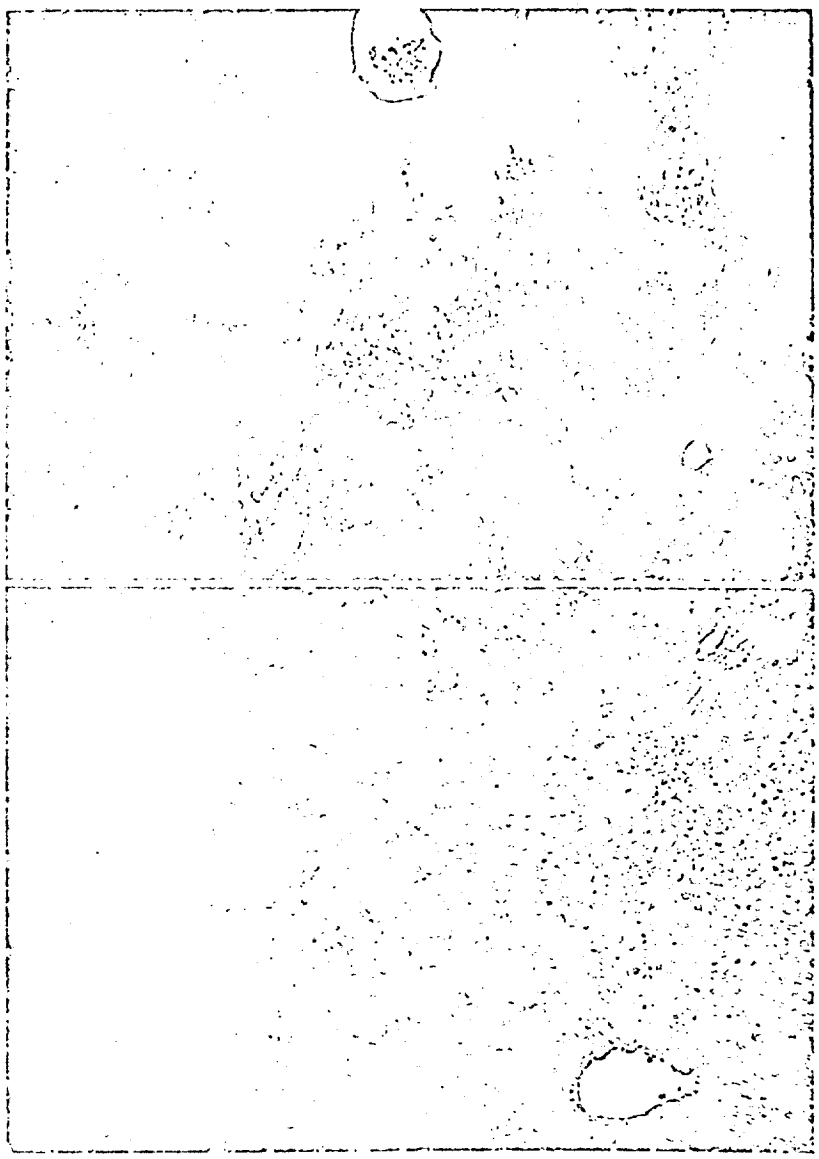

Fig. 2 a. and 2 b.

a. Manganese sulfate -- induced hepatic necrosis in the golden hamster 7 days after start of treatment.
b. Liver of a golden hamster after 7 days' administration of manganese sulfate and zinc sulfate (H.-E.).

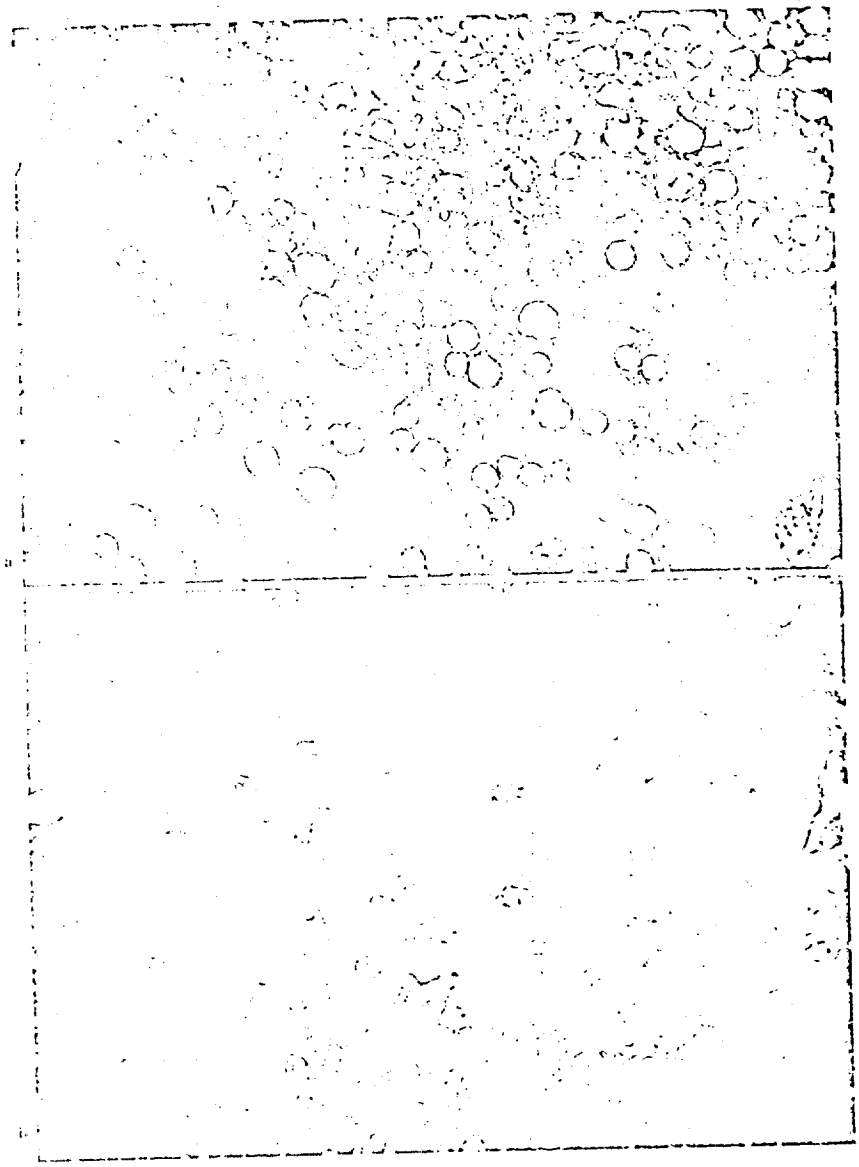

Fig. 3 a. and b.

a. Liver after ten-day treatment with carbon tetrachloride. Intense fatty degeneration of a liver lobule.
b. Liver after ten-day treatment with carbon tetrachloride and zinc chloride. Moderate acinocentral fatty degeneration. (H.-E.)

Carbon tetrachloride

As orientational investigations have shown, in consonance with the findings of WAKIM and MANN; ASHBURN, ENDICOTT, DAFT and LILLIE; and UNGAR, the daily administration of 0.5 ml of a 25% solution of carbon tetrachloride in mineral oil per 100 g body weight caused in rats, after ten days, pronounced fatty degenerations emanating from the center of the acini, and necroses of the liver parenchyma cells.

Forty-six female rats (SPAGUE-DAWLEY strain, Anticimexfarm, Stockholm) were divided up into three groups. As with the golden hamsters in the preceding experiment series, the animals were fed a standard fare (commercial rat pellets) and water as desired. The one group (23 rats) received daily injections of 0.5 ml of the 25% carbon tetrachloride solution in mineral oil 1 m (thigh); the other group (16 rats) received the same dosage of carbon tetrachloride in mineral oil 1 m (thigh) and 4.5 mg zinc chloride subcutaneously per 100 g body weight daily from the second day before commencement of the carbon tetrachloride injections. Group 3 received only zinc chloride. The rats were sacrificed one day after the last injections.

On dissection, it was not possible to establish macroscopically any definite differences in the livers of the animals of the two first groups. On the other hand, histological examination, especially in the case of the animals not treated with zinc, revealed the characteristic changes following injections with carbon tetrachloride. The extent of parenchyma cell fatty degeneration and of the necroses, determined histologically, has been evaluated as follows: (1) only acinocentral damage; (2) damage also in the intermediary zone; and (3) diffuse damage to all the acini. As shown in the table, a clear difference between the two groups became apparent.

The fatty degeneration and the tendency to necrosis running parallel with it has significantly less in the group that had received zinc at the same time. The difference was statistically significant. ($p<0.01$).

Rats that had received only zinc chloride exhibited no liver damage.

Discussion

Following subcutaneous administration over a period of several days, manganese sulfate regularly causes severe liver damage in the Syrian golden hamster. The damage takes the form of a parenchyma cell necrosis emanating from the acinus periphery and accompanied by leukocytic infiltrates, whereas carbon tetrachloride results (1) in a fatty degeneration, well known from numerous investigations, emanating from the center of the hepatic lobules, and (2) in the destruction of the liver parenchyma cells. To judge by the histological picture, a basic difference exists between the mode of action of these two hepatotoxic substances with respect to the reaction site in the liver.

Subcutaneous administration of hydrosoluble zinc salts alone showed no demonstrable morphological changes in the liver.

Administration of hydrosoluble zinc salts simultaneously with manganese sulfate or carbon tetrachloride exerts a distinct protective effect on the liver, an effect deducible from the histological picture. The action mechanism is unknown, but it should be noted that the zinc-activated enzyme systems are significant for this action. In manganese intoxication, zinc is certainly also active in the presence of the myocardial necroses, and it is not impossible that in the action of zinc in the presence of manganese, a "competitive inhibition" similarly

exists, as is assumed for the protective effect of zinc with respect to the testicular necroses induced by cadmium (PARIZEK).

In our research experiments, the question of whether zinc promotes the regeneration of already pathologically altered livers was not examined. Certain observations by VALLEE, LACKER, BARTHOLOMAY and HOCH appear to support this possibility. These authors were able to observe an improvement in liver function in some patients suffering from cirrhosis of the liver, following peroral administration of physiological amounts of zinc. These observations, however, could not be confirmed by HAMMOND, BLACK and COLLISON. As early as 1940, PRABHU was able to report an improvement in the clinical picture in the case of patients with sub-critical infantile liver cirrhosis.

Summary

Repeated administration of manganese sulfate causes in the Syrian golden hamster necroses of the liver emanating from the acinus periphery, whereas carbon tetrachloride in rats produces acinocentral fatty depositions and necroses of the liver. Parenteral dosage of zinc salts simultaneously with manganese sulfate or carbon tetrachloride provides clear protection against development of the liver changes cited.

Zeitschrift für Pathologie 74, 572—578 (1965)

Aus dem Institut für gerichtliche Medizin der Universität Lund (Schweden)
(Vorstand: Prof. Dr. G. E. VOIGT)

Über den Schutzeffekt des Zinks gegenüber Mangansulfat- oder Kohlenstofftetrachlorid-induzierten Leberschäden*

Von

G. E. VOIGT und T. SALDEEN

Mit 3 Textabbildungen

(Eingegangen am 17. Februar 1965)

Zink ist bekanntlich ein wesentlicher Bestandteil verschiedener Enzyme und vermag die Wirksamkeit gewisser Enzymsysteme zu aktivieren.

Dieses Metall ist somit eines der wichtigsten Spurenelemente im Organismus, und es dürfte nicht wunder, wenn man sich in den letzten Jahren in steigendem Maße für den Zinkgehalt der verschiedenen Organe, des Serums und Urins unter normalen und pathologischen Verhältnissen interessiert hat, so besonders bei Fällen von Lebercirrhose. Wie LESNIAK-VIRON und H. GERSTENBERGER, WACKER, BERTHOLOWAY und HOCH gezeigt haben, kann der Zinkgehalt der cirrhotischen Leber gegenüber der Norm vermindert sein.

Der Gedanke lag nahe, zu untersuchen, ob dem Organismus zugeführte größere Zinkmengen einen Einfluß auf degenerative und/oder entzündliche Veränderungen der Leber haben, wie sie experimentell, z. B. durch Mangansulfat oder Kohlenstofftetrachlorid, hervorgerufen werden können. Untersuchungen in dieser Richtung erschienen besonders deshalb berechtigt, nachdem SADASIVAN einen lipotropen Effekt des Zinks in der Leber nachgewiesen hat.

Mangansulfat

Mangansulfat, wie Untersuchungen von KOBERT, HANDOVSKY, SCHULTZ und STAEMMLER u. a. gezeigt haben, nach mehrfacher Injektion beim Versuchstier Lebernekrosen, eventuell eine Leberzirrhose hervor. Es sind auch degenerative Veränderungen im Gehirn und der Niere sowie Gefäßveränderungen in der Milz beschrieben worden (MENON).

Für die durchzuführenden Experimente hat sich nach orientierenden Versuchen der syrische Hamster als geeignetes Versuchstier erwiesen, der gegenüber Mangan bedeutend empfindlicher ist als Ratte, Meerschweinchen oder Maus. Für die Versuche wurden Goldhamster eines institutseigenen Stammes verwendet, die zwischen 60 und 130 g schwer und 3—9 Monate alt waren. Als zweckmäßige Lösis haben sich 4,5 mg Mangansulfat in wäßriger Lösung pro 100 g Körpergewicht herausgestellt, das täglich bis zu 10 Tagen subcutan am Rücken injiziert wurde. Mangansulfat führt lokale Nekrosen hervor, die eine Fortsetzung der Injektionen über die angegebene Zeit hinaus verbieten. Da sich in der Literatur keine Angaben über den Effekt mehrfacher Mangangabe beim Hamster fanden, wurde einer Gruppe von 60 Tieren 10 Tage lang die angegebene tägliche Mangansulfatdosis subcutan gegeben und Verluste eines Monats wurden ab 1. Tag nach Versuchsbeginn täglich 2 Tiere getötet. Die mikroskopischen Untersuchungen hatten folgendes Ergebnis:

* Auszugsweise vorgetragen auf der Tagung der nordischen Gesellschaft für gerichtliche Medizin in Oslo 1964.

Als erstes Zeichen einer Leberschädigung findet sich, in der Nähe der periportalen Räume beginnend, schon einen Tag nach Versuchsbeginn eine großtropfige Verfettung der Parenchymzellen in der Peripherie der Leberacini. Schnell danach zeigen sich deutliche Zeichen einer Nekrose dieser Leberzellen mit pyknotischen Kernen und später eine Karyolyse. Das Cytoplasma wird eosinophil. Diese Nekrose ist in frühen Stadien auf kleinere Bezirke begrenzt. Sie breitet sich jedoch danach bald von der Umgebung der periportalen Räume entlang der Peripherie der Leberacini und von hier aus nach deren Zentrum aus. Die nekrotischen Zellen sind von reichlich polymorphkernigen Leukocyten umgeben, die mitunter bereits vor Einsetzen ausgedehnter Nekrosen in den periportalen Räumen angehäuft sind. Die hauptsächlich in der Peripherie der Acini gelegenen Kupfferschen Sternzellen sind schon wenige Tage nach Versuchsbeginn geschwollen und enthalten Fett. Ab 7. Tag läßt sich in ihnen Eisen nachweisen. Die Lebernekrosen sind am 6./7. Tag nach Versuchsbeginn deutlich ausgebildet. Hiernach setzt allmählich eine Regeneration der Leberparenchymzellen ein. Nur bei einzelnen Tieren, die mehr als 15 Tage überlebt haben, sieht man eine periportale Bindegewebsvermehrung und Zeichen für eine Gallengangsproliferation während bei den Tieren, die 1 Monat nach Versuchsbeginn getötet wurden, lediglich eine vermehrte Anzahl sog. dunkler Leberparenchymzellen in der Läppchenperipherie vorhanden sind. Ab und zu sieht man auch kleine Rundzellherde, meist von den periportalen Räumen abgelegen, in der Läppchenperipherie. Die Kupfferschen Sternzellen enthalten reichlich Eisen.

Parallel mit den Leibernekrosen treten regelmäßig auch in den Nieren Nekrosen der Epithelzellen im distalen Teil der Tubuli cont. I, der Tubuli cont. II und der anfänglichen Sammelröhren auf. Bei längerer Überlebenszeit läßt sich eine Regeneration der untergegangenen Epithelien beobachten, die durch platte Epithelzellen ersetzt werden. Im Myokard sieht man fast regelmäßig kleine Nekrosen mit Ansammlungen von polymorphkernigen Leukocyten.

Zinksulfat

Zinksulfat wurde in einer Dosis von 4,5 mg/100 g Körpergewicht täglich 10 Tage lang subcutan am Ober- Oberschenkeln verabreicht. Noch stärker als Mangansulfat hat Zinksulfat lokale Nekrosen zur Folge.

Bei der mikroskopischen Untersuchung der Leber konnten keinerlei morphologische Veränderungen nach Zinkapplikation beobachtet werden.

Die Letalität nach Applikation von Mangan- und Zinksulfat

Insgesamt 92 Goldhamster wurden in 3 Gruppen aufgeteilt. Davon erhielt die 1. Gruppe (92 Tiere) bis zu 10 Tagen täglich 4,5 mg Mangansulfat/100 g Körpergewicht, die 2. Gruppe von 40 Tieren bis zu 10 Tagen täglich 4,5 mg Zinksulfat und die 3. Gruppe (70 Tiere) bis zu 10 Tagen täglich 4,5 mg Mangansulfat sowie gleichzeitig 4,5 mg Zinksulfat. Die Überlebenszeit wurde registriert. Überlebende Tiere wurden 1 Monat nach Versuchsbeginn dekapitiert. Die Ergebnisse gehen aus Abb. 1 hervor.

Die Todesfälle in Gruppe 1 dürften in erster Linie auf die Leberveränderungen, aber auch auf die Herzmuskelnekrosen sowie Tubulusnekrosen in den Nieren

[illegible] Zusammenhang. Die Todesfälle in der 2. Gruppe dürften wohl mit den [illegible] Nekrosen in der Umgebung der Injektionsstelle zusammenhängen. Bei [illegible] fanden sich Leber-, Nieren- und Herzveränderungen. Bei der 3. Gruppe [illegible] anzunehmen, daß die Tiere infolge der Nierenveränderungen und der ausgedehnten lokalen Nekrosen in der Umgebung der Injektionsstellen verstorben sind. Myokardnekrosen wurden bei der mikroskopischen Untersuchung [illegible] nicht, während Leberveränderungen bedeutend schwächer ausgeprägt oder überhaupt fehlten.

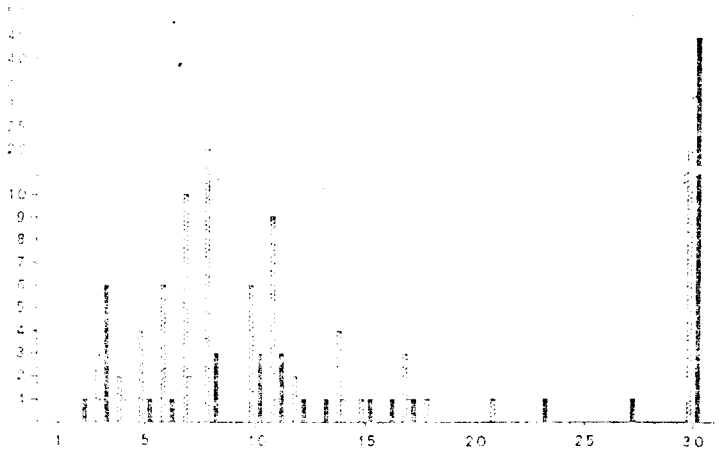

Abb. 1. Überlebende Tiere [illegible] Gruppen gerechnet vom 1. Tag nach Beginn der Behandlung mit Mangansulfat [illegible] Zinksulfat [illegible] Mangansulfat und Zinksulfat [illegible] Überlebende Tiere wurden am 30. Tag getötet.

Wie aus Abb. 1 hervorgeht, ist die Letalität nach mehrfachen Mangansulfatinjektionen am 6.—7. Tage am größten, was mit den Beobachtungen über die Schwere der Leberveränderungen übereinstimmt.

Am meisten auffallend war, daß die Lebernekrosen in der 3. Gruppe bedeutend schwächer ausgeprägt waren als in der 1. Gruppe. Um den Effekt des Zinks gegenüber den Mangan-induzierten Leberveränderungen näher zu studieren, wurden die morphologischen Veränderungen der Leber nach Injektion von Mangansulfat bzw. Mangansulfat und Zinksulfat am 6. und 7. Tage nach Versuchsbeginn untersucht.

Vergleichende Untersuchungen über die Leberveränderungen am 6. und 7. Tage nach Versuchsbeginn

30 Tiere erhielten täglich 4,5 mg Mangansulfat und 45 Tiere 4,5 mg Mangansulfat und 4,5 [illegible] Zinksulfat pro 100 g Körpergewicht. Tiere, die in einem schlechten Allgemeinzustand waren, wurden [illegible] am 6. Tage getötet, die restlichen am 7. Tage.

Die Verfettungen der Leberparenchymzellen und Lebernekrosen [illegible] Keine Veränderungen [illegible]

Nekrose auch in der Intermediärzone der Acini und 3: Diffuse Verfettung und Nekrose der gesamten Acini. Die Veränderungen wurden von 2 verschiedenen

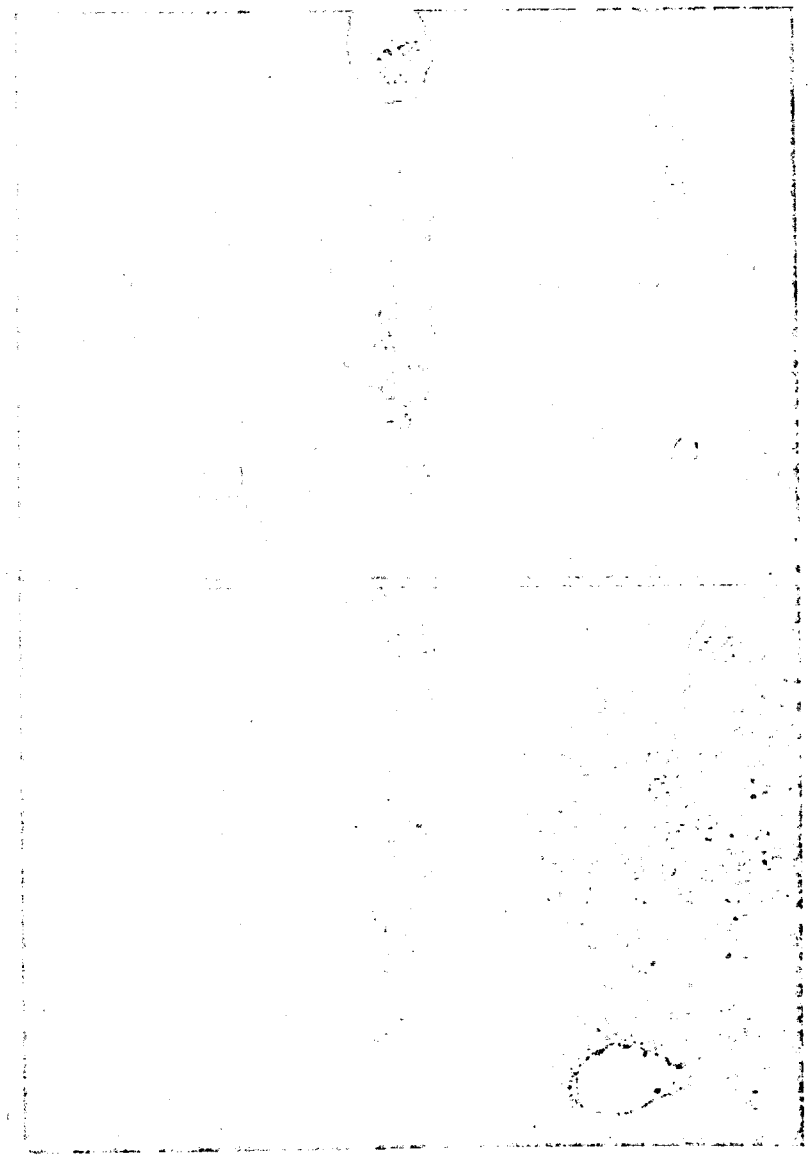

Abb. 2a, b, c Mangansulfat-induzierte [illegible] am 6. bzw. 7. Tage nach Beginn der Behandlung. b Leber eines Gebärmutters nach 7tägiger Applikation von Mangan- und Zinksulfat (H.-E.)

Untersuchern beurteilt. Die Mittelwerte der unabhängigen Beurteilungen ergeben sich aus der Tabelle. Die Unterschiede zwischen den Leberveränderungen waren statistisch signifikant ($p < 0{,}01$).

Tabelle. Der Zinkschutz der Mangansulfat- und Kohlenstofftetrachlorid induzierten Leberverfettungen. Mittelwerte der Verfettungen bei [illegible] Versuchsanordnung (s. Text)

	$MnSO_4$	$MnSO_4$ und $ZnSO_4$	CCl_4	CCl_4 und $ZnCl_2$
Anzahl der Versuchstiere	20	15	23	16
Verfettung	1,8	0,2	2,8	1,0
Nekrose	2,1	0,4		

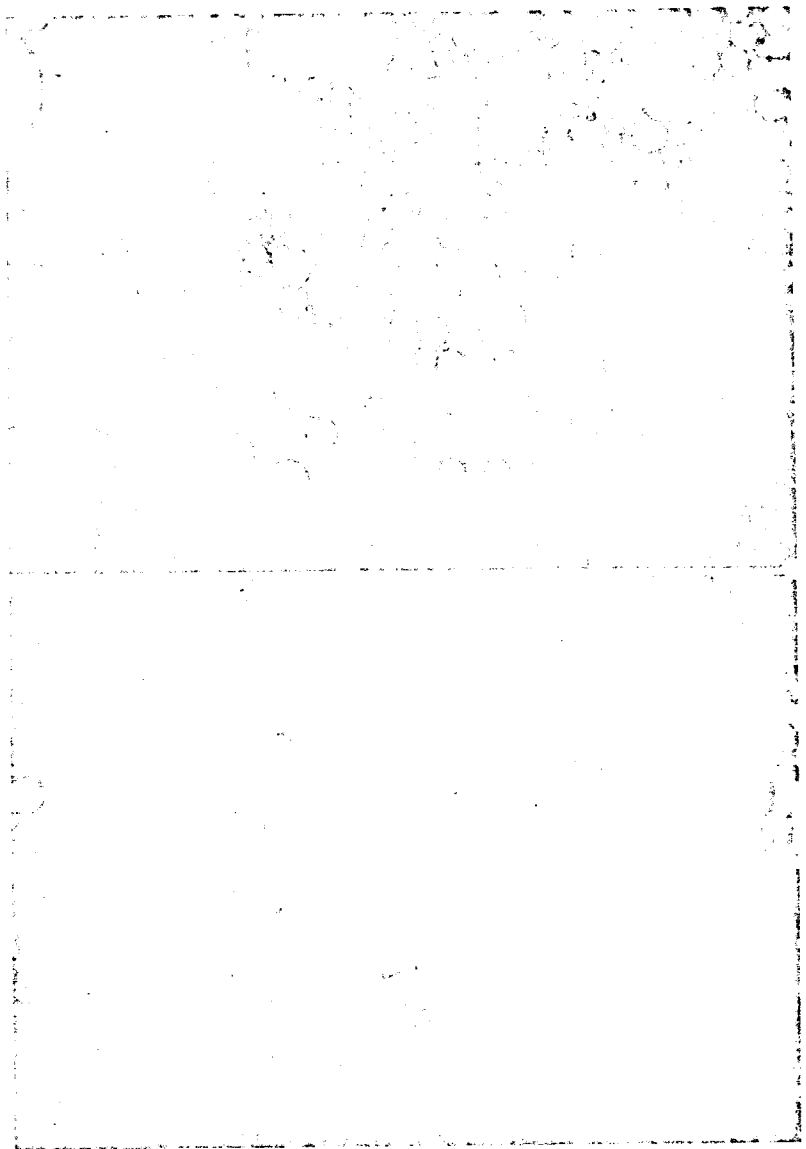

Abb. [illegible] Leber einer Ratte [illegible] Kohlenstofftetrachlorid und Zinkchlorid. Mangelhafte Verfettung (H.E.)

Kohlenstofftetrachlorid

Wie orientierende Untersuchungen ergeben haben, ruft in Übereinstimmung mit den Angaben von Wurzel und Mayer-Ashburn, Endicott, Davey und Lillie; Ungar die tägliche Zufuhr von 0,5 ml einer 25%igen Lösung von Kohlenstofftetrachlorid in Olivenöl per 100 g Körpergewicht bei Ratten nach 10 Tagen ausgeprägte, vom Zentrum der Azini ausgehende Verfettungen und Nekrosen der Leberparenchymzellen hervor.

[illegible] weibliche Ratten (Sprague-Dawley-Stamm, Astra-Lantmanfarm, Stockholm) wurden in 3 Gruppen aufgeteilt. Die Tiere wurden [illegible] wie die Goldhamster in der voranstehenden Versuchsreihe [illegible] einer Standardkost (Astra-spezieller Rattenpelletz) und Wasser ad libitum ernährt. Der [illegible]gruppe (23 Ratten) wurde [illegible] 0,5 ml der 25%igen Kohlenstofftetrachloridlösung in [illegible] (Olivenschenkel), [illegible] (16 Ratten) die gleiche Dosis Kohlenstofftetrachlorid in Mandelöl i. m. (Oberschenkel) und 4,5 mg Zinkchlorid subcutan per 100 g Körpergewicht täglich [illegible] 2 Tage vor [illegible] injiziert. Gruppe 3 bekam nur Zinkchlorid [illegible]. Die Ratten wurden [illegible] Tiere nach der letzten Injektion getötet.

Bei der Sektion ließen sich makroskopisch an den Lebern der Tiere beider ersten Gruppen keine sicheren Unterschiede feststellen. Histologisch dagegen war es insbesondere bei den nicht mit Zink behandelten Tieren die charakteristischen Veränderungen nach Kohlenstofftetrachloridinjektionen zu beobachten. Der histologisch festzustellende Grad der Parenchymzellverfettung und der Nekrosen wurde folgendermaßen beurteilt: 1. Nur acinozentrale Schäden, 2. Schädigung auch in der Intermediärzone und 3. Diffuse Schäden der gesamten Acini. Wie sich aus der Tabelle ergibt, resultierte ein klarer Unterschied zwischen beiden Gruppen. Die Verfettung und damit parallelgehende Nekrosetendenz war in der Gruppe, die gleichzeitig Zink erhalten hatte, bedeutend geringer. Der Unterschied war statistisch signifikant ($p < 0{,}01$).

Ratten, die nur Zinkchlorid bekommen hatten, zeigten keine Leberschäden.

Diskussion

Mangansulfat ruft nach mehrtägiger subcutaner Zufuhr beim syrischen Goldhamster regelmäßig eine schwere Leberschädigung in Form einer von der Acinusperipherie ausgehenden, von Leukocyteninfiltraten begleiteten Parenchymzellnekrose hervor, während Kohlenstofftetrachlorid eine von zahlreichen Untersuchungen her bekannte, vom Zentrum der Leberläppchen ausgehende Verfettung und einen Untergang der Leberparenchymzellen zur Folge hat. Dem histologischen Bild nach zu urteilen, liegt zwischen der Wirkungsweise dieser beiden hepatotoxischen Substanzen ein grundlegender Unterschied bezüglich des Erfolgsortes in der Leber vor.

Die subcutane Applikation von wasserlöslichen Zinksalzen allein hat an der Leber keine morphologischen nachweisbaren Veränderungen gezeigt.

Es hat die Applikation von wasserlöslichen Zinksalzen gleichzeitig mit Mangansulfat oder Kohlenstofftetrachlorid einen deutlichen Schutzeffekt an der Leber, der sich aus dem histologischen Bild ableiten läßt. Der Wirkungsmechanismus ist unbekannt, doch ist daran zu denken, daß die von Zink aktivierten Enzymsysteme hierfür bedeutungsvoll sind. Bei der Manganvergiftung

wirkt das Zink allerdings auch gegenüber der Herzmuskelnekrosen, und es ist nicht ausgeschlossen, daß bei der Zinkwirkung gegenüber dem Mangan in ähnlicher Weise eine "competitive inhibition" verliegt, wie dies für den Schutzeffekt des Zinks gegenüber den durch Cadmium induzierten Hodennekrosen angenommen wird (Pařízek).

In unserer Versuchsreihe ist nicht geprüft worden, ob das Zink die Regeneration bereits pathologisch veränderter Lebern begünstigt. Gewisse Beobachtungen von Vallee, Wacker, Barthorozeny und Hoch scheinen dafür zu sprechen. Diese Verfasser konnten bei einigen Patienten mit Leberzirrhose nach peroraler Zufuhr physiologischer Zinkmengen eine Verbesserung der Leberfunktion beobachten, was allerdings von Hammond, Black und Collison nicht bestätigt werden konnte. Prabhu hatte jedoch bereits 1940 über eine Verbesserung des klinischen Bildes bei Patienten mit sich infantiler Leberzirrhose berichten können.

Zusammenfassung

Mehrfache Applikation von Mangansulfat ruft beim syrischen Goldhamster von der Acinusperipherie ausgehende Nekrosen der Leber hervor, während Kohlenstofftetrachlorid bei Ratten acinozentrale Verfettungen und Nekrosen der Leber erzeugt. Die parenterale Gabe von Zinksalzen gleichzeitig mit Mangansulfat oder Kohlenstofftetrachlorid schützt deutlich vor der Entstehung der genannten Leberveränderungen.

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PHYSIOLOGICAL REVIEWS

VOL. 15 APRIL, 1935 No. 2

MANGANESE: ITS DISTRIBUTION, PHARMACOLOGY AND HEALTH HAZARDS

Physiol. Rev. 15: 175, (1935)

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DISTRIBUTION OF MANGANESE. Manganese occurs as the oxide in the following minerals; Pyrolusite (MnO_2), Braunite ($Mn_2O_3.3Mn_2O_3 \cdot MnSiO_3$), Manganite ($MnOOH$), Hausmannite ($Mn_3O_4$), Franklinite ($FeZnMn(FeMn)_2O_4$); as sulfide in Manganeseblende (MnS) and Hauserite (MnS_2); as carbonate in Manganesespat ($MnCO_3$); as silicate in Tephroite (Mn_2SiO_4), Knebelite ($(FeMn)_2SiO_4$), Heterocline, Helvine, and Rhodonite ($MnSiO_3$ or $MnOSiO_2$). It is characterized by the great number of different oxygen compounds which it may form, of which the following have been described: manganous oxide (MnO), manganous oxide-oxide (Mn_3O_4), manganese sesquioxide (Mn_2O_3), manganese peroxide (MnO_2), manganese trioxide (MnO_3), manganese heptoxide (Mn_2O_7) and manganese tetroxide (MnO_4). It forms two types of salts, of which the manganous salts are derivatives of the bivalent and the manganic salts are derivatives of the trivalent manganese. Manganic acid is a derivative of mangan trioxide and permanganic acid of mangan heptoxide.

Manganese was discovered by Scheele (1774) in pyrolusite and isolated in pure form by Gahn (1807). Scheele found it also in the soil; Leclerc (1872) confirmed this for a great number of soils from different localities.

OCCURRENCE OF MANGANESE IN PLANTS. Manganese appears to be a constant constituent of plants. Von Schroeder (1878) found it in the ashes of pine needles and of pine bark. Its presence in a great variety of plants was established by Maumené (1884) who also found it present in a number of red and white wines to the extent of 0.0001 to 0.0020 gram per liter. Headden (1915) found it in wheat regardless of the type of soil and climate. He found that the wheat kernels contained about

equal quantities of manganese and iron. Bertrand and Rosenblatt (1921) extended the studies of Maumené and found it also in those plants in which the latter was unable to discover it. Pichard (1898) had assumed that manganese is concentrated in those parts of the plant which are in active vegetation and also in very young shoots, and Jardin and Astruc (1913) found that the chlorophyll-containing parts are richer in manganese than the subterranean parts. Bertrand and Rosenblatt (1922) determined the manganese content of different structures of Nicotiana rustica L. and of Lilium lancifolium rubrum and found more manganese in those organs in which chemical reactions (transformations) take place. The reproductive organs, calyx and stamen contained 42.7 and 40.9 mgm. per 100 grams of the ash. They found the wood which has only very little reproductive activity remarkably poor in manganese (4.8 to 6.4 mgm. per 100 grams of ash). The seeds contained 127.7 mgm. per 100 grams of ash. Wester (1921) had made an extensive study of the manganese content of different seeds and had found considerable variations. McHargue (1924) pointed out that with cereals the greater part of the manganese is lost in the polishing and milling. From the work of Czapek (1925) (quoted from Richards) it appears that during the process of ripening the absolute values of manganese in the seeds shows an increase, he quotes that Arendt (1860) reported similar results for Avena sativa and Schjerning (1906) observed the same phenomenon for ripening barley. Bertrand and Rosenblatt also found that young leaves usually contain more manganese than older ones. Bishop (1928) reported similarly that buds, petioles, leaves and twigs contained most, roots less, and small branches, large branches and trunk timber least. In flowers the manganese content of the different organs is decreased in the following order: ovaries, styles, and stigmata, anthers, perianth and peduncles. It appears, therefore, that the reproductive parts have the highest manganese content and the woody sections contain very little. Richards (1930) showed that there is a wide difference in the manganese content of the reproductive organs of different plants even of the same species and assumed that this depends largely upon the abundance of the water supply and its manganese content. This is supported by the statement of Kobert (1883) that true water plants, such as Zostera marina and Trapa natans have a very high percentage of manganese. Bishop assumed that there must be some relation between the manganese content and the formation of chlorophyll and hence with the assimilation of carbon in plants. Richards (1930) determined the manganese content of a great variety of foodstuffs.

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It appears, therefore, that manganese is a constant constituent of plants and that it may have some important functions in their metabolism.

Scheele (1774) had noticed that the manganese of the soil is assimilated by plants. Aso (1902) (quoted from Bishop) found that manganese in small concentrations has a favorable effect on the growth, whereas high concentrations develop toxic effects. Voelker (1903) experimented with manganese salts as fertilizers in field experiments. He found that the chloride and nitrate produce good color in plants and that the iodide retarded distinctly the germination and the growth. Manganese chloride, phosphate and sulfate and also red manganoxide (Mn_3O_4) increased the yield. Bartmann (1910) found manganese dioxide less effective than manganese sulfate in promoting growth of plants. Bertrand (1905) also found that fertilization with manganese increased the yields of the crops and that the individual grains were heavier than those of the control fields. Similar results were also reported by Katayama (1906) and Brenchley (1910), but they noted that larger quantities cause bleaching of the plants. The use of manganese as a fertilizer has been reviewed by Bocher (1922). Toxic effects were observed by Loew and Sawa (1903), Kelley (1909), Guthrie and Cohen (1910), Willcox and Kelley (1912), Skinner and Sullivan (1914) and Deatrick (1916) (quoted from Bishop) and before these Sachs (1865), later Birner and Lucanus (1866) and Wagner (1871) had stated that an excess of manganous and manganic phosphate had an injurious effect on plants.

McHargue (1923) found leguminous plants much more sensitive to the lack of manganese than non-legumes, although only very small quantities of manganese are required for the normal growth of plants. Seeds, which, as demonstrated by Bertrand and others, are especially rich in manganese, do not contain enough to secure normal growth to maturity of the plant; some seeds, however, may have a sufficient amount to maintain a normal development during the first five or six weeks. Several mechanisms for the action of manganese on the growth of plants have been suggested. Hall (1907) postulated an effect of manganese on the soil bases rather than a direct action on the plants. Bernardini (1910) assumed that manganese had some catalytic action on the soil increasing the oxygen absorption. Brown and Miges (1916) claimed that the action of manganese salts consists mainly in their effect on ammonification and nitrification of the soil. The assumption of Johnson (1924) that the toxic effect of manganese is due to a decrease of the assimilation of iron, could not be confirmed by Bishop (1928).

McHargue (1923) found that the toxicity of manganese for plants depends in part upon the acidity of the soil. He reported that the addition of manganese salts to acid soils causes a decrease of the crops; in the same quantity, however, added to an acid soil after its neutralization by means of calcium carbonate, it causes an increase of the yield. He assumed that the toxicity of acid soils may, at least in part, be due to the formation of soluble manganese salts. Bishop found that the addition of calcium carbonate to soils containing manganese salts had an antitoxic effect towards higher concentrations of manganese.

Some of the experiments mentioned indicate that manganese may be involved in the oxygen metabolism of the plant. Bertrand (1897) found that the activity of laccase was increased by manganese salts, the manganese acting presumably as a co-ferment. Aso (1902) observed that the intensity of the color reaction of oxidizing enzymes of plants fertilized with manganese exceeded that of the controls, and Loew and Sawa (1903) found that the juice of plants bleached by an excess of manganese in the soil showed a stronger oxidase and peroxidase reaction than normal plants.

It appears, therefore, that manganese increases the oxidase reaction in plants.

Effect of manganese on tissue activity in animals. Walbum and Moerch (1923) found that the intravenous injection of manganese in goats immunized against diphtheria toxin stimulates the antitoxin formation. In horses they produced an increase of the antitoxin titer even if it was already on the decline. They found oral administration ineffective in this respect and assumed direct stimulation of the antitoxin producing cell mechanism. Later Walbum and Schmidt (1925) found that the injected material disappears rapidly from the circulation and that it is mainly stored in the liver, and they assume that the faculty of this organ to retain manganese is somehow related to the effect on the increased antitoxin formation. Preti (1909) studied the effect of manganese chloride, sulfate, acetate and lactate on autolysis of the liver. When added in small quantities to liver pulp they cause an increase of the noncoaguable nitrogen during autolysis, which progresses with the concentration of the manganese salts. The acetate, however, causes such an increase only within a certain range, larger doses having an inhibiting effect. Bradley and Morse (1915) found with manganese chloride that the stimulation occurs only until a concentration of 1/10 mol is reached, and that further increase of the concentration has an inhibiting effect. They assumed that manganese alters the proteins

in such a way that they become more digestible to the protease. Sato (1929) found that very small doses of manganese stimulate the respiration of tissues, whereas larger doses have a depressant effect.

It appears, therefore, that manganese within certain concentrations stimulates the antitoxin formation, the autolysis of the liver and the respiration of animal tissues.

OCCURRENCE OF MANGANESE IN ANIMAL TISSUES. Marchessaux (1844) and Millon (1847) discovered manganese in the blood. Burin de Buisson (1852) (quoted from Kobert) claimed that manganese is a regular constituent of the blood and suggested that in chlorosis the manganese content of the red blood cells must be increased. But Garrod showed that such an increase is impossible and also Buchheim (1878) was unable to increase the manganese content of erythrocytes of the dog. Glénard (1854) found manganese in the blood of only one person out of forty, and Campani (1872) showed it in the serum and the cells of ox blood. Griffiths (1892) found that the blood of Pinna squamosa (a mollusc) contains as much as 0.19 gram per cent of the ash. Bertrand and Medigreceanu (1912) determined the manganese content of the blood of man, horse, cattle, pig, rabbit, seal, chicken and canary and found from 0.00 to 0.02 mgm. per 1000 cc. of blood, the blood of sheep contained slightly more, namely, 0.06 mgm. per liter. The plasma contained about twice as much as the cells. Reiman and Minot (1920) determined the manganese in the blood of patients suffering from various diseases as 0.004 to 0.025 mgm. per 100 gram, averaging 0.012 mgm. The individual level in the blood was quite constant with repeated examinations. The difference between these figures and those found by Bertrand may be partly due to the improved method of determination used by Reiman and Minot, and partly to the different manganese content of the drinking waters in eastern Massachusetts (0.001 to 0.0015 part per 100,000[3] parts of water). In workers exposed to dust of manganese ores they found 0.013 mgm. per 100 grams of blood which is practically the same as in normal persons.

Manganese appears to be a normal constituent of practically all tissues. Kobert stated that Wurzer (1833) found manganese in human teeth, Weidenbusch and Lehmann (1830) in bile, and Rey, Wurzer and Cottereau (1849) found it in gallstones. Pollacci (1870) detected it in human milk. McHargue (1924) found 5 times as much manganese in colostrum as in milk which was confirmed by Richards (1930) although in these determinations the difference was less marked. Herford (1871) found it in normal urine and Schiaparelli and Peroni (1881) in horse

urine. Bradley (1907) determined the amount of manganese in two clams, Unio and Anodonta, from Madison Lake and found that about 1 per cent of the dried tissue was manganese; it was also present in the eggs of these molluscs to the extent of 0.6 to 0.8 per cent. Later (1910) he found it always present in Modiola modiolus (of the Massachusetts region) even when the animals had been starved for 3 weeks. But in no

TABLE 1

Distribution of manganese in different tissues

(Milligrams per 100 grams wet tissue)

SPECIES	LUNG	LIVER	KIDNEY	AUTHOR
Rabbit		0.237–0.351	0.087–0.101	Bertrand and Medigreceanu, 1913
Rabbit (8)	0.036	0.178	0.147	Lund, Shaw and Drinker, 1921
Cat (15)	0.031	0.341	0.136	Lund, Shaw and Drinker, 1921
Dog	0.010	0.306	0.106	Bertrand and Medigreceanu, 1913
Dog (8)	0.023	0.238	0.087	Lund, Shaw and Drinker, 1921
Guinea pig (2)	0.00		0.000	Lund, Shaw and Drinker, 1921
Rat (2)	0.00		0.110	Lund, Shaw and Drinker, 1921
Macacus rhesus	0.055	0.120	0.155	Mella, 1923
Ox	0.010	0.298	0.084	Bertrand and Medigreceanu, 1913
Calf	0.011	0.290	0.063	Bertrand and Medigreceanu, 1913
Pig	0.023	0.265–0.317	0.109–0.128	Bertrand and Medigreceanu, 1913
Horse	0.006	0.280	0.077	Bertrand and Medigreceanu, 1913
Chicken	0.010	0.416	0.217	Bertrand and Medigreceanu, 1913
Chicken (2)	0.00		0.124	Lund, Shaw and Drinker, 1921
Canary	0.010	0.380	0.238	Bertrand and Medigreceanu, 1913
Turtle (2)	0.00			Lund, Shaw and Drinker, 1921
Man	0.20	0.170	0.061	Reiman and Minot, 1920
Maximal variation	0.00–0.055	0.120–0.351	0.061–0.155	
Median mammals	0.016	0.273	0.104	
Median birds	0.010	0.398	0.217	

instance were the quantities as large as observed with Uniodae. All tissues gave a positive manganese reaction but it was most marked in the nephridial organs. In Pecten the results were less uniform, in some specimens the element was present in rather large quantities, in others only in traces, the content presumably varying with the surroundings. In Mytilus edulis manganese was present in all specimens tested, but only in traces. Bertrand and Medigreceanu (1913) who give the older

literature on the distribution of manganese in the tissue found that there is no considerable variation of the manganese content in the same species. The distribution is within the same range with most mammals, but liver and kidney of birds appear to show distinctly higher values (table 1). In eggs only the yolk appears to contain manganese and the eggwhite was found to be free of this element. Already Pollacci (1870) had detected manganese in chicken eggs and later McHargue (1924); and Richards (1930) found that their manganese content increases during maturation in the ovaries. Reiman and Minot (1920) determined the distribution of manganese in different tissues of man, Lund, Shaw and Drinker (1921) in a number of species and Mella (1923) in the organs of Macacus rhesus. The data for lung, liver and kidney are collected in table 1.

This table indicates that the liver contains normally by far the largest amount of manganese (0.273 mgm. per 100 gm. wet tissue), next ranks the kidney (0.104 mgm.); whereas the spleen contains a median of 0.054 mgm. (Reiman and Minot, 1920; Lund, Shaw and Drinker, 1921; Mella, 1923). From other glandular structures the adrenals of man contained 0.013 mgm., the pancreas 0.076 mgm. and the lymph nodes 0.021 mgm. (Reiman and Minot, 1920). The values for the brain as determined by Lund, Shaw and Drinker (1921) for rabbits, cats and dogs, by Mella (1923) for Macacus rhesus and by Reiman and Minot for man give a median of 0.035 mgm.; according to Lund, Shaw and Drinker the spinal cord of cats contained 0.059 mgm., being, therefore, of the same order as that of the brain. The manganese content of the intestinal tract shows considerable variations in different sections. Lund, Shaw and Drinker (1921) found as median value for the stomach of rabbits, cats and dogs and Reiman and Minot (1920) for that of man 0.036 mgm., for the small intestine it is 0.037 mgm. and for the colon it is 0.083 mgm. From other structures the bone contained 0.101 mgm. (Lund, Shaw and Drinker, 1921), the muscle tissue 0.024 mgm. (Reiman and Minot, 1920; Lund, Shaw and Drinker, 1921), the heart 0.046 mgm. and the lungs 0.016 mgm. Bertrand and Medigreceanu (1913) determined the manganese content of eggs of chicken and canaries as 0.063 and 0.054 mgm. *It appears, therefore, that like other metals manganese is mainly stored in the liver, that the kidney ranks next and that it is partly excreted into the colon.*

Interesting are also the high values for the liver and kidneys of birds which are nearly twice as great as those found for mammals. This variation of the manganese content of different species becomes still

more prominent in table 2 which gives some data taken from a paper of Bertrand and Medigreceanu (1913).

This table shows to which extent the surroundings may affect the manganese content of the animals as indicated by the great variations with some of the fishes, crustacea and molluscs.

THE ABSORPTION OF MANGANESE FROM THE INTESTINAL TRACT. The question whether and to what extent manganese is absorbed from the

TABLE 2

Manganese content of different animal species

(Total amount of managnese in milligrams per 100 grams fresh tissue)

(Bertrand and Medigreceanu, 1913)

A. Mammals:		F. Crustacea:	
White rat (intestine emptied)	0.030	Crawfish	9.144–11.11
White mouse (intestine emptied)	0.036	Shrimp	0.147
White mouse (intestine filled)	0.190	Woodlouse	0.700
B. Birds:		Barnacle	0.180
Turtle dove (intestine filled)	0.207	G. Molluscs:	
C. Amphibia:		Snail (edible)	
Horned viper (intestine empty)	0.180	without shell	2.843
Red frog (intestine empty)	0.390	shell alone	4.236
Green frog (intestine filled)	0.627	Lymnea	0.477–4.145
Salamander	1.017		
D. Fishes:			
Herring	0.121–0.900	Oyster	0.100–0.649
Sardine	0.116	Oyster shell	0.595–0.818
Plaice (flounder)	0.238		
Mackerel	0.056	H. Annelides:	
Whiting	0.094	Leech	<0.040
Shark	0.611	I. Echinoderms:	
E. Insects:		Starfish	0.094
Cabbage butterfly	0.248	Sea cucumber	0.040
Gyrinus	0.702		
Locust	0.381		

intestinal tract is important in regard to the hazards to which workers handling manganese ores are exposed. Orfila (1826), Gmelin (1837) and Huenefeld and Wibmer (1831) (quoted from Harnack) had found that introduction of simple manganese compounds into the stomach causes toxic symptoms only when very large doses are given which produce corrosion of the gastric walls and of the intestine. Kobert (1883) fed manganese citrate to rabbits and observed very little absorption from the intestinal tract. A better absorption was noted with larger doses

which caused gastritis. Cahn (1884) also stated that only very little manganese is absorbed from the intestinal tract. Reiman and Minot (1921) determined the solubility of franklinite, rhodonite and manganese dioxide in gastric juice, they found that all are soluble to a certain extent depending upon the acidity and duration of the contact and that this was sufficient to secure absorption of manganese into the bloodstream. Two persons drank a suspension of 8 grams of franklinite in water alone and followed by the intake of 50 grams of cooked meat. Blood samples were taken at intervals of 1, 3, 6, and 24 hours after the administration. There was no difference in both series; the manganese level in the blood increased rapidly during the first hours and then rapidly returned to normal as indicated by the following data:

Time after administration	0	1	3	6	24 hours
Manganese in mgm. per 100 gm.	0.0105	0.014	0.012	0.0105	0.0105

Similar results were reported by Drinker and Shaw (1921) after intravenous injections of manganese dioxide suspensions into cats.

The reason for this rapid disappearance from the circulation with oral administration is that the solubility in gastric juice is rather slow and that on the other hand the rate of elimination is too rapid to allow an accumulation in the blood. Von Oettingen and Sollmann (1927) observed no toxic effects in birds (pigeons and roosters) after administration of metallic manganese as indicated by the red cell count and the weight curve.

It appears, therefore, that the absorption of manganese from the gastro-intestinal tract is very slow and incomplete under normal conditions and that there is only a temporary increase of the manganese level in the blood.

THE EXCRETION OF MANGANESE. Wichert (1860) (quoted from Harnack) detected manganese in the bile of cats after its oral administration. After the oral administration of manganese citrate to rabbits Kobert (1883) found most of it excreted with the feces and only traces in the urine. Only when large doses were given which developed gastritis was the urinary excretion more marked. Also Cahn (1884) stated that manganese is mostly excreted with the feces. Harnack and Schreiber (1901) with the oral administration of manganese peptonate and manganese phosphate to rabbits and Bargero (1906) who administered manganese orally to dogs confirmed the results of Kobert. After the administration of manganese peptonate to patients with biliary fistula,

manganese could be detected in the bile. According to Bargero (1906) the biliary excretion increases with the manganese content of the liver, but Benedetti (1906) who injected manganese citrate subcutaneously into dogs stated that larger doses tend to decrease the biliary excretion. Reiman and Minot (1921) reported on one person who drank 25 grams of franklinite suspended in water in the course of three days. During this time and several days afterwards 24 hour samples of urine and of feces were collected, and 500 cc. of the urine and 2 grams of the feces were analysed for manganese and the total amount of the excreted material was calculated. They found that it is mainly excreted through the intestine and in no instance could more than a trace be detected in the urine. Administration of franklinite to patients with biliary fistulas showed that the manganese content of the bile which is normally very low, ranging from 0.003 to 0.010 mgm. per 100 grams, was materially increased after the administration of the ore. After the administration of 5 grams in three doses to two patients the manganese content of the bile went up to 0.111 mgm. and 0.120 mgm. per 100 grams on the following day, whereas another patient after the same dose showed only a moderate increase. All three persons showed a slow return to the normal level. Handowsky, Schulz and Staemmler (1925) found in rabbits with subcutaneous injection of manganese chloride, manganese citrate and a manganese pyrocatechol derivative that about 50 per cent were excreted with the feces but only traces with the urine. They found that bile, ascitic fluid and pus contained comparatively large quantities. Orent and McCollum (1931) stated that manganese passes through the placenta.

From these data it appears that after oral and subcutaneous administration manganese is excreted mainly by the feces, partly in the bile, and presumably also through the colon as indicated by the large manganese content of this organ. The urinary excretion is, however, very moderate as may be expected from the rapid disappearance of the metal from the circulation.

DISTRIBUTION OF MANGANESE AFTER ITS ADMINISTRATION. Kobert (1883) stated that in rabbits after the subcutaneous injection of manganese citrate all organs, especially the liver, kidney and the intestine contained manganese. After intravenous injection of the same compound into rabbits Cahn (1884) found most in the intestinal walls and large quantities in the liver and kidneys. Harnack and Schreiber (1901) fed manganese peptonate to rabbits in doses of 2 grams daily for from three to four weeks and detected large quantities in the spleen, liver, stomach, and colon, but less in the kidneys. Benedetti (1906)

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injected manganese citrate subcutaneously into dogs and found the metal in the liver and kidneys as early as twelve hours after the injection. Similar experiments were reported by Bargero (1906) with oral administration of manganese to dogs. Handowsky, Schulz and Staemmler (1925) stated that after the subcutaneous injection of manganese chloride, manganese citrate and of a manganese pyrocatechol derivative large quantities were deposited in the bones, spleen and brain; the content of the liver being variable and the lungs containing comparatively small quantities. Reiman and Minot (1921) determined the manganese content of the organs of dogs which had been fed franklinite, pyrolusite and rhodonite over a long period. Their results and those reported by Mella (1923) with intraperitoneal injections of manganese chloride into monkeys and of Handowsky and his collaborators with subcutaneous injections into rabbits are given in table 3.

Reiman and Minot's experiments show that with oral administration the manganese content of brain and spinal cord is hardly increased as compared with the normal, and that the concentration in the hemispheres, the spinal ganglia and the cord is of the same order. The same holds true for the lungs, spleen and muscle indicating that the manganese in circulation is not filtered off by these organs like colloids. The stomach, the small intestine and the colon show considerable increase which is least with the stomach, more marked with the small intestine and most pronounced with the colon, presumably on account of the excretion of the metal into the latter. *From these data it appears that liver and kidney are the main storage places of the metal.* The storage in the bones as stated by Handowsky and his co-workers is very likely, but a quantitative comparison is impossible, because no comparative data are available. The experiments of Richards (1930) who gave from 0.054 to 0.47 gram of manganese daily with the food to pigs showed, however, no evidence of a very marked accumulation in the liver, which may perhaps be explained by differences in the solubility of the manganese in the gastric juice.

The figures presented by Mella with intraperitoneal injection of manganous chloride show a tremendous increase of the manganese content of both liver and brain as compared with the normal values, but the increase of the liver values is also by far the greater.

The results obtained by Handowsky, Schulz and Staemmler with subcutaneous injection of the manganese pyrocatechol derivative and manganous chloride in rabbits indicate that with this method of administration the distribution of manganese in different organs is quite different

TABLE 3

Distribution of managenese in organs after its administration

(Milligrams per 100 gram wet tissue)

ORGANS	DOGS, ORAL ADMINISTRATION REIMAN AND MINOT, 1920		MACACUS RHESUS, INTRAPERITONEAL INJECTION MELLA, 1923		RABBITS SUBCUTANEOUS INJECTION HANDOWSKY, SCHULZ AND STAEMMLER, 1925		
	Ores*	Normal†	$MnCl_2$	Normal	$MnCl_2$	Mnpyrocatechol	Normal†
Brain (hemispheres)	*0.030* 0.023–0.042	0.044	0.122–0.173	0.032	1.154	1.514, 2.538	0.062
Spinal cord	*0.045* 0.024–0.088	0.044					
Lung	*0.044* 0.024–0.056	0.023			0.287, 1.335	0.124, 0.472	0.036
Liver	*0.600* 0.276–0.900	0.238	4.906–8.88	0.120	0.08, 2.642	0.045, 3.582	0.238
Spleen	*0.037* 0.025–0.112	0.022			3.333, 5.260	12.5, 0.736	0.054
Kidney	*0.151* 0.110–0.216	0.087			0.431, 3.720	0.963, 1.372	0.147
Stomach	*0.098* 0.059–0.211	0.043					
Duodenum	*0.131* 0.051–0.377						
Small intestine	*0.190* 0.062–0.420	0.028					
Colon	*0.308* 0.060–0.396	0.080					
Pancreas	*0.154* 0.089–0.284						
Heart	*0.054* 0.024–0.300				1.076, 0.50	0.362, 0.189	
Bone (marrow)	*0.135* 0.043–0.550	0.101			6.817		0.101
Muscle	*0.024* 0.016–0.042	0.020			0.647, 1.661	0.0, 0.0	0.022
Basal ganglia	*0.049* 0.042–0.138						
Omentum	*0.013* 0.006–0.130						
Bile	*1.60* 0.666–3.68						

* These animals received a total of from 228 to 768 grams of franklinite, rhodonite or manganese dioxide in the course of from 270 to 477 days.

† The normal values are from the paper of Lund, Shaw and Drinker (1921). The figures below the medians indicate the extremes.

from that with oral administration and very irregular especially in regard to the concentration in the liver. Special attention is drawn to the fact that only manganese injected as chloride could be recovered from the muscle, but not if injected as the pyrocatechol derivative.

With the intravenous administration of suspensions of manganese dioxide, as reported by Drinker and Shaw (1921), the distribution is somewhat different. Although the available data are less complete the increase of the concentration in the intestinal tract is considerably less marked than with oral administration. Unfortunately there are no data for the manganese content of lungs and spleen which might indicate a filtration in the hematopoietic system. These experiments do not reveal much retention of manganese in the central nervous system.

TABLE 4

Per cent of injected manganese recovered one hour after the intravenous injection of a suspension of manganese dioxide

(Drinker and Shaw, 1921)

	LIVER	LUNGS	SPLEEN	BLOOD	OTHER ORGANS
20 cats	38.3	47.0	4.7	0.0	10.0
3 rabbits	81.0–85.6	0.2 – 1.2	0.55–2.5	0.0–0.7	13.3–16.5
3 dogs	64.0–83.0	0.52–14.0	2.0 –4.0	0.9–3.9	9.1–16.1
2 guinea pigs	76.0–85.0	0.8 – 2.1	1.4 –1.7	0.3–1.2	12.5–19.0
2 rats	94.0–98.0	0.5 – 0.6	0.8 –1.9	0.0	2.0– 3.6
2 chickens	84.0	0.0 – 0.4	1.7	0.0	13.9–14.3
2 turtles	94.0–88.0	3.0 – 7.5		0.0	3.0– 4.5

Drinker and Shaw (1921) reported that after the intravenous injection of suspensions of manganese dioxide to cats the liver, lungs and spleen contained 90 per cent of the injected material and with other animal species the distribution appears not to be materially different as indicated in table 4.

As already mentioned, the intravenous injection of a suspension must necessarily give a different distribution and for this reason these experiments do not permit conclusions as to the distribution of manganese in the organism after the ingestion of manganese compounds. The same holds true for the results reported with subcutaneous and intraperitoneal administration.

PHARMACOLOGY OF MANGANESE. Gmelin (1824) (quoted from Harnack) found that the oral administration of manganous sulfate in large doses to dogs causes vomiting, gastritis and paralysis, and the intrave-

nous injections produce shocklike symptoms, hyperemia of the organs and also an increase of the excretion of bile. Kobert (1883) also found that in dogs the repeated subcutaneous injection of small doses of manganese citrate causes nausea, vomiting and icterus; most of his animals developed parenchymatous nephritis which passed into partial or complete cirrhosis. According to Kobert, Hoppe (1858) had claimed that manganese sulfate in certain concentrations stimulates the isolated heart and the isolated intestine, and that it causes vasoconstriction and later paralysis. Harnack (1875) reported that manganese sulfate causes a motor depression in frogs, reduction of the sensitivity and of reflex excitability. He found that the muscles were not affected, because they responded promptly to direct and indirect stimulation. With doses up to 10 mgm. the animals recovered from the depression within one to two hours, larger doses of from 10 to 25 mgm. were fatal. Kobert (1884) using manganese citrate confirmed these results and noted also depression of the respiration and of the heart, in which first the motor ganglia and later also the muscle were affected. With intravenous injections into dogs and rabbits he noted a fall of the blood pressure, with a subsequent rise during the convulsive stage. The convulsions were followed by complete depression and the animals died of paralysis of the respiration while the heart continued to beat for some time. This was confirmed by Cervinka (1929). Kobert also noted no effect on the striated muscle. There seems to be a characteristic difference between frogs and mammals inasmuch as in the latter the heart survives the respiratory depression. This was also demonstrated by Cahn (1884) who found that in rabbits death may be postponed with artificial respiration. On the other hand Cervinka (1929) claimed there is a fundamental difference between rabbits and dogs as with intravenous administration the former first show paralysis of the vasomotor mechanism, which caused the fall of the blood pressure and slowing of the pulse and that in this case the asphyxia was secondary. Whereas with dogs the fall of blood pressure is due to cardiac depression. Sato (1929) claimed a difference in the toxicity of bivalent and trivalent manganese. In rabbits the intravenous injection of 0.3 mgm. per kilo of manganous salts was already distinctly toxic, whereas the corresponding manganic compound caused no toxic symptoms in doses as high as 7 mgm. per kilo and even 15 to 20 mgm. were not fatal. A similar difference of the toxicity was also noted in regard to the fall of the blood pressure and it appears that the bivalent manganese is about ten times as toxic as the trivalent. Sato also stated that small doses of manganese stimulate the gas exchange, whereas large doses cause a depression presumably on

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account of the fall of blood pressure and the reduced tissue respiration. Handowsky, Schulz and Staemmler (1925) pointed out a fundamental difference between the acute and the chronic manganese poisoning. The acute poisoning is only caused when suddenly a high concentration of manganese is produced in the blood; it is characterized by an ascending paralysis of the central nervous system. Such symptoms were also observed in rabbits by Findlay (1924) with the subcutaneous injection of from 10 to 16 mgm. of manganous chloride. With lower concentrations in the blood, nervous symptoms are not seen, but the liver shows degeneration. The velocity of these changes depends on the amount of metal absorbed within a certain period. With chronic poisoning the Kupffer cells of the liver are first affected. Handowsky observed his animals only for a few weeks, Mella (1923) injected intraperitoneally small doses of manganese chloride into monkeys (Macacus rhesus) over a long period (up to 18 months). These animals developed movements of the choreic and the choreoathetoid type, later they showed a rigidity of the muscles and disturbances of the motility. In the later stage they developed fine tremors of the hands and finally a peculiar contracture of the hands with the terminal phalages extended.

THE EFFECT OF MANGANESE ON THE BLOOD. As has been stated, Burin de Buisson as early as 1852 had claimed that manganese is an essential constituent of the red blood cells and that its administration is an effective treatment of anemias. Schwarz and Pagels (1923), feeding manganese dioxide and pyrolusite ore to cats, found that after four weeks the animals developed an increase of the hemoglobin and of the red blood cells, followed after further administration for another month by a decrease of both. They noted that the pure preparation was more effective than the ore, presumably on account of the greater solubility in the gastric juice. There was also a considerable variation in the sensitivity of different animals. Titus, Cave and Hughes (1928) working with young rabbits made anemic by an exclusive milk diet, found that daily doses of 0.5 mgm. manganese had a definite favorable effect when added to the milk which contained also 0.5 mgm. of iron. This was, however, not confirmed by Waddell, Steenbock and Hart (1929) and also by Mitchell and Miller (1931) who fed 0.1, 0.05, 0.025, and 0.01 mgm. manganese daily and observed only a negligible supplementary action to iron in the synthesis of hemoglobin. They stated, however, that it may have a slight stimulative effect on growth and food intake. Orent and McCollum (1931) also assume that manganese plays no rôle in the regeneration of blood. Myers and Beard, on the other hand, believed that manganese (lowest optimal dose 0.1 mgm. daily)

when added to the iron-milk diet speeds up the regeneration of blood in anemic young rats. They assumed that iron is the essential element in the process and that manganese has some catalytic action. Beard, Rafferty and Myers (1931) found that addition of manganese to the iron-milk diet is no more effective in preventing nutritional anemia, than is iron alone. The same holds true for development of hemoglobinemia (Myers, Beard and Barnes, 1931). Although addition of manganese to the iron-milk diet does not increase the reticulocytosis in young anemic rats, it causes a marked drop of the reticulocytes on the 5th or 6th day with a more rapid increase of cells and hemoglobin than observed with iron alone. This may suggest that in these doses manganese speeds up the maturation of red blood cells (Beard, Baker, and Myers, 1931).

EFFECT OF MANGANESE ON THE GROWTH. It has been demonstrated before that manganese is essential for the growth of plants. Levine and Sohm (1924) demonstrated that it has also a favorable effect on the growth of young rats. They found that manganous sulfate in concentrations of 1:2000, 1:4000 and 1:10000 given with the food had a marked catalytic action on the growth, and the manganese-fed animals were more active than the controls and the hair was sleeker, longer and thicker. They claimed that the offspring of such animals grew somewhat faster than the average young rat. McCarrison (1926) reported that feeding of manganous chloride to young rats to the extent of 0.037 mgm. daily had a markedly favorable effect on their growth. Larger doses, on the other hand, such as 0.889 mgm. had a progressively increasing retarding effect which became manifest after 37 days of feeding apparently due to accumulation. Later experiments of the same author (1929) seem to indicate that manganese in foodstuffs may act differently than manganese salts. McHargue (1926) assumed that manganese compounds as they occur in green leaves and seeds of mature plants and in the vital organs of animals have much more active biological potency than could be expected from feeding equal proportions of a crystalline salt of manganese in a synthetic diet. Bertrand and Nakamura (1928) confirmed the findings that manganese plays some important rôle in the animal metabolism. Also Kemmerer, Elvehjem and Hart (1931) found that addition of 0.01 mgm. of manganese as manganese chloride per mouse daily to the diet of whole cows' milk, supplemented with iron and copper, has a favorable effect upon growth of mice. On the other hand Orent and McCollum (1931) found that young rats fed with a manganese free diet grow to maturity in an apparently normal manner, but that the offspring of such animals are undersized and inferior in appearance and that they are not fed by

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their mothers. Male rats on a manganese free diet also developed normally but showed degeneration of the testes. Addition of from 0.005 to 0.05 per cent manganese in form of manganese chloride to the diet prevented this degeneration. They assumed that manganese is concerned with the production of a hormone by the anterior lobe of the hypophysis which is essential for the functioning of the testes and for the proper development and functioning of the mammary tissue.

AUTOPSY FINDINGS IN ANIMALS TREATED WITH MANGANESE. Kobert (1883) in some of his animals which had received subcutaneous injections of manganese citrate noted nephritis and cirrhosis of the kidneys. After intravenous injections into dogs Bargero (1906) observed fatty degeneration of the liver. Findlay (1924) injected subcutaneously into rabbits 10 to 16 mgm. of manganous chloride and the animals died within 22 to 48 hours in a semi-comatose state. The urine contained considerable quantities of albumen, red blood cells, casts and traces of bile pigments. The liver was slightly enlarged, friable and of a pale-yellow color often with reddish mottlings. It showed fatty degeneration especially at the periphery of the lobules; a few Kupffer cells were swollen and prominent, but there was no proliferation of the endothelial cells. All organs showed more or less marked congestion, the liver contained small hemorrhagic areas; the glomeruli were also congested and frequently blood was found in the intercapsular space and the kidneys gave evidence occasionally of fatty degeneration. In another series of experiments Findlay injected every second day from 3 to 5 mgm. of manganous chloride and these animals lived from five to nine weeks. At autopsy they showed a biliary type of cirrhosis of the liver, but no fatty degeneration. Similar changes were also observed in rats after the oral administration of 0.3 to 0.4 gram of manganese chloride daily. Handowsky, Schulz and Staemmler (1925), whose animals survived the subcutaneous injections of different manganese compounds for three to four weeks, noted fatty degeneration of the liver beginning in the Kupffer cells and extending to other cell structures; occasionally there were also degenerative changes in the kidneys and in the heart. Mitotic processes observed in the liver cells were interpreted as symptoms of regeneration. They also saw destruction of the erythrocytes, indicated by the formation of hemosiderin. The difference between these findings and those reported by Findlay may be explained by the more rapid course of the poisoning in Hadowsky's series. Mella (1923) who injected monkeys intraperitoneally with small doses of manganous chloride over a period of 18 months found acute hepatitis and areas of liver necrosis. In the nervous system he noted the following changes: The

cortex of the brain showed slight but evenly distributed chromolysis, with vacuolated nerve cells but no gliosis or myelin degeneration. The neurons of the cortex contained fat droplets. In the putamen and caudate nucleus the cells were shrunken and pyknotic, many having lost their nuclei. There were scattered areas of gliosis which occasionally were quite dense. Many of the fibers connecting the caudate nucleus with the putamen had lost their myelin sheath. The globus pallidus showed degenerative changes. In one animal a definite loss of the myelin sheath in the ansa lenticularis was noted. The cerebellum gave essentially a negative picture, the pons as a whole was normal and also the spinal cord. The peripheral nerves exhibited no pathologic changes. Gruenstein and Popowa (1929) fed daily doses of from 2 to 3 grams manganese dioxide to rabbits and after the death of these animals noted degenerative changes in liver, spleen, heart, kidneys and adrenals. All animals showed severe changes of the central nervous system, characterized by a diffuse degeneration of the nerve cells in the corpus striatum and of the small cells of the caudate nucleus and of the pentamen. They also saw degenerative changes of the endothelium of the plexus choroideus and inflammation of the pia. Matsumura (1933) studied the effect of subcutaneous injections of manganese chloride in rabbits and dogs. In rabbits the degenerative changes in the liver were more marked than in dogs. The former species showed hyperemia, hemorrhages, dilatation of the veins and regressive changes in the liver cells of different degree. In severe cases there were also sharply defined degenerative processes in the periphery of the acini which occasionally showed formation of connective tissue. This could also be observed in Glisson's sheath giving the picture of beginning cirrhosis of the liver. In dogs the degenerative changes in the liver were less marked and consisted mainly in stasis, small hemorrhages, and slight regressive changes of the liver cells. On the other hand he found that pathologic changes of the central nervous system were comparatively slight in the rabbit. They consisted of stasis and hemorrhages in the brain and shrinkage of the ganglionic cells in the superficial layers of the cortex of the hemispheres. Similar changes were found in the entire brain and were not especially marked in the interbrain and the corpus striatum. Neither rabbits nor dogs showed the extrapyramidal symptoms observed in man. The central nervous system of the dog exhibited, however, various changes of the ganglionic cells, mainly with edematous changes, weblike shrinkage and swelling, total chromatolysis and vacuole-formation in the ganglionic cells. There was swelling and proliferation of the glia cells and glia nodes were observed. The cells

of the vascular walls showed swelling and proliferation. The ganglionic changes were observed in the superficial layers of the hemispheres as in the rabbits and also in the thalamus and corpus striatum. Glia changes were found in the striatum, thalamus and in the floor of the fourth ventricle. Changes of the vascular walls were most frequent in the thalamus and the striatum.

Although Matsumura does not assume a specific relation between the pathological changes in the liver and the central nervous system, other experiments, especially those with oral administration appear to indicate that the degenerative changes in the liver are the earlier symptoms of manganese poisoning and that pathologic changes of the central nervous system occur at a later stage.

TABLE 5

*Reports on industrial manganese poisoning**

NUMBER	AUTHOR	YEAR	NUMBER OF CASES
1	Couper	1837	5
2	von Jaksch	1901	3
3	Embden	1901	4
4	Seiffer	1904	1
5	von Jaksch	1907	1
6	Casamajor	1909	9
7	Seelert	1913	1
8	Edsal, Wilbur and Drinker	1919	3
9	Davis and Huey	1922	2
10	Charles	1922	3
11	Gayle	1925	6
12	Charles	1927	7
13	Hilpert	1929	1
14	Meyer	1930	1
15	Hiplert	1930	13
16	Schwarz	1931/32	2
			62

* Since this paper went to press, 7 more cases of manganese poisoning have been reported. Baader (1932, 1934) published 1 case of a man engaged in unloading manganese ores, and 2 cases of workmen engaged in a dry cell plant. One case was reported by Ashizawa (1927), and 4 cases by Owen and Cowen (1934), so that there are altogether 70 cases of manganese poisoning on record.

INDUSTRIAL MANGANESE POISONING. The first report on this subject is that of Couper (1837) on five cases of manganese poisoning observed in workers handling powdered manganese dioxide. In three of these the cause of the disease was recognized very early and the patients

recovered after the discontinuation of their occupation. Altogether there are 70 cases of manganese poisoning reported in the literature which are collected in table 5.

Most of these workmen were exposed to the dust of pyrolysite or of manganese ores in mills or in discharging boats. The absorption takes place presumably through the gastro-intestinal tract although it is possible that some is inhaled through the lungs. All observers report a peculiar masklike expression of the face, involuntary laughing and less frequently crying (2, 4, 8, 11), the voice is low and the speech is blurred and scanned. Most patients show rather early a peculiar stiffness of the muscles which results in a spastic gait and in propulsion and retropulsion (jerking forward or backward while walking or stopping). Occasionally also a peculiar gait on the metatarsophalangeal joint is observed (Charles, 1927). Intension tremor especially of the hands is practically always present; micrography is frequently observed (3, 4, 8, 11, 15). The tendon reflexes are often increased (2, 3, 4, 5, 7, 8, 10, 12, 15). There are usually no sensory disturbances aside from paresthesias which have been observed occasionally in the beginning of the disease (3, 6, 11, 16). Psychic changes are not a rule but a certain mental dullness has been observed occasionally (2, 8, 14, 16). Gayle (1925) reported in two out of six cases a pronounced lack of sociability. Insomnia was only reported once (Casamajor, 1909). In a few cases salivation and lacrimation have been noted (4, 10, 12). Visual and acoustic disturbances are not frequent, the latter have only been reported by Casamajor (1909). The function of the intestinal tract and of the bladder is not affected. The urine contains manganese only in fresh cases and is usually without pathologic constituents; von Jaksch (1901) reported a temporary glucosuria in one case. The blood picture if reported is practically always normal, only Schwarz (1932) found in his case 6.28 million erythrocytes, 76 per cent hemoglobin and 40 per cent lymphocytes. The spinal fluid as far as studied never showed pathologic changes.

Although most of the patients show extrapyramidal symptoms the reports of von Jaksch (1907) and Hilpert (1930) indicate that they may develop signs of pyramidal lesions in the latter stage of the disease. The clinical symptoms observed in manganese poisoning have been compared with multiple sclerosis (Embden, 1901), and with epidemic encephalitis (Cohn, 1928). It may be distinguished from the latter by the absence of pathologic changes in the background of eyes (Meyer, 1930). Hilpert (1930) mentions some similarity with metencephalitis, paralysis agitans and chronic carbon monoxide poisoning. The simi-

larity to the latter is illustrated by the report of Schlockow (1879). Stoecker (1913) was the first to direct the attention to the similarity of manganese poisoning with progressive lenticular degeneration (Wilson's disease) which is associated with a peculiar degeneration of the liver. A relation between liver changes and nervous symptoms was also made by Charles (1927) who reported that some of his patients showed symptoms of hepatic insufficiency. In this connection may be mentioned the observation of a temporary alimentary glucosuria in one of the cases of von Jaksch. The animal experiments reported above with acute manganese poisoning as well as with chronic manganese poisoning over long periods showed degenerative changes of the liver. Some of the cases reported in the literature had a history of alcoholism, with most of them this point is not stressed but it appears very likely that workmen of a rather low type as employed in manganese mills and in discharging of boats are accustomed to the use of strong alcohols and in this way a predisposition may be produced for the toxic effects of manganese on the liver. The only autopsy of a case of manganese poisoning in the literature is that of Casamajor (1916) which showed lesions of the medulla and biliary cirrhosis.[1] On the other hand it is quite likely as pointed out by Gayle (1925) that the individual susceptibility may play an important rôle in the development of manganese poisoning.

The course of manganese poisoning is progressive and there seems to be no effective curative treatment. Charles (1927) reported, however, that he saw distinct improvement in the conditions of some of his patients with liver therapy, although in no instance was a cure obtained. It is therefore important that manganese poisoning be recognized at an early stage. Schwarz and Pagels (1923) suggested the periodic control of the erythrocytes of the blood and of the hemoglobin as adequate and considered a rise of the red cell count as the first symptom of manganese toxicosis. But in none of the cases reported in the literature with the exception of that of Schwarz (1932) was a pathologic change of the blood picture reported, and therefore it appears that such control may not be very efficient. On the other hand a periodic determination of the hepatic function may lead to early detection, and abuse of strong alcoholic drinks should be avoided. In view of the lack of therapy the prophylaxis is the most important measure. That this may be quite effective in reducing the incidence of manganese poisoning is shown by the report of Wilbur (1932) that improved dust-collecting

[1] Further reports on autopsies were published by Ashizawa (1927) and by Canavan, Cobb and Drinker (1934).

systems reduce the occurrence of manganese poisoning in ore mills and in similar places.

Von Jaksch (1901) had assumed that bivalent manganese is more toxic than trivalent and, therefore, more liable to produce manganese poisoning, which was confirmed by Sato (1929); but from the history of the cases reported it appears to be only of minor importance in the production of industrial manganese poisoning.

TOXICITY OF MANGANESE. The toxicity of manganese varies for different salts and for the bivalent and trivalent manganese ions.

For *manganous chloride* Reichert (1881) gave the fatal concentration for fishes as 0.3 gram manganese per liter With subcutaneous injections Handowsky, Schulz and Staemmler (1925) gave the fatal dose as 50 mgm. per kilo for mice, guinea pigs and rabbits. With intravenous injections Cervinka (1929) found that the minimal fatal dose was 56 mgm. per kilo in dogs and 18 mgm. per kilo in rabbits.

Manganous citrate injected subcutaneously is, according to Handowsky and his co-workers (1925), more rapidly fatal than manganous chloride. In the same dose of 50 mgm. per kilo there is apparently more rapid absorption from the site of the injection. Kobert (1883) found that the subcutaneous injection of 6 to 8 mgm. (MnO) kills dogs in two days and doses of 13 to 14 mgm. per kilo in 24 hours. The same doses were fatal for cats; rabbits are killed with doses of 12 to 13 mgm. per kilo and guinea pigs with 28 to 30 mgm. per kilo. Rabbits and guinea pigs show no symptoms during the first hours, later thay develop convulsions ending in paralysis, or with smaller but fatal doses they die from progressive depression.

Potassium permanganate is used on account of its oxidizing action as an antiseptic and antidote in alkaloid poisoning. In this compound manganese exists as an anion and one has, therefore, to consider the toxicity of both the potassium and the manganese radicle. The subcutaneous injection of aqueous solutions causes local necrosis of the skin (Harnack and Schreiber, 1901). According to Levine (1929) with oral administration 10 grams are fatal to the horse, 0.24 gram per kilo to rabbits; for man 5 grams may be fatal but larger doses have been tolerated. Pills containing 0.12 gram, given for therapeutic purposes, produced pain of the upper digestive tract, cardiac disturbances, collapse and cyanosis. Several such doses may give rise to abortion. According to De Jongh (1933) this is due to a direct effect on the uterine muscle on account of the oxidizing action of the permanganate and is not caused by the manganese ion. In man poisoning is characterized by vomiting, inability to speak (without loss of consciousness), lividity of lips, chin

and fingers, and swelling and blackening of the tongue. Marked edema of the glottis has been observed and also ulceration and corrosion of the upper digestive tract. Kidney disturbances may occur. In some instances the respiration stopped after the cardiac arrest. At autopsy the following findings were reported: corrosions of the mouth and of the esophagus (not of the stomach), pulmonary edema, broncho-pneumonia, and occasionally degenerative changes of the heart, liver and kidneys.

THERAPEUTIC USE OF MANGANESE COMPOUNDS. Manganese has been used in a great variety of conditions, but so far it has not been generally accepted in any field, except in the form of potassium permanganate as an antiseptic and antidote in alkaloid poisoning. De Buscher (1904) found it to be of limited value in the treatment of morphine poisoning. Widenmann (1915) demonstrated its usefulness in the treatment of snake bites. Recently Taylor recommended manganese sulfate in concentrations of 1:1000 for the treatment of fungus infections of the foot.

Kugler (1831) suggested the use of manganese in scrophulosis. Hannon (1849), Pètrequin (1852), and Burin de Buisson (1852) advocated its administration in chlorosis and in anemic conditions and recently Faludi (1930) reported good results with the administration of a manganese albumen mixture in 17 cases of secondary and 6 cases of pernicious anemia. The former exhibited rapid improvement and five of the patients with pernicious anemia showed complete remission. Camescasse (1923) gave doses of 0.07 gram of manganese dioxide in tablets daily for two weeks to debilitated and undernourished children and claimed a greater speed of recovery, a greater and more rapid gain in weight and apparently also of the growth as compared with untreated children of the same type. Nott (1925) advocated the rectal administration of potassium permanganate (1–1½ grain per pint) combined with the oral administration of thyroid in a great number of pathologic conditions and from the comments on this paper it appears that it is used in this way as a roborant also by other practitioners in England. McDouagh (1925) reported good results from the use of manganese butyrate (1–1.5 cc. of a 1 per cent solution) in boils, carbuncles and a variety of infectious conditions. Although some of the experimental work may indicate that manganese stimulates the self defense mechanism of the organism, more experimental and clinical data must be submitted before its use for such purposes can be accepted.

SUMMARY

From the data presented in the literature it appears that manganese is a regular constituent of plant and animal tissues and that it has important metabolic functions in both.

In animals it is mainly stored in the liver and in the kidneys.

Its absorption from the intestinal tract is very slow and incomplete under normal conditions. The degree of absorption depends upon the acidity of the gastric juice and the solubility of the manganese compounds.

The excretion takes place in the colon, partly also with the bile and to a moderate extent with the urine.

With oral administration to animals toxic symptoms are only observed with large doses which lead to gastric disturbances. With subcutaneous injections central nervous symptoms are reported and with intravenous administration circulatory disturbances are the most characteristic symptoms. Manganese appears to stimulate the growth of young animals, its effect on the generation of blood needs further study. Animals treated with manganese compounds in different ways show degeneration of the liver and other organs, later also degenerative changes of the central nervous system which vary as to their extent and location with different species.

Workmen exposed to the dust of manganese compounds may develop manganese poisoning. This resembles in its clinical picture progressive lenticular degeneration (Wilson's disease). It appears possible that there is a primary affection of the liver and that the symptoms from the central nervous system may be secondary.

The toxicity of manganese varies with different salts. Therapeutically manganese compounds have been advocated in a great variety of conditions, but further studies and clinical observations appear necessary before these can be accepted.

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Zeitschr. Immunitätsf.
42:32-43, 1925

The importance of metal salts for the formation of amboreceptors

by

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As is known, it is possible to influence the antibody concentration of the actively immunized organism by means of various procedures. Salomonsen and Madsen (1) induced an increase of diphtheria antitoxin in horses immunized against diphtheria by injecting pilocarpine. Roux and Vaillard (2), Salomonsen and Madsen (3), Friedberger and Dorner (4), Schröder (5), Pfeiffer (6), Reymann (7) and others have shown that a large phlebotomy or several small phlebotomies can stimulate the formation of antitoxin. Similar changes in antibody production can be induced by injection of pyrodine or pyrogallol (Madsen and Tallquist, 8), hetol (Müller, 9), methylene blue (Fürst, 10), salvarsan (Walker, 11), thorium (Hektoen and Corper, 12). Recently Walbum (13) and Walbum and Mørch (14) have studied metal salts with regard to their importance in antibody formation and their effect on its formation and found that several among these substances have an unusually strong effect. Walbum , who initiated these studies on the basis of various theoretical considerations, has reported the following:

"It is difficult with our present knowledge to enter into the problem of the nature of the process which takes place during the formation of antitoxin in the organism, but regardless of the nature of these processes (synthetic processes, splitting, intramolecular changes, etc.) it can probably be assumed that changes of an enzymatic nature play a varyingly important role in this case as in the case of all cellular activity.

For enzymes to unfold their full activity, as is known, certain conditions must exist, of the nature of which very little is known. The observation has been made, however, that the presence of certain metals salts has a considerable and sometimes decisive effect (catalysts, coenzymes) on many enzyme activities (several oxydases), which suggests that possibly certain enzyme salts might also be of importance for antitoxin-forming processes in the animal organism, and, if this proved to be correct, that the nature and quantity of such salts in the organism might be one of the causes of the often considerable individual variations in the antitoxin-forming capacity of animals. It can be assumed that every animal that is capable of reacting to a toxin injection by formation of antitoxin possesses the (specific?) enzymes necessary for such a reaction, which thus assume decisive importance for the qualitative conditions of antitoxin formation, while the nature and quantity of the metal salts present (catalysts?) determine the quantitative conditions of these processes to a varying degree.

If this were the case, it could be assumed that the administration of the metal salts in question to an organism that was in

an immunized state would in many cases intensify the process of antitoxin formation, that is to say it would be expressed by an increase in the antitoxin concentration in the blood".

The first studies dealt with the effect of manganese chloride, nickel chloride, cobalt chloride and zinc chloride on the formation of diphtheria antitoxin in actively immunized horses and on the formation of coli agglutinins in goats. The injections were given intravenously and it was found that all the salts studied resulted in an increased antibody production, although to a varying degree.

Horses immunized against diphtheria, who revealed a constant decrease in antitoxin strength, could in many cases be brought to an antitoxin concentration, by injection of manganese chloride, which was higher than the concentration origianlly achieved by ordinary immunization with toxin alone. Injections of manganese chloride were introduced into immunization techniques at the Statens Serum Institute in the preparation of diphtheria antitoxin and other sera.

Walbum and Mørch (14), who pursued and enlarged these investigations, have shown amongst other things that it is possible in this manner to increase the antitoxin concentration in the blood of goats immunized against diphtheria to a considerably higher level than is generally possible with toxin treatment alone, which has a certain practical interest. In the immunization of humans it is often desirable to be able to use goat diphtheria antitoxin,

but until now as a rule it was impossible to raise the antitoxin level in the blood of the goats higher than ca. 60-80 IU per cc by ordinary immunization, which is a rather low antitoxin strength for practical use. In our experiments with manganese chloride we were able to produce serum containing 165 IU per cc.

The injected manganese chloride very quickly disappears from the circulation and almost the entire quantity of manganese eliminated by the animal is secreted through the intestinal mucosa. A fairly considerable portion is retained by the various organs, although the capacity to store manganese is very variable. As a rule the manganese content of organs gradually increases with the injected quantity, but in this respect the liver is an exception.

It appears that the capacity of the liver to retain manganese is linked in some way to the antitoxin-producing capacity of the animal, so that animals that are good antitoxin producers have an increased manganese content in the liver, while the manganese content in the liver of poor antitoxin producers is much reduced. This manganese depot in the animal liver may be of some importance for the formation of antitoxin (catalyst?).

To compare the effect of the various metal salts, extensive experiments were made on animals immunized against coli (goats and rabbits). It was found that the effect of the different metal salts was extremely variable. There does not appear to exist any concordance between the effect of metals and their

place in the periodic table. If, on the other hand, one adheres to somewhat earlier approaches, it will be found that within these groups there is indeed a definite concordance in most cases between the atomic number and the effect, in such a way that the effect in different groups (alkali metals? and calcium group) intensifies with rising atomic number, while in other groups the situation is reversed (magnesium group, silver group, iron group? and possibly platinum group).

It was also found that beryllium chloride is the most effective of all salts included in the study.

Since it is apparent that these factors, aside from their purely practical interest, may be of considerable importance from the theoretical standpoint of immunity, it is our intention progressively to study the importance of metal salts for other processes of the organism which may be more or less directly linked to immunology, primarily in order to determine whether the characteristic dependence of the effect upon the atomic number of the substances is a generally valid law of immunology.

Thus Walbum (15) has studied the effect of different metal salts on the bactericidal substances of the blood and found that the intensity of action of these substances in many cases was affected to a considerable degree by metal salts, just as here again there was a certain concordance between the atomic number and the effect.

In the present work we have studied the importance of metal salts for the formation of amboreceptors.

The method used was as follows:

Defibrinated sheep's blood was centrifuged and the centrifuged blood cells were washed 3 times with physiologic sodium chloride solution. One part of these washed blood cells was dissolved in 2 parts sterile distilled water and 3 cc of this solution (= 1cc blood cells) was injected intravenously to the experimental animal (rabbit). A blood sample was drawn before the injection in order to determine whether normally occurring amboreceptors were present, which was not the case in any of the animals used.

The injection of blood cell solution as a rule did not apparently give rise to any signs of poisoning in the rabbits. From the 5th day after the injection blood samples were drawn daily and on the 16th day of the injection, following which the amboreceptor curve would presumably have dropped steeply, 1 cc of a 0.001 molar solution of the metal salt in question per kg rabbit was injected intravenously in physiologic saline. According to the studies of Walbum and Mørch this is the largest quantity of the most toxic salt (cadmium) that can be injected to a rabbit intravenously without eliciting signs of poisoning.

Blood samples were drawn 10 min, 30 min, 1, 2, 3, 5, 7 and 24 hours after the injection of the metal salt. The amboreceptor concentration of these serum samples was measured as follows:

First a preliminary experiment was carried out. The serum was diluted 1:250-fold and from this dilution 1.0-0.3-0.1-0.06-0.03-0.01 doses were used, together with a definitely dissolving complement dose (double the dissolving dose determined by experimentation) and 0.25 cc of a 5% suspension of sheep erythocytes

in a total volume of 1.25 cc. After careful shaking the glasses were placed for 1 hour in a 37° water bath, shaken again and stored in the refrigerator at ca. 2° until the following day. The experiments were read by making a comparison of all series in a glass with 30% hemolysis in such a way that the quantity was determined for each serum sample which just gave rise to such hemolysis. The reciprocal value of this quantity gave the number of amboreceptor units in 1 cc of undiluted serum. The main experiment was of course carried out with much smaller dose intervals than in the preliminary experiment.

The effect of the individual metal salt injections was determined by the level to which they were capable of raising the amboreceptor concentration in the serum and in relation to the maximum amboreceptor concentration previously obtained with antigen alone. The percentile increase in relation to the first peak of the curve should be interpreted by taking it as equal to 100. This eliminates individual differences - as far as feasible - with the different experimental animals with regard to antibody formation and the results achieved are thus directly comparable with one another.

It should be noted here that only those rabbits were used for the experiments which showed strong amboreceptor formation after the blood injection, since it was found that animals that revealed a sluggish amboreceptor formation also reacted weakly to metal salt injections. Among rabbits with a good amboreceptor formation there were some, however, (ca. 10-15% of the animals used) which did not react to the subsequent metal salt injection at all.

These animals were of course disregarded.

Table I (pp. 38-39) shows all the results of this investigation, with the metals arranged according to their effects. Three rabbits were used for each metal. We have naturally not included all the results of the measurements, but only those that are relevant to this investigation. The last column of the table shows the average percentual increase produced by the metal salt injection.

Based on some investigations by Walbum (16) according to which very small quantities of various metal salts often had a stimulating effect in cultures on the formation of bacterial toxins, Helena Purdy and Walbum (17) carried out a number of investigations on the effect of metal salts on the course of various hemolytic processes, including complement-amboreceptor hemolysis (sheep's blood). Since it was found that many of these salts had an enhancing or reducing effect on hemolysis in minute quantities, it was feared that the quantities of metal salts introduced into the hemolytic system with the rabbit serum would be large enought to cause a disturbing effect on the reaction. A simple calculation showed, however, that the quantities introduced in this manner are so small that they are far below the limit of action of the various metal salts.

Table II shows the metals arranged in groups according to their chemical properties and within individual groups according to their atomic numbers. The third, fourth and fifth columns of the table show the effect of salts on amboreceptor formation,

agglutinin formation and on bactericide substances occurring in the plasma. It is apparent that in amboreceptor formation there is also a certain correlation between the stimulating effect of the metals and their atomic number.

In the magnesium group the effect decreases from beryllium to zinc, and increases again up to cadmium and mercury.

In the calcium group the effect decreases evenly with rising atomic numbers, while in the silver group the situation is apparently reversed.

In the iron group there can only be a question of a comparison between the three divalent Co, Ni and Mn, and it would seem that there was an increased effect with decreasing atomic number.

If we study the effect of the individual metals salts on amboreceptor formation, agglutinin formation and the bactericide substances of the blood in different groups, the following will be found:

Magnesium group. The effect of metal salts on agglutinin formation and on bactericide substances decreases with increasing atomic number and conditions with regard to amboreceptor formation are apparently partly (or possibly entirely) the same. The values for Cu, Zn and Cd (12, 11 and 13) are so close that the difference lies within the limits of experimental error.

The silver group also runs evenly, the effect decreasing with rising atomic number.

In the calcium group conditions are the same for amboreceptors and agglutinin formation, the effect increasing with rising atomic numbers, while the situation is reversed for bactericide

substances.

It seems to us premature at this stage to attempt to explain these phenomena on a theoretical basis.

As has been shown by Walbum and Mørch (14), the concentration of the injected metal salt plays an extraordinarily important role in the intensity of its effect, since an optimal concentration and decreasing effect appear to exist with both higher and lower concentrations.

The strongest effect on agglutinin formation was achieved with a 0.001 molar solution.

We have studied this situation in amboreceptor formation by injecting beryllium chloride solutions in the concentrations cited to a number of rabbits with a steeply descending amboreceptor curve. Blood samples were drawn before the injection and 10 min, 2 hours and 7 hours after it. Table III shows the results of the study, the figures indicating the number of amboreceptor units per 1 cc serum. The curve is shown in the graph below.

It is apparent that while both 1.0 and 0.1 molar solutions give rise to a pronounced drop in the amboreceptor concentration of the blood, 0.01, 0.001 and 0.0003 molar solutions give rise to increases, while the 0.0001 molar dilution leads to the slightest decrease.

As in the case of agglutinin formation, here again a ca. 0.001 solution appears to have the strongest effect.

Summary

This report, in which the effect of various metal salts on the amboreceptor formation of the rabbit is investigated, is one link in the continuing study of the effect of metal salts on the immunologic and other processes of the animal organism. As in works published earlier, the authors again find a certain concordance in the different groups between the atomic numbers of the metals and their effects.

TABLE 1

1 Salz	2 Die erste Akme	3 Amboceptoreinheiten pr. ccm: 4 vor der Inj. von Salz	3 Amboceptoreinheiten pr. ccm: 5 die zweite Akme	6 Steigerung in %	7 Durchschnittliche Steigerung
$MnCl_2$	1920	833	1470	33	28
	1470	1000	1470	32	
	1430	714	1000	20	
$PbCl_2$	2500	1250	1920	27	24
	3850	3000	4050	27	
	1000	455	630	17,5	
$BeCl_2$	1670	556	1090	32	27
	1460	1000	1430	29	
	1250	833	1110	22,2	
$HgCl_2$	385	192	217	6,5	20
	250	135	217	32,5	
	217	111	156	20	
$BaCl_2$	2780	1920	2780	30	20
	455	313	357	10	
	1000	500	714	21	
$MgCl_2$	417	167	313	35	18
	1250	625	769	11,5	
	200	110	125	7,5	
$SrCl_2$	3130	1470	2170	22	16
	625	250	313	10	
	3850	2500	3130	16,5	
$CaCl_2$	2500	1670	2220	22	15
	2500	1330	1670	14	
	3850	2860	3210	9	
AgCl	3850	3130	3850	18	14
	2500	1000	1370	15	
	833	435	500	7,8	
$CdCl_2$	556	278	345	12	13
	278	147	192	16	
	1000	385	500	11,5	
$CrCl_3$	500	278	400	24	13
	1920	1470	1670	10	
	3130	1470	1670	6	
$CuCl_2$	1000	833	909	7,6	12
	3850	1220	2000	20	
	313	125	147	7	
$ZnCl_2$	1250	1090	1250	13	11
	909	556	625	8	
	625	426	500	12	
$OsCl_4$	1250	1250	1470	17	9
	909	125	155	3	
	1670	714	833	7	
$CoCl_2$	556	250	313	11	7
	833	385	435	6	
	833	313	350	4,5	
$NiCl_2$	1470	625	700	5	7
	2500	1470	1670	8	
	1920	833	1000	8,6	
$FeCl_3$	2780	1670	1920	9	7
	5560	3130	3350	5,75	
	1110	630	709	7	
$HAuCl_4$	1470	1000	1110	7	7
	1920	1470	1590	6	
	833	500	556	6,7	
H_2PtCl_6	1250	500	556	4,5	4
	1250	714	769	4,4	
	833	435	455	2,4	
$AlCl_3$	250	192	192	0	0
	2500	1920	1920	0	
	1000	500	500	0	

I. Key: 1. Salt. 2. First peak. 3. Amboreceptor units per cc. 4. Before the injection of salt. 5. Second peak. 6. Increase %. 7. Average increase.

TABLE II

	1 Atomzahl	2 Ambozeptor	Agglutinin	3 Bakterizide Stoffe
		4 Magnesiumgruppe		
Be	9,1	27	1147	63,0
Mg	24,3	18	565	4,2
Cu	63,6	12	334	1,7
Zn	65,4	11	188	1,3
Cd	112,4	13	44	1,2
(Hg)	200,6	20	181	
		5 Kalzium (baryum)-Gruppe		
Ca	40.1	15	27	0.44
Sr	87,6	16	43	0,26
Ba	137,4	20	233	0
Pb	207,2	24	402	0
		6 Silbergruppe		
Cu	63,6	12	334	1.7
Ag	107,9	14	95	1,3
Au	197,2	7	30	0,83
		7 Eisengruppe		
Co	59,0	7	20	5.6
Ni	58,7	7	30	0,71
Mn	54,9	28	142	0,44
Fe	55,8	7	0	0,13
Cr	52,0	13	48	0,67
		8 Platingruppe		
Os	190,1	9	268	0,59
Pt	195,2	4	36	0,87
Al	27,1	0	166	0

II. Key: 1. Atomic number. 2. Amboreceptor. 3. Bactericide substances. 4. Magnesium group. 5. Calcium (barium) group. 6. Silver group. 7. Iron group. 8. Platinum group.

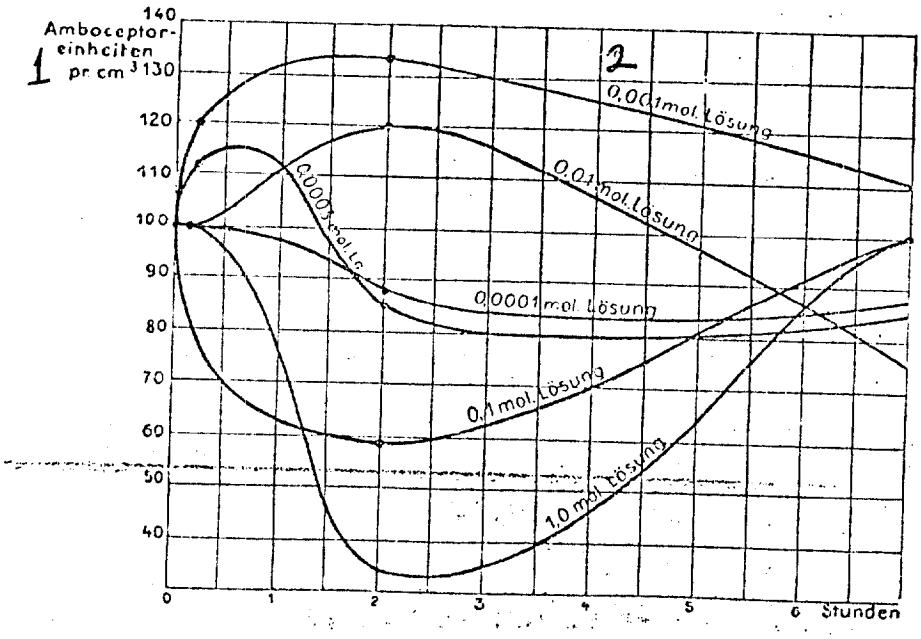
TABLE III

Amboceptoreinheiten pr. ccm.

$BeCl^2$	1 Vor Injektion	2 10 Minuten n. Injektion	3 2 Stunden n. Injektion	4 7 Stunden n. Injektion	5 Gestorben
1,0 mol.	100	100	33	100	6 n. 15 Std.
0,1 „	100	77	50	100	„ 18 „
0,01 „	100	100	120	75	
0,001 „	100	120	133	110	
0,0003 „	100	113	85	85	
0,0001 „	100	100	87	87	

III. Amboreceptor units per cc. Key: 1. Before injection. 2. 10 min after injection. 3. 2 hours after injection. 4. 7 hours after injection. 5. died. 6. After 15 hours.

GRAPH



Graph

Key: 1. Amboreceptor units per cc. 2. 0.001 mol. solution.

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Zeitsch. Immunitäts-42, 32-43 (1925)

Nachdruck verboten.

[Aus dem Statens Seruminstitut Kopenhagen
(Direktor: Dr. Th. Madsen).]

Die Bedeutung der Metallsalze für die Ambozeptorbildung.

Von L. E. Walbum und S. Schmidt.

Mit 1 Kurve im Text.

(Eingegangen bei der Redaktion am 29. September 1924.)

Es ist bekanntlich gelungen, mittels verschiedenartiger Eingriffe die Antikörperkonzentration im aktiv immunisierten Organismus zu beeinflussen. Salomonsen und Madsen (1) riefen durch Injektion von Pilocarpin eine Steigerung des Diphtherieantitoxins bei diphtherieimmunisierten Pferden hervor. Roux und Vaillard (2), Salomonsen und Madsen (3), Friedberger und Dorner (4), Schröder (5), Pfeiffer (6), Reymann (7) und andere zeigten, daß ein großer Aderlaß oder mehrere kleine Aderlässe die Antitoxinbildung stimulisieren können. Durch Injektion von Pyrodin und Pyrogallol (Madsen und Tallquist, 8), Hetol (Müller, 9), Methylenblau (Fürst, 10), Salvarsan (Walker, 11), Thorium (Hektoen und Corper, 12) lassen sich ähnliche Verschiebungen in der Antikörperproduktion bewerkstelligen. Neuerdings haben Walbum (13) und Walbum und Mørch (14) die Metallsalze in bezug auf ihre Bedeutung für die Antikörperbildung und ihren Einfluß auf diese näher studiert und gefunden, daß sich unter diesen Stoffen mehrere finden, die sogar eine ungewöhnlich starke Wirkung in dieser Beziehung haben. Walbum, der diese Untersuchungen auf Basis verschiedener theoretischer Ueberlegungen anfing, teilt hierüber folgendes mit:

„Es ist mit unserem jetzigen Wissen schwierig, näher auf die Frage nach dem Charakter der Prozesse einzugehen, welche während der Bildung des Antitoxins im Organismus vor sich gehen, aber von welcher Art die Prozesse auch sein mögen (synthetische Prozesse, Spaltungen, intramolekuläre Umlagerungen usw.), darf man wohl annehmen, daß Umbildungen enzymatischer Natur hier wie bei aller Zelltätigkeit eine mehr oder weniger hervorragende Rolle spielen.

Damit Enzyme ihre volle Tätigkeit entfalten können, müssen, wie bekannt, gewisse Bedingungen vorhanden sein, Bedingungen, betreffs deren Natur unser Wissen sehr gering ist. Unter anderem hat man indessen die Beobachtung gemacht, daß das Vorhandensein gewisser Metallsalze einen beträchtlichen und mitunter entscheidenden Einfluß (Katalysatoren, Ko-Enzyme) auf viele Enzymwirkungen (mehrere Oxydasen) ausüben kann, was mich vermuten ließ, daß gewisse Metallsalze vielleicht auch für die antitoxinbildenden Prozesse im tierischen Organismus von Bedeutung wären, und — falls dieses sich als richtig herausstellte — daß die Art und Menge derartiger Salze im Organismus vielleicht eine der Ursachen der oft beträchtlichen individuellen Unterschiede des Antitoxinbildungsvermögens der Tiere sein könnte. Es wäre zu vermuten, daß jedes Tier, das überhaupt imstande ist, auf eine Toxininjektion mittels Antitoxinbildung zu reagieren, die für eine derartige Reaktion nötigen Enzyme (spezifische?) besitzt, welche somit für die qualitativen Verhältnisse bei der Antitoxinbildung entscheidend werden, während die Art und Menge der vorhandenen Metallsalze (Katalysatoren?) die quantitativen Verhältnisse dieser Prozesse mehr oder weniger bestimmen.

Falls die Sachlage so wäre, dürfte man annehmen, daß eine Zufuhr des betreffenden Metallsalzes zu dem sich unter Immunisierung befindenden Organismus in vielen Fällen den Umfang des Antitoxinbildungsprozesses vermehren würde, d. h. sich in einer Steigerung der Antitoxinkonzentration des Blutes äußern müßte."

Die zuerst vorgenommenen Untersuchungen bezogen sich auf die Einwirkung des Manganochlorids, Nickelchlorids, Kobaltchlorids und Zinkchlorids auf die Bildung des Diphtherieantitoxins bei aktiv immunisierten Pferden und auf die Coliagglutininbildung bei Ziegen. Die Injektionen wurden intravenös vorgenommen und es stellte sich heraus, daß die untersuchten Salze alle — obwohl in verschiedenem Grade — eine Vermehrung der Antikörperproduktion zur Folge hatten.

Gegen Diphtherie immunisierte Pferde, die eine ständige Abnahme der Antitoxinstärke aufwiesen, ließen sich in mehreren Fällen durch Injektion von Manganochlorid auf eine Antitoxinkonzentration bringen, die größer war als die, welche ursprünglich durch die gewöhnliche Immunisierung mit Toxin allein erzielt worden war. Injektionen von Manganochlorid sind im Statens Serum Institut in die Immunisierungstechnik bei der Darstellung von Diphtherieantitoxin und anderen Seren eingeführt worden.

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Walbum und Mørch (14), die diese Untersuchungen fortgesetzt und weiter vertieft haben, zeigten unter anderem, daß es auf diese Weise auch bei diphtherieimmunisierten Ziegen möglich ist, die Antitoxinkonzentration des Blutes bedeutend höher zu treiben, als es gewöhnlich durch Toxinbehandlung allein möglich ist, was ein gewisses praktisches Interesse beanspruchen kann. Für die Immunisierung von Menschen ist es oft wünschenswert, Ziegen-Diphtherieantitoxin verwenden zu können; bis jetzt war es aber in der Regel unmöglich, durch gewöhnliche Immunisierung das Antitoxin im Blute der Ziegen höher als auf ca. 60—80 IE. per ccm zu bringen, eine Antitoxinstärke, die ziemlich niedrig für die praktische Verwendung ist. Es gelang bei unseren Versuchen, mit Manganochlorid Serum darzustellen, das 165 IE. per ccm enthielt.

Das injizierte Manganochlorid verschwindet sehr schnell aus dem Blutkreislauf und fast die ganze vom Versuchstier abgegebene Manganmenge wird durch die Darmschleimhaut ausgeschieden. Ein nicht geringer Teil wird in verschiedenen Organen zurückgehalten, die Fähigkeit dieser zur Aufspeicherung des Mangans ist jedoch sehr verschieden. Gewöhnlich steigt der Mangangehalt der Organe allmählich mit der injizierten Menge, jedoch bildet die Leber in dieser Beziehung eine Ausnahme.

Es scheint, daß die Fähigkeit dieses Organs, das Mangan zurückzuhalten, in einem gewissen Verhältnis zum Antitoxinproduktionsvermögen des Tieres steht, so daß die guten Antitoxinbildner einen vermehrten Mangangehalt in der Leber haben, während derselbe in der Leber der schlechten Antitoxinbildner stark herabgesetzt ist. Dieses Mangandepot in der Leber des Tieres ist vielleicht von Bedeutung für die Antitoxinbildung (Katalysatoren?).

Um die Wirkung der verschiedenen Metallsalze zu vergleichen, wurden ausgedehnte Versuche mit Coli-immunisierten Tieren (Ziegen und Kaninchen) vorgenommen. Es stellte sich hierbei heraus, daß die Wirkung der verschiedenen Metallsalze außerordentlich variierend ist. Eine Uebereinstimmung

zwischen der Wirkung der Metalle und deren Platz im periodischen System scheint nicht vorhanden zu sein. Falls man sie dagegen nach den etwas älteren Gesichtspunkten ordnet, wird man finden, daß innerhalb der so aufgestellten Gruppen in den meisten Fällen eine sogar ausgesprochene Uebereinstimmung zwischen Atomzahl und Wirkung in der Weise vorliegt, daß die Wirkung innerhalb einzelner Gruppen (Alkalimetalle? und Kalziumgruppe) mit steigender Atomzahl zunimmt, während innerhalb anderer Gruppen das entgegengesetzte Verhältnis waltet (Magnesiumgruppe, Silbergruppe, Eisengruppe? und vielleicht Platingruppe).

Man fand ferner, daß Berylliumchlorid von sämtlichen in die Untersuchung einbezogenen Salzen am wirksamsten ist.

Da es einleuchtend ist, daß diese Verhältnisse außer dem rein praktischen Interesse ein nicht geringes immunitätstheoretisches Interesse beanspruchen können, besteht die Absicht, die Bedeutung der Metallsalze für andere Prozesse im Organismus, welche größere oder geringere unmittelbare Anknüpfung an die Immunitätswissenschaft haben, allmählich zu untersuchen und zwar in erster Linie, um zu konstatieren, ob die eigentümliche Abhängigkeit der Wirkung von den Atomzahlen der Stoffe innerhalb dieses Gebietes ein Gesetz von allgemeiner Gültigkeit ist.

So hat Walbum (15) die Einwirkung der verschiedenen Metallsalze auf die bakteriziden Stoffe des Blutes untersucht und gefunden, daß auch der Wirkungsgrad dieser in mehreren Fällen und in nicht geringem Grade von den Metallsalzen beeinflußt wird, ebenso wie sich auch hier eine gewisse Uebereinstimmung zwischen Atomzahl und Wirkung findet.

In der vorliegenden Arbeit haben wir die Bedeutung der Metallsalze für die Ambozeptorbildung untersucht.

Die angewandte Technik war die folgende:

Durch Schütteln defibriniertes Hammelblut wurde zentrifugiert und die abzentrifugierten Blutkörperchen 3mal mittels physiologischer Natriumchloridlösung gewaschen. 1 Teil dieser gewaschenen Blutkörperchen wurde in 2 Teilen sterilem destillierten Wasser gelöst, und von dieser Lösung wurden

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3 ccm (= 1 ccm Blutkörperchen) dem Versuchstier (Kaninchen) intravenös injiziert. Vor der Injektion wurde eine Blutprobe entnommen, um zu untersuchen, ob in dieser normal vorkommende Ambozeptoren vorhanden waren, was bei keinem der benutzten Tiere der Fall war.

Die Injektion der Blutkörperchenlösung rief gewöhnlich — jedenfalls anscheinend — keine Vergiftungssymptome bei den Kaninchen hervor. Vom 5. Tage ab nach der Injektion wurden täglich Blutproben entnommen und am 16. Tage nach der Injektion, an dem die Ambozeptorkurve vermutlich schroff absteigend sein würde, wurde per Kilogramm Kaninchen 1 ccm einer 0,001 molaren Lösung des betreffenden Metallsalzes (in physiologischer Natriumchloridlösung) intravenös injiziert. Nach den Untersuchungen von Walbum und Mørch ist dieses die größte Menge des am stärksten toxischen Metallsalzes (Kadmium), die man einem Kaninchen intravenös injizieren kann, ohne sichtbare Vergiftungssymptome hervorzurufen.

Blutproben wurden 10 Minuten, 30 Minuten, 1, 2, 3, 5, 7 und 24 Stunden nach der Metallsalzinjektion entnommen. Die Messung der Ambozeptorkonzentration dieser Serumproben wurde folgendermaßen ausgeführt:

Zuerst wurde ein Vorversuch gemacht. Das Serum wurde 1:250 verdünnt, und aus dieser Verdünnung wurden die Dosen 1,0—0,3—0,1—0,06—0,03—0,01 verwandt, zusammen mit einer sicher lösenden Komplementdosis (das Doppelte der durch Versuch gefundenen lösenden Dosis) und 0,25 ccm einer 5-proz. Aufschwemmung von Hammelblutkörperchen in einem Totalvolumen von 1,25 ccm. Nach sorgfältigem Schütteln wurden die Gläser 1 Stunde in ein Wasserbad von 37° gestellt, wieder geschüttelt und bis zum nächsten Tage bei ca. 2° im Eiskeller aufbewahrt. Die Ablesung des Versuches wurde in der Weise ausgeführt, das mittels eines Glases mit 30-proz. Hämolyse ein Vergleich sämtlicher Serien so vorgenommen wurde, daß für jede Serumprobe die Menge, welche eben eine derartige Hämolyse hervorrief, festgestellt wurde. Der reziproke Wert dieser Menge gab die Anzahl von Ambozeptoreinheiten in 1 ccm des unverdünnten Serums an. Der Hauptversuch wurde natürlich mit weit kleineren Sprüngen der Dosen als in dem angegebenen Vorversuch ausgeführt.

Die Wirkung der einzelnen Metallsalzinjektion wurde durch die Höhe bestimmt, auf welche sie die Ambozeptorkonzentration des Serums zu treiben vermochte und im Verhältnis zu der durch das Antigen allein im voraus erzielten maximalen Ambozeptorkonzentration. Die angegebene prozentuale Steigerung ist im Verhältnis zur ersten Akme der Kurve zu beurteilen, indem diese gleich 100 gesetzt wird. In dieser Weise werden — soweit dieses überhaupt möglich ist — die individuellen Unterschiede der einzelnen Versuchstiere betreffs Antikörperbildung eliminiert, und die erzielten Resultate sind somit untereinander direkt vergleichbar.

Es sei hier bemerkt, daß für die Versuche nur die Kaninchen verwandt wurden, die nach der Blutinjektion eine kräftige Ambozeptorbildung zeigten, da es sich herausstellte, daß die Tiere, welche eine träge Ambozeptorbildung aufwiesen, auch auf die Metallsalzinjektion schwach reagierten. Unter den Kaninchen, deren Ambozeptorbildung gut war, fanden sich jedoch einzelne (etwa 10—15 Proz. der verwandten Tiere), die auf die nachfolgende Metallsalzinjektion überhaupt nicht reagierten. Diese Tiere haben wir natürlich unberücksichtigt gelassen.

In der beigefügten Tabelle I (S. 38/39) sind sämtliche Resultate dieser Untersuchungen angeführt, indem die Metalle nach ihren Wirkungen geordnet sind. Zu jedem Metall wurden drei Kaninchen verwendet. Selbstverständlich sind nicht sämtliche Meßresultate angeführt, sondern nur die wenigen, die in dieser Beziehung von Bedeutung sind. In der letzten Spalte der Tabelle wird die durchschnittliche prozentuale, von der Metallsalzinjektion hervorgerufene Steigerung angeführt.

Durch einige Untersuchungen von Walbum (16) veranlaßt, denen zufolge sehr kleine Mengen verschiedener Metallsalze oft in den Kulturen eine fördernde Wirkung auf die Bildung der bakteriellen Toxine ausüben, unternahmen Helena Purdy und Walbum (17) eine Reihe Untersuchungen über die Einwirkung der Metallsalze auf den Verlauf verschiedener hämolytischer Prozesse u. a. auch auf die Komplement-Ambozeptor-Hämolyse (Hammelblut). Da es sich hierbei herausstellte, daß viele dieser Salze in winzigen Mengen eine för-

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Tabelle I.

Salz	Die erste Akme	Ambozeptoreinheiten pr. ccm		Steigerung in %	Durchschnittliche Steigerung
		vor der Inj. von Salz	die zweite Akme		
$MnCl_2$	1920	833	1470	33	28
	1470	1000	1470	32	
	1430	714	1000	20	
$PbCl_2$	2500	1250	1920	27	24
	3850	3000	4050	27	
	1000	455	630	17,5	
$BeCl_2$	1670	556	1090	32	27
	1460	1000	1430	29	
	1250	833	1110	22,2	
$HgCl_2$	385	192	217	6,5	20
	250	135	217	32,5	
	217	111	156	20	
$BaCl_2$	2780	1920	2780	30	20
	455	313	357	10	
	1000	500	714	21	
$MgCl_2$	417	167	313	35	18
	1250	625	769	11,5	
	200	110	125	7,5	
$SrCl_2$	3130	1470	2170	22	16
	625	250	313	10	
	3850	2500	3130	16,5	
$CaCl_2$	2500	1670	2220	22	15
	2500	1330	1670	14	
	3850	2860	3210	9	
AgCl	3850	3130	3850	18	14
	2500	1000	1370	15	
	833	435	500	7,8	
$CdCl_2$	556	278	345	12	13
	278	147	192	16	
	1000	385	500	11,5	
$CrCl_3$	500	278	400	24	13
	1920	1470	1670	10	
	3130	1470	1670	6	
$CuCl_2$	1000	833	909	7,6	12
	3850	1220	2000	20	
	313	125	147	7	
$ZnCl_2$	1250	1090	1250	13	11
	909	556	625	8	
	625	426	500	12	
$OsCl_4$	1250	1250	1470	17	9
	909	125	155	3	
	1670	714	833	7	

Salz	Die erste Akme	Ambozeptoreinheiten pr. ccm		Steigerung in %	Durchschnittliche Steigerung
		vor der Inj. von Salz	die zweite Akme		
$CoCl_2$	556	250	313	11	7
	833	385	435	6	
	833	313	350	4,5	
$NiCl_2$	1470	625	700	5	7
	2500	1470	1670	8	
	1920	833	1000	8,6	
$FeCl_3$	2780	1670	1920	9	7
	5560	3130	3450	5,75	
	1110	630	709	7	
$HAuCl_4$	1470	1000	1110	7	7
	1920	1470	1590	6	
	833	500	556	6,7	
H_2PtCl_6	1250	500	556	4,5	4
	1250	714	769	4,4	
	833	435	455	2,4	
$AlCl_3$	250	192	192	0	0
	2500	1920	1920	0	
	1000	500	500	0	

dernde oder hemmende Einwirkung auf die Hämolyse ausübten, könnte man befürchten, daß die Metallsalzmengen, die mit dem Kaninchenserum ins hämolytische System überführt wurden, so groß waren, daß sie in die Reaktion störend eingriffen. Durch einfaches Rechnen sieht man aber leicht, daß die Mengen, die auf diese Weise überführt werden können, so klein sind, daß sie weit unterhalb der Wirkungsgrenze der verschiedenen Metallsalze liegen.

In Tabelle II sind die Metalle gruppenweise nach ihren chemischen Eigenschaften und innerhalb der einzelnen Gruppen nach ihren Atomzahlen geordnet. In der dritten, vierten und fünften Spalte des Tabelle ist die Wirkung der Salze bzw. auf die Ambozeptorbildung, Agglutininbildung und auf die im Plasma vorkommenden bakteriziden Stoffe angeführt. Es geht daraus hervor, daß auch für die Ambozeptorbildung ein gewisses Verhältnis zwischen der stimulierenden Wirkung der Metalle und ihren Atomzahlen vorzuliegen scheint.

Tabelle II.

	Atomzahl	Ambozeptor	Agglutinin	Bakterizide Stoffe
		Magnesiumgruppe		
Be	9,1	27	1147	63,0
Mg	24,3	18	565	4,2
Cu	63,6	12	334	1,7
Zn	65,4	11	188	1,3
Cd	112,4	13	44	1,2
(Hg)	200,6	20	181	
		Kalzium (baryum)-Gruppe		
Ca	40,1	15	27	0,44
Sr	87,6	16	43	0,26
Ba	137,4	20	233	0
Pb	207,2	24	402	0
		Silbergruppe		
Cu	63,6	12	334	1,7
Ag	107,9	14	95	1,3
Au	197,2	7	30	0,83
		Eisengruppe		
Co	59,0	7	20	5,6
Ni	58,7	7	30	0,71
Mn	54,9	28	142	0,44
Fe	55,8	7	0	0,13
Cr	52,0	13	48	0,67
		Platingruppe		
Os	190,1	9	268	0,59
Pt	195,2	4	36	0,87
Al	27,1	0	166	0

In der Magnesiumgruppe nimmt die Wirkung von Beryllium bis Zink ab, worauf sie wieder bis Kadmium und Quecksilber steigt.

In der Kalziumgruppe ist die Wirkung mit steigender Atomzahl gleichmäßig abnehmend, während das Verhalten der Silbergruppe anscheinend umgekehrt ist.

Innerhalb der Eisengruppe kann wohl nur die Rede von einem Vergleich zwischen den drei divalenten Co, Ni und Mn sein, und es scheint, als ob hier eine zunehmende Wirkung mit abnehmender Atomzahl vorliegt.

Untersucht man innerhalb verschiedener Gruppen die Wirkung der einzelnen Metallsalze auf die Ambozeptorbildung, die Agglutininbildung und die bakteriziden Stoffe des Blutes, so wird man folgendes finden.

Die Magnesiumgruppe. Die Wirkung der Metallsalze auf die Agglutininbildung und auf die bakteriziden Stoffe nimmt mit steigender Atomzahl ab, und die Verhältnisse sind betreffs der Ambozeptorbildung anscheinend zum Teil (oder vielleicht völlig) gleichartig. Die Zahlen für Cu, Zn und Cd (12, 11 und 13) liegen einander so nahe, daß der Unterschied sicher innerhalb des Versuchsfehlers liegt.

In der Silbergruppe ist ebenfalls Gleichmäßigkeit vorhanden, indem auch hier die Wirkung mit steigender Atomzahl abnimmt.

Innerhalb des Kalziumgruppe sind die Verhältnisse bei der Ambozeptor- und Agglutininbildung gleich, indem die Wirkung mit steigender Atomzahl zunimmt, während die bakteriziden Stoffe sich umgekehrt verhalten.

In der Eisengruppe ist ebenfalls zwischen Ambozeptor- und Agglutininbildung Uebereinstimmung vorhanden. Hier ist aber die Wirkung mit abnehmender Atomzahl zunehmend, und auch hier verhalten sich die bakteriziden Stoffe umgekehrt.

Von den beiden trivalenten Fe und Cr ist die Wirkung des Cr in sämtlichen untersuchten Fällen am größten.

Von den Platinmetallen wirkt Osmium auf die Bildung von Ambozeptoren und Agglutininen stärker stimulierend als Platin, während die Wirkung bei den bakteriziden Stoffen anscheinend in entgegengesetzter Richtung verläuft.

Es scheint uns übereilt, schon jetzt zu versuchen, diese Phänomene theoretisch erklären zu wollen.

Wie von Walbum und Mørch (14) gezeigt, spielt die Konzentration des injizierten Metallsalzes für seinen Wirkungsgrad eine außerordentlich große Rolle, da eine Optimalkonzentration und abnehmende Wirkung von sowohl größeren als kleineren Konzentrationen vorzuliegen scheint.

Die größte Wirkung auf die Agglutininbildung wurde mittels einer Lösung von 0,001 molar erzielt.

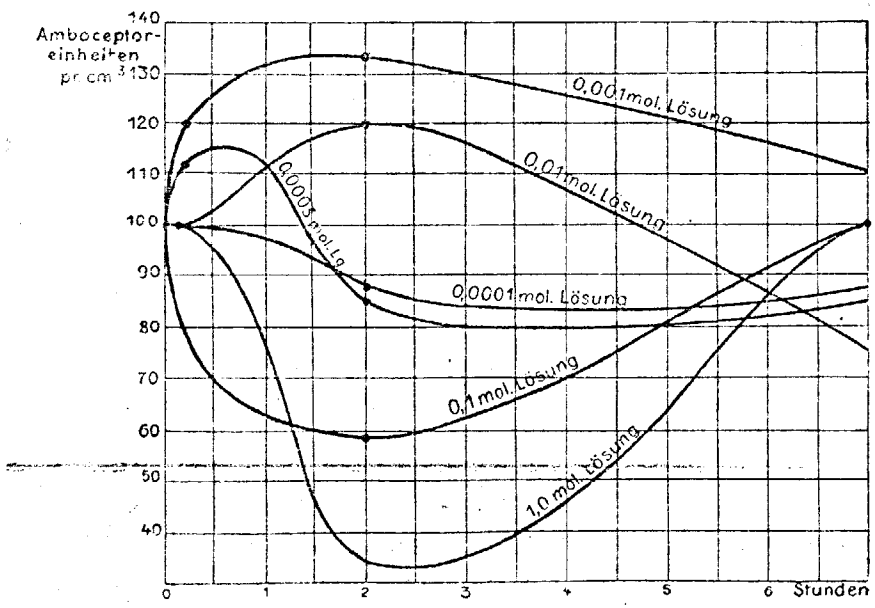
Wir haben dieses Verhältnis bei der Ambozeptorbildung untersucht, indem wir Berylliumchloridlösungen in den angeführten Konzentrationen einer Reihe Kaninchen injiziert haben, deren Ambozeptorkurve stark absteigend war. Die

Blutprobeentnahme fand vor der Injektion und 10 Minuten, 2 Stunden und 7 Stunden nach derselben statt. In Tabelle III ist das Resultat der Untersuchung in der Weise angeführt, daß die Zahlen die Anzahl von Ambozeptoreinheiten pro 1 ccm Serum bezeichnen. Die graphische Darstellung findet sich auf der beigefügten Kurve.

Tabelle III.

Ambozeptoreinheiten pr. ccm.

$BeCl^2$	Vor Injektion	10 Minuten n. Injektion	2 Stunden n. Injektion	7 Stunden n. Injektion	Gestorben
1,0 mol.	100	100	33	100	n. 15 Std.
0,1 „	100	77	59	100	„ 18 „
0,01 „	100	100	120	75	
0,001 „	100	120	133	110	
0,0003 „	100	113	85	85	
0,0001 „	100	100	87	87	



Hieraus geht hervor, daß während sowohl 1,0 wie 0,1 molarer Lösung starkes Fallen der Ambozeptorkonzentration des Blutes hervorruft, sowohl 0,01, 0,001 wie 0,0003 molarer Lösungen Steigerungen verursachen, während die größere

Verdünnung 0,0001 molar am ehesten eine geringe Herabminderung zur Folge hat.

Ebenso wie für die Agglutininbildung scheint auch hier eine ca. 0,001 molare Lösung die größte Wirkung auszuüben.

Zusammenfassung.

Diese Abhandlung, in welcher der Einfluß verschiedener Metallsalze auf die Ambozeptorbildung des Kaninchens untersucht wird, ist ein Glied in den ständig fortgesetzten Untersuchungen über die Einwirkung der Metallsalze auf die immunologischen und anderen Prozesse im tierischen Organismus. Ebenso wie in früher erschienenen Arbeiten finden die Verfasser auch hier innerhalb der einzelnen Gruppen eine gewisse Uebereinstimmung unter den Atomzahlen der Metalle und ihren Wirkungen.

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