GLUTAMATES VOL 6 #39
COPIES OF ARTICLES CITED BUT NOT
USED IN MONOGRAPH SUMMARY Part 2

Glutamates-Volume 6 #39 2/8/74

VOLUME 6

GRAS MONOGRAPH SERIES

GLUTAMATES

COPIES OF ARTICLES CITED BUT NOT
USED IN MONOGRAPH SUMMARY
(PART 2)

prepared for

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

APRIL 30, 1973

prepared by

Tracor Jitco, Inc.

To the Editor: The letter from Dr. Kwok in the April 4 issue has interested me greatly. For about a year and a half I have been afflicted with an illness quite similar to his "Chinese-restaurant syndrome." My cry was a voice in the wilderness until I met within the past year two friends who have loved Chinese food but have suffered postprandially with this peculiar illness.

The symptomatology that we suffer is numbness, which arises in the jaw but which spreads rapidly to the back of the neck. Dull headache follows, and palpitation and weakness are inconstant findings.

In a completely uncontrolled fashion we have identified, quite tentatively, "duck sauce" as the etiologic agent. We have ruled out most of the other dishes as causative, but we know too little about Chinese cuisine to make as sophisticated a statement as was made by Dr. Kwok.

I have been a devotee of Chinese cooking for a long time, and gorge myself frequently on repasts in Cantonese, Mandarin and other fashions. It is only within recent times that the "Chinese-restaurant syndrome" has made its appearance. I feel strongly that an epidemiologic study of recent additives to the Chinese culinary armamentarium is in order.

I offer my services to Dr. Kwok in tracking down this agent of dietary unrest.

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(3831)

Hoppe-Seylers Zeitschr. physiol.Chem.
300(1/3):107-112, 1955

Studies on the Metabolism of L(+)-Glutamic Acid.

2. Shift in Plasma Level of Free Amino Acids after the Administration of Glutamic Acid

by

V. Klingmüller, J. Gayer and F. Bramstedt

From: Physiological and Chemical Institute of the University of Hamburg (Director: Prof. J. Kühnau, M.D.)

Received: Jan. 13, 1955.

In our first report (1) an account was given of the behavior of the two keto acids α-ketoglutaric acid and pyruvic acid after intravenous administration of neutralized glutamic acid to rabbits. A distinct rise of α-ketoglutaric acid and a corresponding drop in pyruvic acid was observed.

In this report an account will be given of the investigations intended to determine the fate of amino nitrogen, since no free ammonia could be detected.

1. Paper chromatographic studies

Full-grown rabbits weighing 3000 to 3500 g received 2 mMol/kg glutamic acid i.v. as a 10% solution neutralized with sodium hydroxide. This quantity corresponds to that used in our first report and to a dose of 20 g in an adult weighing 70 kg administered orally or intravenously for therapeutic purposes. After taking a blank value before the injection,

c. 2 ml blood was drawn from the ear vein of the animals 10, 20, 30, 45, 60, 75, 90, 120, 150 and 180 min after the injection. The blood was rendered incoagulable with heparin, centrifuged as quickly as possible and the plasma obtained precipitated with a 5-fold quantity of absolute alcohol. After a further centrifugation the alcohol was shaken out with chloroform and the clear supernatant fluid was pipetted off (2). 0.05 ml of this was subjected to two-dimensional paper chromatography (Schleicher and Schüll 2043b mgl) in water-saturated phenol in one direction, and in the other in butanol-glacial acetic acid-water (4:1:5) and after careful drying with 0.2% ninhydrin solution, developed in methanol. The series of 11 chromatograms were always developed simultaneously, allowing clear assessment of the changes in amino acids in comparison with the blank value and during the experiment until normalization.

Fig. 1 shows the 3 first chromatograms from two experiments. In addition to the anticipated pronounced increase in glutamic acid itself, the level of which reverted to normal within 120 min in agreement with the microbioloigcal investigations of our first report, there was a distinct increase in alanine (ala) amounting to roughly 2 to 3 times the initial value. The paper chromatographic findings revealed no increase in aspartic acid (asp), which could have been produced from oxalacetic acid. On the other hand Fig. 1b' and 1c' show another spot (glm) between glutamic acid (glu) and aspartic acid, which we have not so far been able to identify and the significance of which

is being further studied. After 120 min the alanine value reverted to normal and the glm spot disappeared.

2. Methods of Microbiological determination

For further confirmation of our paper chromatographic findings and in order to obtain a more precise idea of the degree of alanine increase, microbiological L-alanine determinations were made following the administration of glutamic acid, using Leuconostic citrovorum 8081 according to Baumann and Sauberlich (3). Blood was drawn before and 10, 20, 30, 45, 60 and 120 min after injection of sodium glutamate. The plasma obtained was deproteinated with tungstate according to Hier and Bergeim (4) and a quantity equivalent to 0.6 ml plasma was used for the microbiological test. The growth of the microorganism was determined with an Eppendorf photometer by nephelometry against a calibration curve.

Fig. 2 shows the findings in 5 rabbits after intravenous injection of 2 mMol/kg sodium glutamate; the dots on the curves represent means of double determinations. The size of the alanine increase accords with our estimates on the ninhydrin spots of the paper chromatographic findings.

Since the decrease in pyruvic acid did not exceed 1.0 to 1.2 mg%, corresponding to 0.11 to 0.14 mMol per l (1), the pyruvate necessary for the formation of alanine cannot originate solely from the blood, for the increase in alanine in the plasma, with an average of 1.1 mMol per l, is about 5 to 10 times the decrease in pyruvic acid. It seemed worthwhile in this connection to

investigate whther by increasing the amount of glutamic acid injected or by simultaneous injection of pyruvate it is possible to intensify the hyperalaninemia. Fig. 3 shows the behavior of L-alanine after intravenous administration of 4 mMol/kg glutamate. The rise in alanine corresponds to the lower limit of the curve shown in Fig. 2, that is to say is not increased compared to the rise observed after the injection of 2 mMol/kg glutamate. The broken curve gives the mean values. In comparison with Fig. 2, the considerably slower fall in the alanine level is striking.

In a further experiment the influence of the simultaneous administration of equimolar quantities of pyruvate and glutamate on the alanine level was investigated. Fig. 4 gives the results of this experiment, in which 2 mMol/kg glutamate and 2 mMol/kg pyruvate were injected simultaneously in 2 different veins. Here again there is no difference compared to the experiment without added pyruvate. The blood sugar examined at the same time according to Hagedorn-Jensen showed a slight increase from 123 to 142 mg%, which accords with our own and other (5,6) experiments on the behavior of the blood sugar after the administration of glutamic acid.

After oral administration of 2 mMol/kg glutamic acid an alanine increase between 4 and 6 mg% was observed, the maximum being reached between 45 and 75 min after administration and reverting to the initial values after 3-4 hours.

Discussion

Our in vivo experiments confirm the present experiences with transamination. In in vitro experiments Braunstein and Kritzmann have demonstrated the capacity of glutamic acid to form alanine from pyruvic acid by transamination. Aspartic acid can also be formed in vitro by transamination. Kamin and Handler (7) found, for instance, that in the dog, after continuous infusion of glutamate, particularly large quantities of aspartic acid are formed and excreted in the urine. Even if we were unable to observe any appreciable increase of aspartic acid, this scarcely means disagreement with Kamin and Handler, since we administered glutamic acid in a single injection. Cohen and Hekhuis (8) also found that the liver forms less aspartic acid than alanine from glutamate.

The increase in alanine demonstrated by us exceeds the supply of pyruvic acid in the serum, so that the pyruvic acid required for this considerable increase probably originates from carbohydrate metabolism. The fact that neither the doubling of the quantity of glutamic acid injected nor the simultaneous administration of pyruvate led to a further increase in the alanine quantity, and that the oral administration of glutamic acid, after the delay caused by absorption, gives rise to the same increase in the blood alanine level as parenteral administration, may be explained by the surmise that the transaminase system of the entire organism has a

limited capacity, which is completely exhausted by the 2 mMol/kg glutamic acid quantity administered.

Summary

See English summary on p. 112.

Figures

1. Free amino acids in the plasma after administration of glutamic acid. Paper chromatography, rabbit, fasting for 12 hours. 1a) and 1a') fasting; 1b) and 1b') 10 min after injection of 2 mMol/kg Na-glutamate; 1c) and 1c') 20 min after injection. ph = water-saturated phenol, Bu-E = Butanol – glacial acetic acid - water (4:1:5).

2. Change in the L-alanine level in the plasma after intravenous administration of 2 mMol/kg Na-glutamate, rabbits.

2. Change in L-alanine level in the plasma after injection of 4 mMol/kg Na-glutamate, rabbits.

4. Change in L-alanine level in the plasma after simultaneous administration of 2 mMol/kg glutamate and 2 mMol/kg pyruvate, rabbits.

Bibliography

See footnote references.

Acknowledgement

We thank Mrs. L. von Hein for carrying out the microbiological determinations.

Untersuchungen über den Stoffwechsel der L(-)-Glutaminsäure

II. Mitt.: Verschiebungen im Plasmaspiegel freier Aminosäuren nach Glutaminsäuregaben

Von

V. Klingmüller, J. Gayer und **F. Bramstedt**

Aus dem Physiologisch-Chemischen Institut der Universität Hamburg
(Direktor: Prof. Dr. J. Kuhnau)

(Der Schriftleitung zugegangen am 14. Januar 1955)

In der I. Mitteilung[1] wurde über das Verhalten der beiden Ketosäuren α-Ketoglutarsäure und Brenztraubensäure nach intravenöser Verabfolgung von neutralisierter Glutaminsäure an Kaninchen berichtet. Es wurde ein deutlicher Anstieg von α-Ketoglutarsäure sowie ein entsprechender Abfall von Brenztraubensäure beobachtet.

In dieser Mitteilung berichten wir über Untersuchungen, die zur Klärung des Verbleibs des Aminostickstoffs dienen sollten, da kein freies Ammoniak nachgewiesen werden konnte.

1. Papierchromatographische Untersuchungen

Ausgewachsenen Kaninchen im Gewicht zwischen 3000 und 3500 g wurden 2 mMol/kg Glutaminsäure als 10-proz. mit Natronlauge neutralisierte Lösung intravenös verabreicht. Diese Menge entspricht der in der I. Mitteil. verwendeten sowie einer Dosis von 20 g beim etwa 70 kg schweren Erwachsenen, wie sie in therapeutischer Absicht intravenös und oral gegeben wird. Nach Abnahme eines Blindwerts vor der Injektion wurden den Tieren nach 10, 20, 30, 45, 60, 75, 90, 120, 150 und 180 Min. post injectionem etwa 2 ml Blut aus der Ohrvene entnommen, mit Heparin ungerinnbar gemacht, möglichst schnell zentrifugiert und das gewonnene Plasma mit der 5fachen Menge absol. Alkohols gefällt. Nach nochmaligem Zentrifugieren wurde der Alkohol mit Chloroform ausgeschüttelt und die klare überstehende Flüssigkeit abpipettiert[2]. Hiervon wurden jeweils 0,05 ml der zweidimensionalen Chromatographie auf Papier (Schleicher & Schüll 2043b mgl) in der einen Richtung in wassergesättigtem Phenol, in der anderen in Butanol-Eisessig-Wasser (4:1:5) unterworfen und nach schonendem Trocknen mit 0,2-proz. Ninhydrinlösung in Methanol entwickelt. Die Entwicklung der Serien von jeweils 11 Chromatogrammen erfolgte dabei stets gleichzeitig, so daß eine gute Beurteilung der Veränderung der Aminosäuren im Vergleich zum Blindwert und während des Versuches bis zur Normalisierung gewährleistet wurde.

In Abb. 1 sind die 3 ersten Chromatogramme zweier Versuche wiedergegeben. Sie zeigen neben der erwarteten starken Vermehrung von Glutaminsäure (Glu) selbst, deren Niveau sich entsprechend den mikro-

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[2] J. Awapara, Arch. Biochemistry Biophysics 19, 172 [1948].

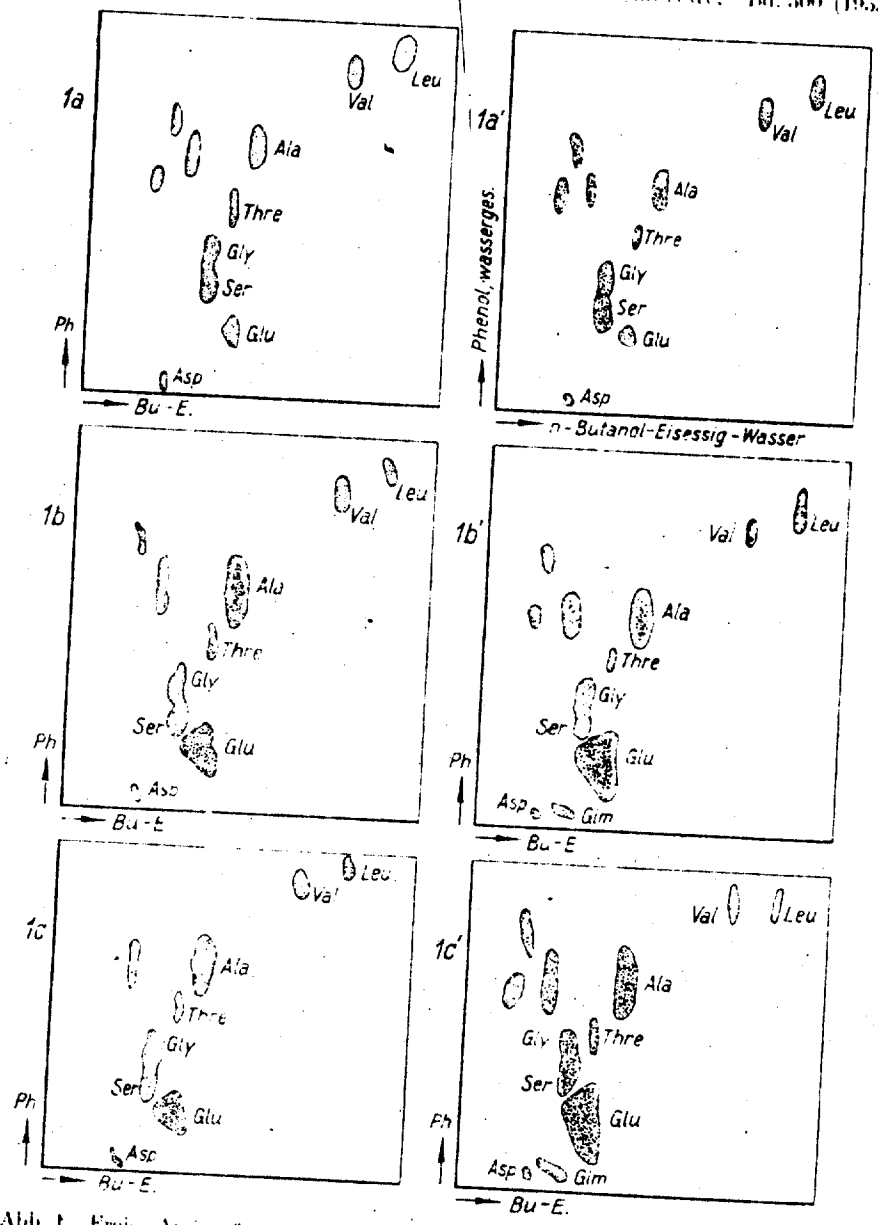

Abb. 1. Freie Aminosäuren des Plasmas nach Glutaminsäure-Verabreichung. Papierchromatographie: Kaninchen: 12 Stdn. nüchtern. 1a) und 1a') Nüchtern; 1b) und 1b') 10 Min. nach der Injektion von 2 mMol/kg Na-Glutaminat; 1c) und 1c') 20 Min. nach Injektion. Ph = wassergesätt. Phenol; Bu-E = Butanol-Eisessig-Wasser (4:1:5).

biologischen Untersuchungen der I. Mitteil. innerhalb von 120 Min wieder normalisiert, eine deutliche Zunahme des Alanins (Ala), die etwa die 2- bis 3fache Menge des Ausgangswertes betragen mag. Eine Vermehrung von Asparaginsäure (Asp), die aus Oxalessigsäure ebenfalls hätte entstehen können, ist aus den papierchromatographischen Befunden nicht zu erkennen. In Abb. 1b′ und 1c′ tritt dafür ein neuer Fleck (Glm) zwischen Glutaminsäure und Asparaginsäure auf, den wir bisher nicht

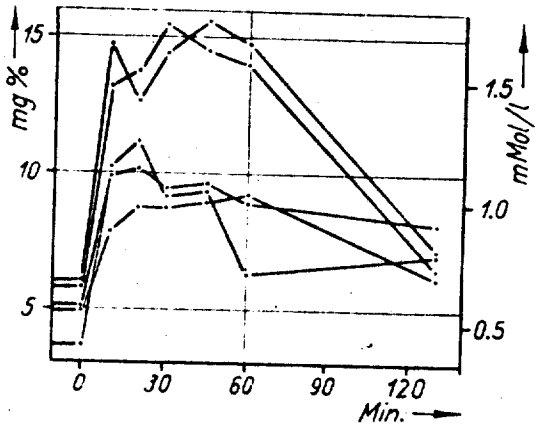

Abb. 2. Änderung des L-Alaninspiegels im Plasma nach i. v. Injektion von 2 mMol/kg Na-Glutaminat; Kaninchen.

identifizieren konnten und dessen Bedeutung weiter untersucht wird. Nach 120 Min. ist der Alaninwert wieder normal und der Fleck (Glm) verschwunden.

2. Mikrobiologische Bestimmungsmethoden

Zur weiteren Sicherung unserer papierchromatographischen Befunde und um genauere Vorstellungen über die Größe der Alaninvermehrung zu bekommen, bestimmten wir L-Alanin im Anschluß an Glutaminsäuregaben mikrobiologisch mit Hilfe von *Leuconostoc citrovorum 8081* nach Baumann und Sauberlich[3]. Die Blutentnahmen erfolgten vor sowie 10, 20, 30, 45, 60 und 120 Min. nach der Injektion von Natriumglutaminat. Das gewonnene Plasma wurde nach Hier und Bergeim[4] mit Wolframat enteiweißt und eine 0,6 ml Plasma entsprechende Menge zum mikrobiologischen Test verwendet. Das Wachstum des Mikroorganismus wurde am Photometer Eppendorf nephelometrisch gegen eine Eichkurve bestimmt.

Abb. 2 gibt die Befunde bei 5 Kaninchen nach intravenöser Injektion von 2 mMol/kg Natriumglutaminat wieder; die Punkte der Kurven stellen Mittelwerte von Doppelbestimmungen dar. Die Größe der Alaninvermehrung entspricht unseren Schätzungen an den Ninhydrinflecken der papierchromatographischen Befunde.

Da die beobachtete Brenztraubensäure-Abnahme 1,0 bis 1,2 mg % entspr. 0,11 bis 0,14 mMol pro *l*, nicht übersteigt[1], kann das zum Aufbau des Alanins benötigte Pyruvat nicht allein aus dem Blut stammen, denn

[3] C. A. Baumann u. H. E. Sauberlich, J. biol. Chemistry 177, 545 [1949].
[4] S. Hier u. O. Bergeim, J. biol. Chemistry 161, 717 [1945].

der Alaninanstieg im Plasma beträgt mit im Durchschnitt 1,1 mMol pro l das etwa 5- bis 10fache der Brenztraubensäureabnahme. Es schien in diesem Zusammenhang von Interesse zu prüfen, ob durch Erhöhung der injizierten Glutaminsäuremenge oder durch gleichzeitige Injektion

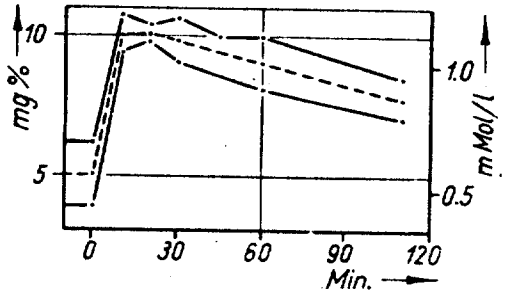

Abb. 3. Änderung des L-Alaninspiegels im Plasma nach Injektion von 4 mMol/kg Na-Glutaminat; Kaninchen.

von Pyruvat die Hyperalaninämie verstärkt werden kann. Abb. 3 zeigt das Verhalten von L-Alanin nach intravenöser Verabfolgung von 4 mMol/kg Glutaminat. Der Anstieg des Alanins entspricht der unteren Grenze der in Abb. 2 dargestellten Kurven, ist also gegenüber dem nach Injektion von 2 mMol/kg Glutaminat nicht erhöht. Die gestrichelte Kurve gibt die Mittelwerte an. Im Vergleich zu Abb. 2 fällt der wesentlich langsamere Abfall des Alaninspiegels auf.

In einem weiteren Versuch prüften wir den Einfluß gleichzeitiger Verabfolgung äquimolarer Mengen von Pyruvat und Glutaminat auf den Alaninspiegel. In Abb. 4 ist das Ergebnis dieses Versuches wiedergegeben,

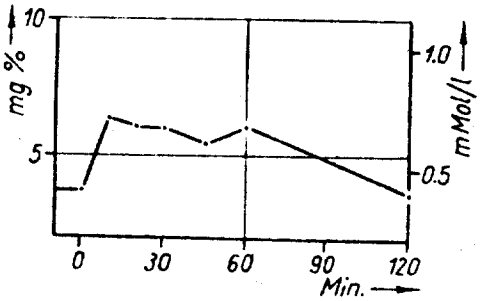

Abb. 4. Änderung des L-Alanin-Spiegels im Plasma nach gleichzeitiger Injektion von 2 mMol/kg Glutaminat und 2 mMol/kg Pyruvat; Kaninchen.

bei dem 2 mMol/kg Glutaminat und 2 mMol/kg Pyruvat gleichzeitig in 2 verschiedene Venen injiziert wurden. Auch hier besteht kein Unterschied gegenüber dem Versuch ohne zusätzliche Pyruvatgabe. Der gleichzeitig nach Hagedorn-Jensen untersuchte Blutzucker zeigte einen geringen Anstieg von 123 auf 142 mg%, der eigenen und anderen Versuchen[5,6] über das Verhalten des Blutzuckers nach Glutaminsäureverabfolgung entspricht.

[5] V. Klingmüller u. J. Gayer, Verh. dtsch. Ges. inn. Med. 60, 960 [1954].
[6] W. A. Himwich, Amer. J. Physiol. 1954, persönl. Mitteilung.

Nach oraler Verabfolgung von 2 mMol/kg Glutaminsäure konnten wir Alaninvermehrungen in der Größenordnung zwischen 4 und 6 mg% beobachten, deren Maximum zwischen 45 und 75 Min. nach der Zufuhr erreicht war und die nach 3–4 Stdn. zur Ausgangslage zurückgekehrt waren.

Diskussion

Unsere Versuche in vivo bestätigen die vorliegenden Erfahrungen über die Transaminierungen. Bei Versuchen in vitro wurde von Braunstein und Kritzmann die Fähigkeit der Glutaminsäure nachgewiesen, durch Transaminierung Alanin aus Brenztraubensäure zu bilden. Andererseits kann in vitro auch Asparaginsäure durch Transaminierung gebildet werden. Kamin und Handler[7] fanden beispielsweise, daß beim Hund nach Dauerinfusion von Glutaminat besonders große Mengen von Asparaginsäure gebildet und im Harn ausgeschieden werden. Wenn wir keine wesentliche Vermehrung von Asparaginsäure feststellen konnten, so bedeutet das kaum einen Gegensatz zu Kamin und Handler, weil wir Glutaminsäure in einmaliger Injektion verabreicht haben. Auch Cohen und Hekhuis[8] fanden, daß die Leber aus Glutaminat weniger Asparaginsäure bildet als Alanin.

Der von uns nachgewiesene Alaninanstieg übersteigt den im Serum vorhandenen Vorrat an Brenztraubensäure, so daß die für diesen erheblichen Anstieg benötigte Brenztraubensäure wahrscheinlich aus dem Kohlenhydratstoffwechsel stammt. Die Tatsache, daß weder die Verdoppelung der injizierten Glutaminsäuremenge noch die gleichzeitige Verabfolgung von Pyruvat zu einer weiteren Vermehrung der Alaninmenge führt, und auch die orale Applikation von Glutaminsäure nach der durch die Resorption bedingten Verzögerung den gleichen Anstieg des Blutalaninspiegels bewirkt wie parenterale Gabe, mag ihre Erklärung in der Annahme finden, daß das Transaminasesystem des Gesamtorganismus eine begrenzte Kapazität besitzt, die durch die zugeführte Menge von 2 mMol/kg Glutaminsäure vollständig ausgenutzt wird.

Frau L. von Hein danken wir sehr für die Ausführung der mikrobiologischen Bestimmungen.

Zusammenfassung

Nach intravenöser Verabfolgung von Glutaminat an Kaninchen wurde das Verhalten der freien Aminosäuren des Blutes auf zweidimensional-papierchromatographischem und auf mikrobiologischem Wege untersucht.

Durch Transaminierung entsteht Alanin aus Brenztraubensäure, während eine Zunahme von Asparaginsäure papierchromatographisch ausgeschlossen wurde.

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[7] H. Kamin u. P. Handler, Amer. J. Physiol. **164**, 654 [1951].
[8] P. P. Cohen u. G. L. Hekhuis, J. biol. Chemistry **140**, 711 [1941].

Die Gesamtmenge des gebildeten Alanins übersteigt den Brenztraubensäurevorrat des Blutes um das 5—10-fache.

Der Alaninanstieg ist in diesen Dosierungen unabhängig von der zugeführten Menge Glutaminsäure, von ihrer Applikationsweise und von der gleichzeitigen Verabreichung von Brenztraubensäure. Diese Tatsache spricht für eine begrenzte Kapazität des Glutaminsäure-Transaminasesystems in vivo.

Summary

Glutamate was administered intravenously to rabbits and the behaviour of the free amino acids in the blood subsequently investigated by means of two-dimensional paper chromatography and by microbiological methods.

Alanine is formed from pyruvic acid by transamination while the paper chromatography measurements excluded any increase of aspartic acid. The total amount of alanine formed is 5—10 times greater than the pyruvic acid content of the blood.

The increase in alanine in these experiments does not depend on the amount of glutamic acid administered, nor on the method of application, nor on the simultaneous administration of pyruvic acid. These facts point to a limited capacity of the glutamic acid-transaminase system in vivo.

4. Der Gehalt an freiem Ammonium im Blut ist nach parenteraler Injektion von Glutaminaten nicht erhöht.

5. Etwa gleichzeitig mit den Veränderungen des Blutspiegels der Glutaminsäure steigt der von α-Ketoglutarsäure, während der von Brenztraubensäure abfällt.

Summary

1. Glutamic acid administered intravenously disappears rapidly from the blood stream.

2. In the case of larger amounts and more rapid injection the ability of the metabolism to transform glutamic acid can be exceeded so that symptoms of intolerance appear.

3. On account of their free ammonium ions ammonium glutamate and ammonium chloride are approximately twice as toxic as sodium glutamate.

4. The free ammonium content in the blood is not increased after parenteral injection of glutaminate.

5. The α-ketoglutaric acid level in the blood increases more or less simultaneously with the changes in the glutamic acid level while that of pyruvic acid falls.

Untersuchungen über den Stoffwechsel der L(+)-Glutaminsäure

II. Mitt.: Verschiebungen im Plasmaspiegel freier Aminosäuren nach Glutaminsäuregaben

Von

V. Klingmüller, J. Gayer und F. Bramstedt

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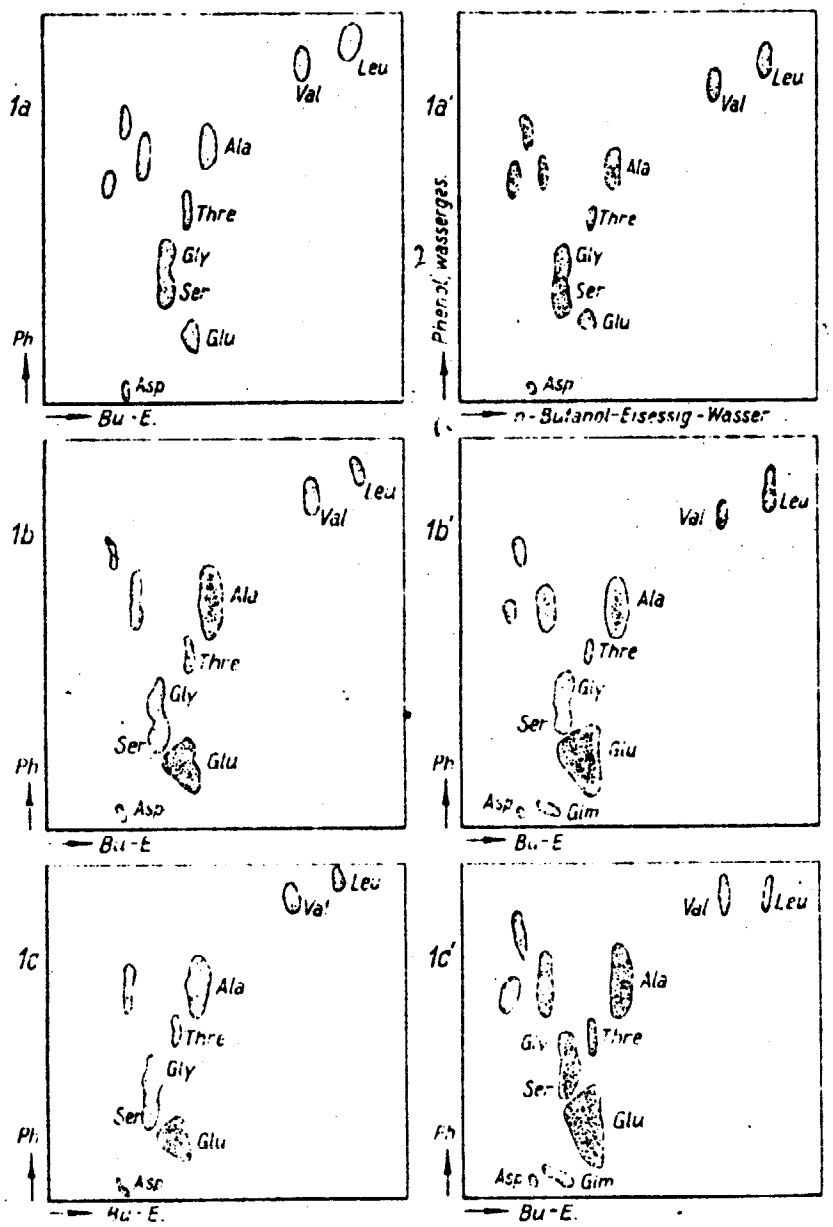

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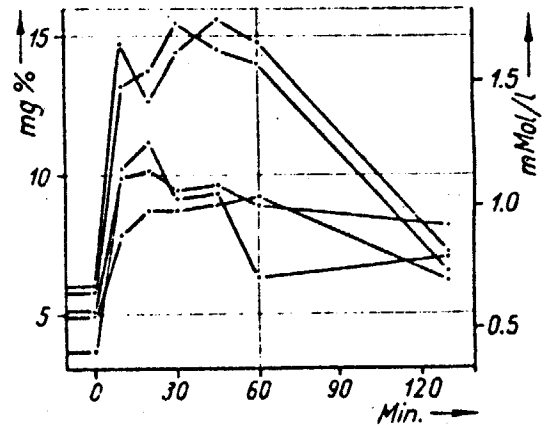

Abb. 2. Änderung des L-Alaninspiegels im Plasma nach i. v. Injektion von 2 mMol/kg Na-Glutaminat; Kaninchen.

identifizieren konnten und dessen Bedeutung weiter untersucht wird. Nach 120 Min. ist der Alaninwert wieder normal und der Fleck (Glm) verschwunden.

2. Mikrobiologische Bestimmungsmethoden

Zur weiteren Sicherung unserer papierchromatographischen Befunde und um genauere Vorstellungen über die Größe der Alaninvermehrung zu bekommen, bestimmten wir L-Alanin im Anschluß an Glutaminsäuregaben mikrobiologisch mit Hilfe von *Leuconostoc citrovorum NRRL* nach Baumann und Sauberlich[2]. Die Blutentnahmen erfolgten vor sowie 10, 20, 30, 45, 60 und 120 Min. nach der Injektion von Natriumglutaminat. Das gewonnene Plasma wurde nach Hier und Bergeim[1] mit Wolframat enteiweißt und eine 0,6 ml Plasma entsprechende Menge zum mikrobiologischen Test verwendet. Das Wachstum des Mikroorganismus wurde am Photometer Eppendorf nephelometrisch gegen eine Eichkurve bestimmt.

Abb. 2 gibt die Befunde bei 5 Kaninchen nach intravenöser Injektion von 2 mMol/kg Natriumglutaminat wieder; die Punkte der Kurven stellen Mittelwerte von Doppelbestimmungen dar. Die Größe der Alaninvermehrung entspricht unseren Schätzungen an den Ninhydrinflecken der papierchromatographischen Befunde.

Da die beobachtete Brenztraubensäure-Abnahme 1,0 bis 1,2 mg % entspr. 0,11 bis 0,14 mMol pro *l*, nicht übersteigt[1], kann das zum Aufbau des Alanins benötigte Pyruvat nicht allein aus dem Blut stammen, denn

[2] C. A. Baumann u. H. E. Sauberlich, J. biol. Chemistry 177, 545 [1949].
[1] S. Hier u. O. Bergeim, J. biol. Chemistry 161, 717 [1945].

der Alaninanstieg im Plasma beträgt mit im Durchschnitt 1,1 mMol pro l das etwa 5- bis 10fache der Brenztraubensäureabnahme. Es schien in diesem Zusammenhang von Interesse zu prüfen, ob durch Erhöhung der injizierten Glutaminsäuremenge oder durch gleichzeitige Injektion

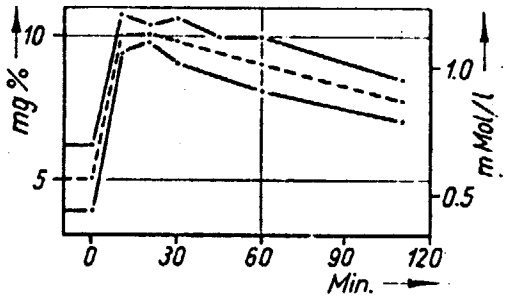

Abb. 3. Änderung des L-Alaninspiegels im Plasma nach Injektion von 4 mMol/kg Na Glutaminat: Kaninchen.

von Pyruvat die Hyperalaninämie verstärkt werden kann. Abb. 3 zeigt das Verhalten von L-Alanin nach intravenöser Verabfolgung von 4 mMol/kg Glutaminat. Der Anstieg des Alanins entspricht der unteren Grenze der in Abb. 2 dargestellten Kurven, ist also gegenüber dem nach Injektion von 2 mMol/kg Glutaminat nicht erhöht. Die gestrichelte Kurve gibt die Mittelwerte an. Im Vergleich zu Abb. 2 fällt der wesentlich langsamere Abfall des Alaninspiegels auf.

In einem weiteren Versuch prüften wir den Einfluß gleichzeitiger Verabfolgung äquimolarer Mengen von Pyruvat und Glutaminat auf den Alaninspiegel. In Abb. 4 ist das Ergebnis dieses Versuches wiedergegeben,

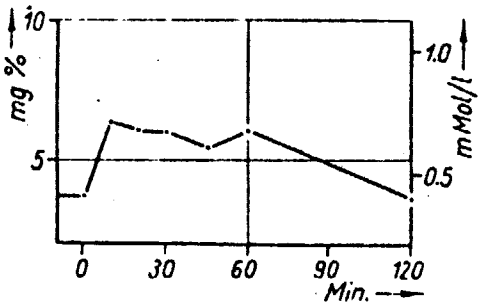

Abb. 4. Änderung des L-Alanin-Spiegels im Plasma nach gleichzeitiger Injektion von 2 mMol/kg Glutaminat und 2 mMol/kg Pyruvat; Kaninchen.

bei dem 2 mMol/kg Glutaminat und 2 mMol/kg Pyruvat gleichzeitig in 2 verschiedene Venen injiziert wurden. Auch hier besteht kein Unterschied gegenüber dem Versuch ohne zusätzliche Pyruvatgabe. Der gleichzeitig nach Hagedorn-Jensen untersuchte Blutzucker zeigte einen geringen Anstieg von 123 auf 142 mg%, der eigenen und anderen Versuchen[5,6] über das Verhalten des Blutzuckers nach Glutaminsäureverabfolgung entspricht.

[5] V. Klingmüller u. J. Gayer, Verh. dtsch. Ges. inn. Med. 60, 960 [1954].
[6] W. A. Himwich, Amer. J. Physiol. 1954, persönl. Mitteilung.

Nach oraler Verabfolgung von 2 mMol/kg Glutaminsäure konnten wir Alaninvermehrungen in der Größenordnung zwischen 4 und 6 mg% beobachten, deren Maximum zwischen 45 und 75 Min. nach der Zufuhr erreicht war und die nach 3—4 Std. zur Ausgangslage zurückgekehrt waren.

Diskussion

Unsere Versuche in vivo bestätigen die vorliegenden Erfahrungen über die Transaminierungen. Bei Versuchen in vitro wurde von Braunstein und Kritzmann die Fähigkeit der Glutaminsäure nachgewiesen, durch Transaminierung Alanin aus Brenztraubensäure zu bilden. Andererseits kann in vitro auch Asparaginsäure durch Transaminierung gebildet werden. Kamin und Handler[7] fanden beispielsweise, daß beim Hund nach Dauerinfusion von Glutaminat besonders große Mengen von Asparaginsäure gebildet und im Harn ausgeschieden werden. Wenn wir keine wesentliche Vermehrung von Asparaginsäure feststellen konnten, so bedeutet das kaum einen Gegensatz zu Kamin und Handler, weil wir Glutaminsäure in einmaliger Injektion verabreicht haben. Auch Cohen und Hekhuis[8] fanden, daß die Leber aus Glutaminat weniger Asparaginsäure bildet als Alanin.

Der von uns nachgewiesene Alaninanstieg übersteigt den im Serum vorhandenen Vorrat an Brenztraubensäure, so daß die für diesen erheblichen Anstieg benötigte Brenztraubensäure wahrscheinlich aus dem Kohlenhydratstoffwechsel stammt. Die Tatsache, daß weder die Verdoppelung der injizierten Glutaminsäuremenge noch die gleichzeitige Verabfolgung von Pyruvat zu einer weiteren Vermehrung der Alaninmenge führt, und auch die orale Applikation von Glutaminsäure nach der durch die Resorption bedingten Verzögerung den gleichen Anstieg des Blutalaninspiegels bewirkt wie parenterale Gabe, mag ihre Erklärung in der Annahme finden, daß das Transaminasesystem des Gesamtorganismus eine begrenzte Kapazität besitzt, die durch die zugeführte Menge von 2 mMol/kg Glutaminsäure vollständig ausgenutzt wird.

Frau L. von Hein danken wir sehr für die Ausführung der mikrobiologischen Bestimmungen.

Zusammenfassung

Nach intravenöser Verabfolgung von Glutaminat an Kaninchen wurde das Verhalten der freien Aminosäuren des Blutes auf zweidimensional-papierchromatographischem und auf mikrobiologischem Wege untersucht.

Durch Transaminierung entsteht Alanin aus Brenztraubensäure, während eine Zunahme von Asparaginsäure papierchromatographisch ausgeschlossen wurde.

[7] H. Kamin u. P. Handler, Amer. J. Physiol. 164, 654 [1951].
[8] P. P. Cohen u. G. L. Hekhuis, J. biol. Chemistry 140, 711 [1941].

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Die Gesamtmenge des gebildeten Alanins übersteigt den Brenztraubensäurevorrat des Blutes um das 5 - 10-fache.

Der Alaninanstieg ist in diesen Dosierungen unabhängig von der zugeführten Menge Glutaminsäure, von ihrer Applikationsweise und von der gleichzeitigen Verabreichung von Brenztraubensäure. Diese Tatsache spricht für eine begrenzte Kapazität des Glutaminsäure-Transaminasesystems in vivo.

Summary

Glutamate was administered intravenously to rabbits and the behaviour of the free amino acids in the blood subsequently investigated by means of two-dimensional paper chromatography and by microbiological methods.

Alanine is formed from pyruvic acid by transamination while the paper chromatography measurements excluded any increase of aspartic acid. The total amount of alanine formed is 5 - 10 times greater than the pyruvic acid content of the blood.

The increase in alanine in these experiments does not depend on the amount of glutamic acid administered, nor on the method of application, nor on the simultaneous administration of pyruvic acid. These facts point to a limited capacity of the glutamic acid-transaminase system in vivo.

Über die Polyenfettsäuren der Leberphosphatide

Von

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(Der Schriftleitung zugegangen am 11. Januar 1955)

Die bisherige Untersuchung[1,2] der C_{20}- und C_{22}-Polyensäuren der Glycerinphosphatide des Gehirns (Mensch) hat ergeben, daß es sich hier um ein sehr komplexes Säuregemisch handelt, daß aber die Doppelbindungen in der Kohlenstoffkette der einzelnen Komponenten durchweg in der Divinylmethangruppierung angeordnet sind. Außerdem ließen die Befunde darauf schließen, daß, von der endständigen Methylgruppe aus gerechnet, die erste Doppelbindung in allen diesen Säuren in Stellung 3, 6 oder 9, also an derselben Stelle wie in Linolensäure, Linolsäure oder Ölsäure sich befindet. Bis jetzt konnte das Vorhandensein einer Polyensäure vom Linolensäuretyp* ($\Delta^{4,7,10,13,16,19}$-Dokosahexaensäure) und von drei verschiedenen Vertretern vom Linolsäuretyp** ($\Delta^{6,8,11,14}$-Eikosatetraensäure, $\Delta^{7,10,13,16}$-Dokosatetraensäure und $\Delta^{4,7,10,13,16}$-Dokosapentaensäure) sichergestellt werden.

Um der hier erkennbar werdenden Eigenart im chemischen Aufbau dieser im Tierreich sehr verbreiteten Polyensäuren weiter nachzugehen, haben wir jetzt die Untersuchung auch auf die Polyensäuren der Leberphosphatide (Rind) ausgedehnt, in welchen sie den Hauptanteil der ungesättigten Fettsäuren ausmachen. Schon vor längerer Zeit[3] konnte gezeigt werden, daß unter diesen ebenfalls C_{20}- und C_{22}-Polyensäuren nebeneinander vorkommen. Jedoch fanden sich in nachweisbaren Mengen auch C_{18}-Polyensäuren, welche in den Gehirnphosphatiden fast vollständig fehlten.

Wir haben zunächst eine größere Menge (53 g) des Gemischs der C_{20}- und C_{22}-Polyensäuren, von welchen die C_{18}-Säuren durch fraktionierte Esterdestillation so vollständig wie möglich abgetrennt waren, durch Ozonidspaltung nach der Methode von Klenk und Bongard[4] abgebaut. Nach Trennung der Abbausäuren in die Monocarbonsäuren

* d. h. Polyensäuren, in welchen sich die erste Doppelbindung, von der endständigen Methylgruppe aus gerechnet, ebenso wie in der Linolensäure in Stellung 3 befindet.

** d. h. Polyensäuren, in welchen sich die erste Doppelbindung, von der endständigen Methylgruppe aus gerechnet, ebenso wie in der Linolsäure in Stellung 6 befindet.

[1] E. Klenk u. W. Bongard, diese Z. 291, 104 [1952].
[2] E. Klenk u. F. Lindlar, diese Z. 299, 74 [1955].
[3] E. Klenk u. O. v. Schoenebeck, diese Z. 209, 112 [1932].
[4] E. Klenk u. W. Bongard, diese Z. 290, 181 [1952].

German translation

Synergism by vitamins of glutamic acid action

by

Josef Klosa

Pharmazeutische Zeitung 100, 1298-9, 1955)

According to Burgi's principle, the effect exerted by two drugs is not only additive, but in some cases can also be a synergistic effect. Special drug combinations have been prepared according to this principle in the field of mixed analgesics, sleep-inducing drugs and recently also in sulfonamide therapy. It is therefore understandable that attempts have been made to achieve a synergistic effect also with amino acids, and in particular with glutamic acid, by combination with suitable active principles. In many respects, a certain necessity can be noted here, first, in order to reduce the huge dosages of glutamic acid required for successful therapy, and thereby to increase also the digestibility, and second, in order to achieve a definite effect of glutamic acid, since the successful results obtained with this acid are, as we know, still disputed in spite of the large amount of work done in this field. This fact is not surprising, in view of the lack of an objective animal test allowing us to judge the value (or lack of value) of glutamic acid, since the known intelligence tests are to a great extent dominated by suggestive influence and illusions.

In our search for a suitable objective test, we have concentrated our attention on the cramp symptoms observed in animals which have been fed isonicotinic acid hydrazide. It is known that ammonia is formed as a metabolic product in the organism after administration of isonicotinic acid hydrazide; nervous side effects, and primarily convulsions, are attributed to ammonia. In animal experiments, Curei and del Torre(1) were able to suppress these nervous symptoms and to reduce the toxicity of isonicotinic acid hydrazide by about 41% by simultaneous administration of glutamic acid. The question of major interest to us was to what extent vitamins B_{12}, B_1 and B_6, and finally folic acid, when combined with glutamic

acid are capable of influencing the toxic side effects of isonicotinic acid hydrazide. We followed the procedure used by Curei and del Torre, modifying it in such a way that the following preparations were given per os to 5 guinea pigs* for 5 days prior to administration of the known toxic dose (600 mg/kg) of isonicotinic acid hydrazide.

Table on p.1298

1. L-glutamic acid (salt-free)
2. Monosodium glutamate
3. Disodium glutamate
4. L-glutamic acid hydrochloride
5. L-glutamic acid with 5 ug B_{12}
6. L-glutamic acid with 5 ug B_{12}, 100 ug folic acid
7. L-glutamic acid with 300 ug B_1
8. L-glutamic acid with 800 ug B_1, 5 ug B_{12}, 600 ug B_6 and 100 ug folic acid
9. Bioglutan (2) (Contains the entire vitamin B complex and vitamin B_{12})
10. Monosodium glutamate and 400 ug B_6

The amounts of glutamic acid given above represent the per os dosage which prevented death or cramps after administration of the lethal dose of isonicotinic acid hydrazide. Accordingly, salts of glutamic acid exert a weaker effect than pure L-glutamic acid. By combination with B_{12} and particularly B_6, as well as with B-complex vitamins (e.g. Bioglutan), the toxicity of isonicotinic acid hydrazide is considerably reduced. Therefore, vitamins appear to play an important role for a better utilization of glutamic acid in the organism; they exert a potentiating (synergistic) action or the glutamic acid effect (3).

Summary

In the anti-cramp test, glutamic acid salts exert a different effect. Simultaneous feeding of vitamins of the B-complex, and particularly B_6, increases the action of glutamic acid.

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* Rabbits received a dose of 700-800 mg/kg isonicotinic acid hydrazide. To avoid cramps, a 3 to 10-fold amount of glutamic acid preparation was required.

Potenzierung der Glutaminsäurewirkung durch Vitamine

Von Dr. rer. nat. Josef Klosa

Nach dem Bürgischen Prinzip braucht sich die Wirkung zweier Heilmittel nicht allein additiv zu verhalten, sondern kann sich in besonderen Fällen auch potenzieren. Besondere Kombinationsschöpfungen sind nach diesem Prinzip unter den analgetischen Mischpräparaten, auch bei Schlafmitteln und neuerdings in der Sulfonamidtherapie geschaffen worden. Es ist nur verständlich, wenn man versucht, auch bei den Aminosäuren und insbesondere der Glutaminsäure durch Kombination mit geeigneten Wirkstoffen einen potenzierten Effekt zu erreichen. Hier zeigt sich in vieler Hinsicht eine gewisse Notwendigkeit, einmal um die enormen Dosen von Glutaminsäure, welche für eine erfolgreiche Therapie nötig sind, reduzieren zu können und damit auch die Bekömmlichkeit zu steigern, ein andermal, um einen sicheren Effekt der Glutaminsäure zu erreichen, da ja bekanntlich trotz der intensiven Bearbeitung dieses Gebietes die Erfolge mit Glutaminsäure immer noch umstritten sind. Und dies ist bei dem Mangel eines objektiven Tiertestes zwecks Aussage über den Wert oder Unwert der Glutaminsäure nicht zu verwundern, denn die bekannten Intelligenzteste werden weitgehend von suggestiven Einflüssen und Täuschungen beherrscht.

Bei der Suche nach einem geeigneten objektiven Test konzentrierten wir uns auf die Krampferscheinungen, welche bei Tieren nach Verfütterung von Isonikotinsäurehydrazid auftreten. Bekanntlich tritt im Organismus als Stoffwechselprodukt nach Isonikotinsäurehydrazidzuführung Ammoniak auf. Diesem werden in erster Linie die Nebenwirkungen nervöser Art, vor allem konvulsive Erscheinungen, zugeschrieben. Curei und del Torre (1) konnten im Tierversuch durch gleichzeitige Zuführung von Glutaminsäure diese nervösen Erscheinungen unterdrücken und die toxische Wirkung des Isonikotinsäurehydrazids um ca. 41% senken. Für uns war besonders die Frage bedeutungsvoll, inwiefern Vitamin B_{12} sowie B_1 und B_6 und schließlich Folsäure in Kombination mit Glutaminsäure die toxischen Nebenwirkungen des Isonikotinsäurehydrazids zu beeinflussen vermögen. Wir hielten uns an die Versuchsanordnung von Curei und del Torre und modifizierten dieselbe in der Weise, daß je fünf Meerschweinchen*) fünf Tage hindurch vor der Zuführung der bekannten toxischen Dosis des Isonikotinsäurehydrazids von 600 mg/kg folgende Präparate peroral erhielten:

Nr.	Dosierung mg/kg	Substanz
I	600	L-Glutaminsäure (salzfrei)
II	580	Mononatriumsalz derselben
III	720	Dinatriumsalz derselben
IV	720	Hydrochlorid derselben
V	430	L-Glutaminsäure mit 5 γ B_{12}
VI	420	L-Glutaminsäure mit 5 γ B_{12}, 100 γ Folsäure
VII	480	L-Glutaminsäure mit 300 γ B_1
VIII	340	L-Glutaminsäure mit 800 γ B_1, 5 γ B_{12}, 600 γ B_6 und 100 γ Folsäure
IX	410	Bioglutan²) (enthält den gesamten Vitamin-B-Komplex sowie B_{12})
X	400	Mononatriumglutaminat und 400 γ B_6

*) Bei Kaninchen wurden 700—800 mg/kg verabreicht. Zur Verhütung der Krämpfe war die 3—10fache Menge an Glutaminsäurepräparaten nötig.

Die angegebenen Glutaminsäuremengen stellen diejenige Dosis per os dar, durch welche der Tod sowie Krämpfe nach Zuführung der letalen Dosis von Isonikotinsäurehydrazid unterblieben. Danach sind also die Salze der Glutaminsäure schwächer wirksam als die reine L-Glutaminsäure. Durch Kombination mit B_{12} und ganz besonders B_6 sowie den Vitaminen des B-Komplexes (z. B. Bioglutan) wird die Toxizität des Isonikotinsäurehydrazids erheblich gesenkt. Danach scheinen die Vitamine für eine bessere Verwertung der Glutaminsäure im Organismus eine Bedeutung zu besitzen, sie entfalten auf dem Glutaminsäureeffekt eine potenzierte Wirkung (3).

Zusammenfassung

Im Antikrampftest sind die Salze der Glutaminsäure unterschiedlich wirkend. Durch gleichzeitige Verfütterung von Vitaminen des B-Komplexes — insbesondere B_6 — wird die Wirkung der Glutaminsäure gesteigert.

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Electron Microscopic Observation on the Rabbit Retina Treated with Long Term Daily Administration of Small Dose of Sodium Iodate or Sodium l-Glutamate

By Hideji Kobayashi

I. Introduction

There have been many reports of the study of the experimental change of the retina by sodium iodate(afterwards abbreviated as I) or the selective retinal lesion by sodium l-glutamate(afterwards abbreviated as G). But the effect of long administration of G or I on the retina was only studied histologically and electrophysiologically by Hamatsu[1] and Imaizumi et al[2] and Imaizumi[3] in our laboratory, but it has not been investigated microscopically.

The author made this experiment in order to clarify what electron microscopic changes happen to the adult rabbit retina, when long term administration of small dose of I, which brings a lesion on the pigment epithelial layer of the retina at the biginning of administration, and of G, which selectively brings a lesion on the bipolar cell layer and the ganglion cell layer under a certain condition, causes electrophysiological changes.

II. Experiment

1) The method of the drug administration

Adult albino rabbits, heavier than 2.0kg, were used for the experiment after they had been kept under a certain con-

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74: 902-908 (Aug. 19[illegible])

dition for more than a week and it had been made sure that their ERG and EOG, and eyegrounds had been normal.

A daily dose of I (1.0 mg/kg of 2.0% solution) was administered into the auricular vein as I group, and a daily dose of G (0.1g/kg of 25.0% solution) as G group, for consecutive 50 days.

On the 10th day after the administration, the eyes of three rabbits, and on the 30th day, the eyes of three rabbits, and on the 50th day, the eyes of three rabbits were taken out to be observed. The date to take out those eyeballs was decided according to Imaizumi's[3] result that on these days, the changes in amplitude of ERG and EOG were intense.

2) The preparation method of the sample for electron microscopy

After these eyeballs were soaked in 2.0% glutaraldehyde (phosphoric acid buffur solution, pH 7.3), the cornea and the crystalline lens were taken out, and they were fixed for one and a half hours at 4°C, and then they were fixed by 2.0% osmium tetra oxide solution for one hour at 4°C.

The retina was cut into ten pieces, their length 5mm, which were dried by alcohol and put into Epon 812 by Luft[4] method.

Ultra-thin pieces were doubly stained by uranium acetate and lead citrate, and examined with Hitachi HU-11 DS electron microscope, of which picture was taken in 2800-3500 magnification.

To count synaptic vesicle, triad and a synaptic body were chosen and observed. The former is the nearest to the nuclei

of the visual cells and has the process of the bipolar cells and the process of the horizontal cell. In the latter, one synaptic ribbon was observed. The size of the synaptic, vesicle was determined by measuring the largest diameter of indiscriminately chosen 500 synaptic vesicles, and the number was determined by taking the average from the 25 squares with 4.5cm of side.in the electromicroscopic picture of 90000 magnification.

III. Results

A. Electron Microscopical Findings in I Group

1) The Findings on the 10th Day of Administration (See Fig 1 and 7, and Table 1)

The Bruch membrane was a little unclear, but no change was observed on the pigment epitherial cell layer and the internal and external ganglion cells. (Fig 1) The external prexiform layer was almost normal, and the membrane, ribbon, and vesicle of the synaptic body were all clear. (Fig 7)

79% of synaptic vesicles were 41-50nm in size, and big diametered ones increased. The number of the synaptic vesicles were 35, and it was a little more than normal.

2) The Findings on the 30th Day of the Aministration (See Fig. 2,8 and Table)

It was impossible to distinguish five layers in the Bruch membrane, and it seemed it was one band, and in the center of it, there was a form of blank cell. The basalin-

folding in the pigment epitherial cell layer was comparatively distinct, and relatively a lot of the free ribsomes and coarse vesicles in the cytoplasm were observed. But the cristae of the mitochondria near the ground part had vanished, and a part of the limiting membrane had vanished.

The micrvilli in the pigment epitherial cells and the point of the outer segment of the visual cells are apt to separate from each other, and the membrane of the outer segment vanishes where it separates from the micrvolli.

In the inner segment of the visual cells, the mitochondria, the cristae of which was not distinct, was observed in a part of ellipsoid, and there were a lot of small coarse vesicles. The shape of the vesicles in the inner segment of the external limiting membrane was not circle.(Fig. 2)

As for the synaptic body in the external prexiform layer, the membrane, ribbon, and vesicle were all distinct.(Fig. 8) 42% of the vesicles were 51-60nm in size, big vesicles were more than small ones. The number was about 28, and it decreased less than before.(Table 1)

3) The Findings on the 50th Day of the Administration (See Fig 3,9 and Table 1)

It was impossible to distinguish five layers in the Bruch membrane in which electron density decreased. A little basal-infoldings in the pigment epitherial cell layer were just unclearly observed. The smooth vesicles in the basic cytoplasm were broken, and the electron density was not homogenious. But the existence of free ribosome and coarse vesicles was comparatively remarkable. Most of the mitochondria had lost their

2-r-6

original shapes, and there became less electrons in the base, and it turned into vacancies. The microvilli was separate from the external segment.

Only part of the membrane of the outer segment of the visual cell remained, and the line of the layer bodies were disordered, and the spaces between them were unequal. In the layers, small vacancies were formed. (Fig. 3) In the inner segment of the external limiting membrane were circle or oval, but the number was small.

The filament of Müler cell in the external prexiform layer or glycogen glanules were hardly observed. This finding is very different from that of the 10th and 30th day after the administration. Within the region from Bruch membrane to the external limiting membrane, there were observed a lot of changes on the Bruch membrane, the pigment epithelial cell, mitochondria, basal-infolding, smooth vesicles and layer bodies of the inner segment of the visual cells. On the other hand, the shapes of the membrane of the synaptic bodies, the ribbons, and the vesicles were kept well.(Fig. 9) About 43% of the vesicles were 41-50nm. and 48% of the vesicles were 51-60nm in size. Most of them became bigger in size and the number was 25, which was less than normal. (Fig. 1)

B. Electron Microscopical Findings in G Group

1) The Findings on the 10th Day after the Administration (See Fig 9 and Table 1)

The cytoplasm of the Bruch membrane and the pigment epi-

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the ial cell were almost the same as normal ones. The electron density in the basic substance of mitochondria in the epithelial cell became less. In the mitochondria, vacancies were observed. There were few coarse vesicles. The inner and outer segments of the visual cells were normal.

The Muller cells in the external plexiform layers were normal, and the membrane, ribbons, and vesicles of the synaptic bodies were distinct.(Fig 9)

About 76% of vesicles were 41-50mm and 20% of vesicles were 51-60mm in size. There was a tendency that the number of the vesicles of a small diameter increased. The number was about 32, which was a little more than normal.(Table 1)

2) The Findings on the 30th Day after the Administration (See Fig. 4, 11 and Table 1)

The five layers of the Bruch membrane became a little indistinct. The basal-infolding on the pigment epithelial layers was distinct, and the cytoplasm was normal. There scattered mitochondria with substances of layer structure and vacancies in their center.

Microvilli was inclined to separate from the outer segment of the visual cells. There was a formation of vacancies near free surface. A part of the outer segment membrane had vanished, and a part of it ran in waves,(Fig. 4) The inner segment of the visual cells were almost normal. The shape of the vesicles in the inner segment in the external limiting membrane remained circular, but the number was a little less than normal.

The cytoplasm of the Müler cells in the external prexiform layers was almost normal. The membrae, ribbone, and the vesicles of the synaptic bodies were all distinct. (Fig. 11) About 34% of the vesicles were 31-40nm and about 52% of the vesicles were 41-50nm in size. Most of them were small in size, and the number also decreased. (Table 1)

3) Findings on the 50th Day of the Administraion
(See Fig 5,6 and 12, Table 1)

Five layers of the Bruch membrane were indistinct. In the center of the layer, a little vacancies were formed. The basal-infolding in the pigment epithelial cells were quite indistinct. The electron density in the base of the cell bodies was inhomogeneous and the normal form was broken. Free ribosome and coarse vesicles increased. The cristae, and limiting membrane of mitochondria had vanished, and it was turned into vacancies. The microvilli was also all separate.

The lines of the layer bodies in the outer segment of the visual cell were disordered, and the limiting membrane was indistinct. The ellipsoid in the inner segment of the visual cells were filled with more indistinct mitochondria and coarse vesicles than in the I group on the 50th day of the administration. The shape of the vesicle of the inner segment in the external prexiform layer remained circle but the number was less. (Fig 5)

The electron density was small in the cytoplasm of the Müler cell in the external prexiform layers. The glycogen granules,

2,3

and the basic cell bodies were indistinct. (Fig. 6) The limits of the synaptic membrane and ribbons on this layer were a little indistinct. The form of the most synaptic vesicle was especially quite indistinct. The order was disordered, too.(Fig. 12) The number of the vesicles was about 12, and it was less than a half of the normal. About 73% of the vesicles were 31-40mm in size. Clearly the size became smaller.

IV. Discussion

Suyama[5] and Grignolo et al[6] reported after the electron microscopical examination that the retina change of the adult rabbits by a large dose of I by the intravenous injection occurred in the pigment epitherial cells and gradually developed progressively into the internal layer.

On the other hand, Wald et al[7] electrophysiologically examined how G acted on each layer of the retina, after incubating the turtles retina into the mixture solution of isotonic Ringer's solution and G, and they indicated that the synapse in the internal plexiform layer and the nerve elements clearly swelled, and that mitochondria turned into vacancies, and that vesicles almost completely vanished, but that other retinal layers remained normal completely.

Cohen[6] injected a daily large dose of G to infant mice for consecutive ten days, and examined the retinal electron-microscopically, and got the result that the inner layers from the inner molecular layers were selectively broken, but that each tissue of the remaining retinal inner layer, for example, the

bipolar cells or synaptic body, quite hardly changed.

These above investigators electron microscopically examined about the retinal change prduced by a large dose of I and G administration. So naturally it is quite different from the author's experiment of the change by a long term small dose of administration.

By the author's histological findings on the 10th and 30th day of the drug administration, most are similar in I group and G. There can be observed only a little change in the Bruch membranes and the pigment epithelial cells. But on the 30th day, a little more change can be observed in Group I than in group G.

On the 50th day, the changes by I and G are very distinct in the Bruch membrane, the pigment epithelial cells, and the outer and inner segments of the visual cells, and the external plexiform layers.

It is remarkably interesting that there is a tendency that the changes by I are intense in the Bruch membrane, the pigment epithelial cells and the outer segment of the visual cells, and those by G are intense in the inner segment of the visual cells and the external plexiform layer.

According to Imaizumi's[3] experiment about the electrophysiological change of the rabbit eyes on administration of G and I under the same condition as the author's, the ERG and EOG on I administration became very different from those on G administration after the 40th day. Before the 40th day, a daily change in amplitude of ERG and EOG goes paralell both by G and I, but on the other hand, after the 40th day, they go on quite a different process ~~by I and G.~~

(10)

A daily process of ERG and EOG by I after the 40th day coincides with the histological findings, but it is impossible to explain that by G histologically. Probably the electrophysiological changes by G have almost nothing to do with the histological changes in the organs of cells, and they are supposed to be caused by the decrease of the activity of various kinds of enzymes in metabolic system.

The cause of the retinal change by I is said to be not only the disturbance of catabolism of glucose and breathing but also the disturbance of the metabolism of maltiglucose and nucleic acid. That by G is assumed to be, as Potts suggested, the decrease of activity of five enzymes concerning G metabolism. So the changes of the pigment epithelial cells and the small organs in the inner segment of the visual cells, which require high enzyme activity, are noticeable.

A daily change of the synaptic body on the external plexiform layers is a noticeable phenomena. The number of the vesicles temporally increases administration, but thereafter, it decreases. The rate of the decrease by G is higher than that by I. The size of each vesicle becomes bigger daily by I, but by G, it becomes smaller. Generally, the change of the vesicle by G is intenser than by I.

There are two kinds ~~in~~ of synaptic bodies: one belongs to the conical and the other belongs to the cylindrical.[10-12] But as Akitani[13], it was difficult also for the author to distinguish the two kinds.

About the change of the synaptic vesicles in the external plexiform layers after the drug administration, Suyama,[5] who

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(11)

I for his experiment, and Lasanski et al[14] and Akitani,[13] who used Iodoacetic acid, only reported that the vesicles decreased, As far as the author looked for, there is no report about a daily change of the vesicles by drugs. Wald,[7] who used G, reported that the synaptic bodies in the external plexiform were almost normal, but those on the internal plexiform layers changed a lot, and the vesicle vanished. But the conditions of the experiment are different, so it is impossible to compare with the author's. The physiological significance of the synaptic vesicles is not fully clear according to McLennan[15], Kurtz[10], Heinz,[11] and Yamada.[12] But it is almost considered a chemical substance which transmit nervous excitement, that is, the substance with a transmitter. As a kind of transmitter within the vesicles, there is a monoamine like catecholamine.[16][17] It is noticeable that the change of the vesicle by G, which is an acidic aminoacid existing naturally in the retina, is intenser than that by I.

In Imaizumi's[3] experiment by I and G, on the 12th to 15th day after the administration, when the retinal change can't be observed histologically, a remarkable change in amplitude of ERG and EOG was observed. This is an interesting coincidance with the author's result of the increase of the number of the synaptic vesicles in the external plexiform layers on the 10th day.

It is an important new finding that the vesicle with a transmitter is systemtically and progressively changed in size and in number by the consecutive administration of I and G. But in this experiment, the meaning of it can't be clarified. It is the question to be fully examined in a future studies.

V. Conclusion

Comparing with Imaizumi's[3] electrophysiological findings, the retinal changes of albino adult rabbits from the Bruch membrane to the external plexiform by the non-oral daily administration of small dose of I and G for consecutive 50 days, were electron microscopically examined, and the following conclusions were obtained.

1) The histological change caused by I and G developed progressively daily from the outer layer to the inner layer of the retina, and the initial appearance and the progressing speed of the change by I were earlier and more quickly than by G.

2) On the 10th and 30th day of the administration, the histological appearence of the retina, as well as the electrophysiological results, were similar in I and G group. However, on the 50th day, in the I group, electron microscopically, the Bruch's membrane, the pigment epithelial cells and outer segment of the visual cells were affected markedly. Also electrophysiologically, abnormal ERG and EOG were recorded. On the other hand, in the G group, the inner segment of the visual cells and the external plexiform layer were affected severely in spite of recording nearly normal ERG and EOG.

3) On the 10th day of the administration of I or G, the number of the vesicles within the synaptic bodies in the external plexiform layer increased. During this period, there were remarkable fluctuations in amplitudes of ERG and EOG. On the 30th and 50th day decrease in number of the vesicles and their morphological changes were found. These findings of the vesicles were more remarkable in the group G than in group I.

Symposium

D. Yonemura:

Please tell me whether the presynaptic terminal in which you observed the presynaptic vesicle was connected to bipolar cell or horizontal cell.

K. Honda: About the connection of synapse

1. Bipolar cell is direct connection
2. Horizontal cell is indirect connection

I think I heard so.

Mizuno:

Have you ever done the experiment of the same kind using some metabolites or derivatives of amino acid? If you use glutathione or GABA, it will be useful to elucidate the mechanism of the degeneration.

H. Kobayashi (Answer):

To Yonemura

In the present experiment presynaptic body was examined electron microscopically. But it is very hard to distinguish them.

To Mizuno

Besides two drugs which were used in this experiment, sodium asparaginate is being used for the experiment. I am going to examine about various drugs in future.

LIterature

1) T. Hamatsu, Nippon Ganka Gakkai Zasshi,

2) K. Imaizumi,

3) S. Imaizumi, to be published.

4) T. Suyama,

12) E. Yamada, Picture Book of Cell Tissue by Electron Microscope, Vol. IV, Igaku Shoin,

13) S. Akitani,

Explanation of Pictures

The scale in each picture represents 1μ, if nothing is indicated.

Fig. 1 : On the 30 th day of I administration.

Fig. 4 : On the 30 th day of G administration.

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41. 薬物長期小量継続投与家兎網膜の電子顕微鏡的観察（図12，表1）

小林秀樹（岩手医科大学医学部眼科学教室 主任：今泉亀撥教授）

Electron Microscopic Observation on the Rabbit Retina Treated with Long Term Daily Administration of Small Dose of Sodium Iodate or Sodium l-Glutamate

Hideji KOBAYASHI

Department of Ophthalmology, School of Medicine, Iwate Medical University.

(Director: Prof. K. IMAIZUMI)

Morioka-shi

A study was made with the purpose of attempting to correlate the electron microscopic and electrophysiological changes in the retina of the adult rabbits induced by daily administration of small dose of sodium iodate (I) (0.1 mg/kg of 2.0% solution) or sodium l-glutamate (G) (0.1 gr/kg of 25% solution) for 50 days.

1) The retinal lesion caused by I or G was noted to develop progressively from the outer to inner layer of the retina. The initial appearance and degree of the retinal destruction were earlier and more severe in the rabbits treated with I than with G. This fact coincided with the findings obtained from the electrophysiological studies such as ERG and EOG.

2) On the 10th and 30th day of the injection, the histological appearance of the retina, as well as the electrophysiological results, were similar in I and G group. On the 50th day, however, the I group showed different features from the G group in electron microscopic and electrophysiological examinations. Namely, in the I group, electron microscopically, the Bruch's membrane, the pigment epithelial cells and the outer segment of the receptor, cells were damaged markedly. Also electrophysiologically, abnormal ERG and EOG were recorded. On the other hand, in the G group, the inner segment of the receptor cells and the outer plexiform layer were affected severely in spite of recording nearly normal ERG and EOG.

3) On the 10th day of the administration of I or G, the number of the vesicles within the synaptic bodies in the outer plexiform layer increased more than in the normal retina. During this period, there were conspicious fluctuations in amplitudes of ERG and EOG. However, decrease in number of the vesicles and their morphological changes were found in the retina treated for 30 and 50 days. These findings of the vesicles were more remarkable in the group G than in group I.

I 緒言

sodium iodate（以下I）による実験的網膜変性の研究，或は sodium l-glutamate（以下G）による選択的網膜障害の研究に就ての報告は多数みられる．しかし，小量のI或はGの長期継続投与が網膜に与える影響に関しては，教室の浜津[1]，今泉ら[2]及び今泉[3]によつて光学顕微鏡的並びに電気生理学的に追求されているにすぎなく，その電子顕微鏡的所見の詳細は不明である．

網膜の色素上皮細胞層を投与初期に障害するIと，一定条件下で両極細胞層と神経節細胞層とを選択的に障害するGの，小量長期継続投与が，電気生理学的に異常を来す時点で，成熟家兎網膜に，電顕的に如何なる障害を与えるかの問題を確かめるため，著者は本実験を行つた

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II 実験方法

1) 薬物投与方法

実験動物は，2.0kg以上の白色成熟家兎を用い，1週間以上一定条件下で飼育し，ERG，EOG及び眼底に異常がないことを確認してから実験に供した．

I投与は，2.0%I水溶液 1.0mg/kgを耳静脈内に，G投与は，25.0%G水溶液 0.1g/kgを腹腔内に，1日1回，連日50日間注射した．

観察眼は，薬物投与10日目（3羽），30日目（3羽）及び50日目（3羽）に摘出した．摘出日の決定は，その時点でERG及びEOGの振巾変動が著明であることを見出した今泉[3]の成績によつた．

2) 検査資料作製法

摘出眼球は，2.0% glutaraldehyde（燐酸緩衝液，pH 7.3）に浸して角膜及び水晶体を除去し，4℃で1.5時間，次いで，4℃の2.0%四酸化オスミウム液で1時間，後固定した．

網膜は，乳頭より外側へ8mmの点を中心として，5mmの正方形小片を，強膜を付けて10個採り，アルコール系列で脱水し，Luft[4]法に従つてEpon 812に包埋した．

超薄切片は，酢酸ウラニールとクエン酸鉛で二重染色し，日立HU-11DS電子顕微鏡で検査し，2,800～3,500倍で撮影した．

synaptic vesicleの算定には，視細胞核に最も近接し，両極細胞の樹状突起とその両側の水平細胞の突起とを伴う所謂triadと，1個のsynaptic ribbonを確認出来るsynaptic bodyとを選び観察した．synaptic vesicleの大きさは，無差別に選んだ500個の最大径を計測することにより求め，その数は，倍率90,000倍の電顕写真上の1辺4.5cmの正方形25カ所の平均値をもつて表わした．

III 実験成績

A．I群の電顕所見

1) 投与10日目の所見（第1，7図及び第1表参照）

Bruch膜が稍々不鮮明である以外には，色素上皮細胞層，視細胞外及び内節に殆んど変化はない（第1図）．外網状層も正常に近く，synaptic bodyのmembrane，ribbon及びvesicleのいずれも明瞭である（第7図）．

synaptic vesicleの大きさは，41～50nmのものが全数の約79%を占め，大径のものが少し増している．数は約35で，正常より僅かに増加している．

Table 1 Distribution of the synaptic vesicles in outer plexiform layer

subjects		number of the vesicles in 0.5μ²	diameter and percentage of the vesicle					
			21~30 nm	31~40 nm	41~50 nm	51~60 nm	61~70 nm	71~80 nm
normal		29	– (%)	23 (%)	72 (%)	5 (%)	– (%)	– (%)
I group	10th day	35	–	17	79	4	–	–
	30th day	28	–	–	37	43	19	1
	50th day	25	–	6	43	48	3	–
G group	10th day	32	–	4	76	20	–	–
	30th day	25	–	34	52	14	–	–
	50th day	12	12	73	10	5	–	–

2) 投与30日目の所見（第2，8図及び第1表参照）

Bruch膜は5層の判別が不可能で，一層の帯状をなし，中央部に空胞状形象がある．色素上皮細胞のbasal-infoldingは比較的鮮明で，胞形質内の遊離のribosomeと粗面小胞体が，比較的多く見られる．しかし，基底部近くのmitochondriaのcristaeは，消失し，中心に層状構造物を含み，境界膜は，一部消失しているものもある．

色素上皮細胞のmicrovilliと視細胞外節先端とは離開する傾向が強く，外節の膜はmicrovilliと離開する部位で消失している．

視細胞内節では，ellipsoidの一部に，cristaeの判然としないmitochondriaがみられ，myoidのribosomeと粗面小胞体は，豊富である．外境界膜部の内節小胞体の形は，円形を崩している（第2図）．

外網状層のsynaptic bodyは，membrane，ribbon及びvesicle共に明瞭である（第8図）．vesicleの大きさは51～60nmのものが約42%を占め，大径のものが多く，その数は約28で，稍々減少している（第1表）．

3) 投与50日目の所見（第3，9図及び第1表参照）

Bruch膜の5層の識別は不可能となり，電子密度が低くなつた膜内に空胞が出現している．色素上皮細胞のbasal-infoldingは，不鮮明に散見されるにすぎない．基礎胞形質内の滑面小胞体が崩れて，電子密度も不均一となるが，遊離のribosomeと粗面小胞体の存在が比較的目につく．mitochondriaは，完全な形態を残すものが殆んどなく，その基質は電子疎となり，空胞化している．microvilliは外節と離開している．

視細胞外節の膜は，一部残存しているのみで，層板体の走行は乱れ，その間隙も不均となり，層板体中に小空胞形成を見る（第3図）．視細胞内節では，ellipsoidのmitochondria内外境界膜が一部消失して，cristaeが

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不明瞭のものがある．外境界膜部の内節小胞体の形は，円形又は楕円形を呈するが，数は少い．

外網状層の Müller 細胞の filament 或はグリコゲーン顆粒は，10日目及び30日目の所見と異り，ほとんど見られない．Bruch 膜から外境界膜までの範囲で，Bruch 膜，色素上皮細胞，mitochondria, basal-infolding，滑面小胞体，視細胞外節層板体，並びに内節糸粒体等に，可成りの変化が存在するのに比べて，synaptic body の membrane, ribbon, vesicle 等の形が良く保たれている（第9図）．vesicle の大きさは，41～50 nm のものが約43%，51～60 nm のものが48%を占め，正常と異なり大径化の傾向が窺われ，その数は約25で，正常より減少している（第1表）．

B．G群の電顕所見

1）投与10日目の所見（第9図及び第1表参照）

Bruch 膜と色素上皮細胞の胞形質は略々正常である．上皮細胞の mitochondria はその基質が電子疎となり，空胞を含むものが散見され，粗面小胞体は少い．視細胞外及び内節も正常である．

外網状層の Müller 細胞は正常で，synaptic body の membrane, ribbone 及び vesicle は明瞭である（第9図）．vesicle の大きさは，41～50 nm のものが約76%，51～60 nm のものが20%で，小径のものが増す傾向を示し，その数は約32で，正常よりも稍々増加している（第1表）．

2）投与30日目の所見（第4，11図及び第1表参照）

Bruch 膜の5層は稍々不鮮明となる．色素上皮細胞の basal-infolding は明瞭で，胞形質は正常であるが，中央部に層状構造物と小空胞を含む mitochondria が散在している．

microvilli は視細胞外節と離開する傾向にあつて，自由表面近傍に空胞形成がみられる．外節膜の一部は消失し，一部は波状に走る（第4図）．視細胞内節は略々正常で，外境界膜部の内節小胞体は円形を保つが稍々少ない．

外網状層の Müller 細胞の胞形質は殆んど正常で，synaptic body の meinbrane, ribbone, vesicle 等も凡て明瞭である（第11図）．vesicle の大きさは，31～40 nm のものが約41%，41～50 nm のものが約52%で，小径のものが多く，その数も減少している（第1表）．

3）投与50日目の所見（第5，6，12図及び第1表参照）

Bruch膜の5層の識別は不明瞭で，層の中央部に若干の空胞を形成している．色素上皮細胞のbasal-infoldin
は極めて不鮮明となり，その基質胞形質は電子密度が
均一で，正常形態を崩しているが，遊離の ribosome
粗面小胞体が増加している．mitochondria の基質
cristae 及び境界膜は殆んど消失して，空胞化し，mi
rovilli も凡て離開している．

視細胞外節の層板体の走行は乱れ，境界膜は不明瞭
ある．視細胞内節の ellipsoid は，I群50日目の場合
りも不鮮明な mitochondria と滑面小胞体で満され，
外境界膜部の内節小胞体は円形に保たれているが，そ
数は少い（第5図）．

外網状層の Müller 細胞の胞形質は電子密度が軽く
細糸，グリコゲーン顆粒及び基質胞形質は不鮮明であ
（第6図）．この層の synaptic membrane と ribbo
の境界は稍々不明の場所があつて，特に synaptic ves
cle の形態は甚だ不明瞭のものが多く，その配列も乱
ている（第12図）．vesicle の数は約12で，正常の半数
下になり，その大きさは，31～40 nm のものが約73%
占め，著明に小径化している（第1表）．

IV　総括並びに考按

須田[5]及び Grignolo ら[6]は，大量のIを家兎に静
して起る網膜の変性を，電顕的に観察して，経時的に
素上皮細胞より次第に内層に進展すると述べている．

他方，Wald ら[7]は，亀の網膜を等張リンゲルとG
の混合液中に incubate して，Gが網膜の各層に如何
作用するかを電顕的に調べた結果，内網状層の synaps
並びに神経要素が著明に膨大し，mitochondria が空
化すると同時に，vesicle が殆んど完全に消失するが，
他の網膜層は極めて良く正常に保存されていることを
摘した．

また，Cohen[8]は，幼若マウスに大量のGを10日間
日注射して，その網膜を光顕的及び電顕的に調査して，
内顆粒層より内方の層が，選択的に破壊されるが遺残
た網膜内層の個々の組織，例えば両極細胞或はsynapti
body の変化は極めて少ないことを強調している．

以上はI及びGの大量を投与することによつて惹起
た網膜変化を，電顕レベルで検討したものであつて，
者の実験のように，小量の長期連続投与による変化と
自ら異なることは当然である．

著者の薬物投与10日目と30日目の組織所見は，I と
で略々類似し，Bruch 膜及び色素上皮細胞に軽度の
常がみられるのみである．しかし，30日目では，I に

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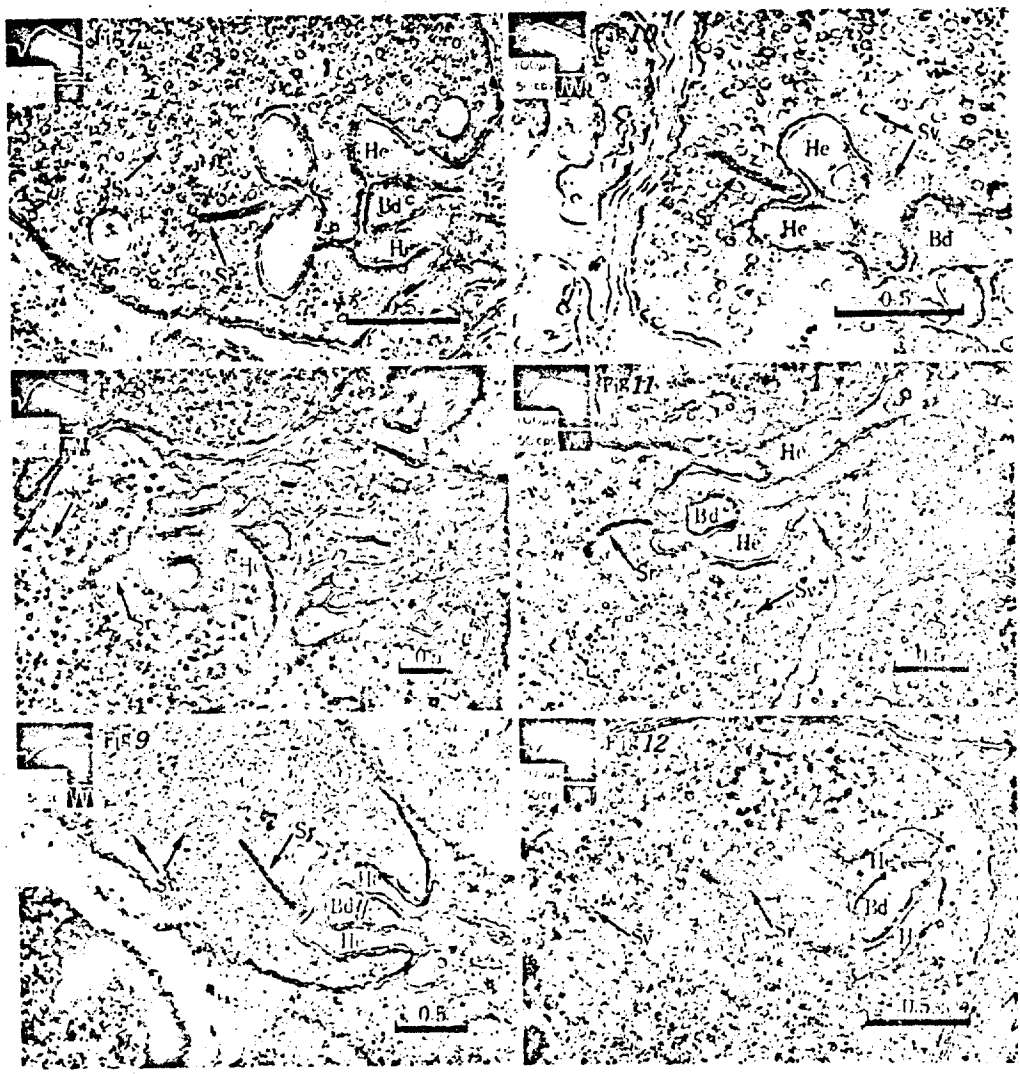

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る変化がGよりも多少強い感じを受けた．

50日目になると，ＩとＧによる変化は，Bruch 膜，色素上皮細胞，視細胞外内節及び外網状層にも波及して明瞭になる．

興味深いことは，両剤を総体的に比較すれば，Ｉによる変化は Bruch 膜，色素上皮細胞及び視細胞外節に強く，Ｇでは視細胞内節及び外網状層に強く発現される傾向があることである．

ＩとＧを著者と同様の条件で負荷した家兎眼の電気生理学的変化を追求した教室の今泉[3]の実験によれば，ＥＲＧ及びＥＯＧの応答は，薬物投与40日目を境として，両剤の関係が全く異る様相であつて，40日目以前のＥＲＧ及びＥＯＧの振巾は両剤とも殆んど平行した経日的変動を呈するのに反して，40日目以後は両剤が全く異つた経過をたどると云う．

即ち，40日目以後のＩによるＥＲＧとＥＯＧの経日的過程は，組織所見と一致して変動するが，Ｇのそれは組織学的に説明不可能である．Ｇによる電気生理学的異常は，恐らく電顕的にみる細胞内器官の変化には関係が少く，諸種の代謝酵素レベルの活性変調によるものと推定される．

Ｉによる網膜変化の原因は，組織における呼吸及び解糖作用の障害のみならず，多糖類及び核酸代謝の障害によるとされ，Ｇでは，Potts[7] の推測通りＧ代謝に関与する５種の酵素の活性障害が問題であると思う．従つて，高い酵素活性が要求される色素上皮細胞や視細胞内節の細胞内小器官の変化が意義深いものと考えられる．

外網状層に於ける synaptic body の経日的変化が注目すべき現象で，Ｉ及びＧ投与10日目には vesicle の数が一時的に増加するが，それ以後は減少する．その減少の割合は，ＩよりもＧの方が高い．vesicle 個々の大きさは，Ｉでは経日的に大きくなるのに対し，Ｇでは次第に小さくなる．即ち，総体的に見て，Ｇによる vesicle の変化は，Ｉよりも顕著である．

synaptic body は，錐体及び桿体に属する二種類が区別されるが[10~12]，秋谷[13]の意見と同様に，著者の場合もその区別が困難であつた．

薬物投与後の外網状層の synaptic vesicle の変動については，須山[5]はＩ，Lasansky ら[14]及び秋谷[13]はヨード酢酸を使用しての実験報告中で，共に vesicle は減少するとのみ述べているにすぎない．薬物による vesicle の経日的変化に言及した報告は，著者の調べた限りでは見当らない．Wald ら[9]のＧによる成績では，外網状層の synaptic body は殆んど正常であるが，内網状層のそれらは極めて高度に変化し，vesicle は消失すると観じている．但し，実験条件が全く異るので，著者の実験と同等に比較出来ない．

synaptic vesicle の生理学的意義に関しては，Mc Lennan[15]，Kurtz[16]，Heinz[11] 及び山田[17]によると，現在，不明の点が多いが，神経興奮を伝導する化学物質，即ち transmitter を vesicle 内に包含しているものと見做されている．vesicle 内の transmitter の一種として，catecholamine 等の monoamin 類があるが[14][17]，生理的に網膜内に存在する酸性アミノ酸であるＧの投与による vesicle の変化が，Ｉよりも強いことは，注目すべきことである．

今泉[3]のＩ及びＧによる実験では，光顕的に網膜変化が確認し難い薬物投与12乃至15日目に，ＥＲＧ及びＥＯＧの著明な振巾変動が証明されるが，著者の実験における10日目の外網状層の synaptic vesicle 数の増加と考え合わせると，甚だ興味深い成績の一致である．transmitter を包含すると云う vesicle が，Ｉ及びＧの連続投与によつて，系統的に而も経過的に，その大きさ及び数を変動することは，貴重な新知見であるが，今回の実験の範囲内からは，その意義づけは不明であり，今後の研究によつて充分検討すべき事項である．

Ⅴ 結　論

小量のＩ及びＧを成熟白色家兎に50日間連日非経口的に投与し，Bruch 膜から外網状層に到る網膜に生じた変化を，今泉[3]によつて得られた電気生理学的所見と対比しつつ，電顕的に観察して，次の如き結論に達した．

1) Ｉ及びＧ投与による組織変化は，一般に，網膜の外層から内層へと経日的に次第に進展するが，その変化の初発時期と進展速度とは，Ｉの方がＧよりも早い．

2) Ｉ及びＧ投与が電気生理学的に共に相似した経過をたどる10日目及び30日目には，電顕的所見も略々類似している．然し，ＩではＥＲＧ及びＥＯＧに異常を来し，Ｇでは両者が殆んど正常を保つ50日目の電顕的所見では，Ｉの場合は，Bruch 膜，色素上皮細胞及び視細胞外節に，Ｇの場合は，視細胞内節及び外網状層に，夫々変化が強く発現する．

3) Ｉ及びＧ投与によつて，外網状層の synaptic body 内の vesicle は，ＥＲＧ及びＥＯＧの振巾変動の激しい10日目にその数を増し，30日目及び50日目に減少し，経過的に形態にも変化を来すが，その変化は，Ｉよ

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りもGにより強く表現される傾向がある．

写真説明

各図のスケールは，特に表示しない限り1μである．

第1図：ヨード酸ソーダ投与10日目．Bruch膜(Br)の各層が不鮮明である以外は著変がない．×11,600

第2図：ヨード酸ソーダ投与30日目．mitochondria (Mit) は cristae が一部判然としない．粗面小胞体 (Er) は10日目に比べて豊富に出現し，外境界膜部の内節小胞体は円形を崩している．×11,600

第3図：ヨード酸ソーダ投与50日目．Bruch膜(Br)の各層は識別困難で，空胞が出現し，basal-infolding (Bi) は不鮮明で，色素上皮細胞の基礎胞形質は電子密度が不均一である．遊離の ribosome と粗面小胞体 (Er) が目につく．mitochondria (Mit) の cristae は消失し，その基質は電子疎となる．外節 (Os) 層板体の走行は乱れている．×20,000

第4図：グルタミン酸ソーダ投与30日目．Bruch膜(Br) は稍ゝ不鮮明で，basal-infolding (Bi) は鮮明である．mitochondria (Mit) は中央部に層状構造物を含有し，microvilli (Mv) の自由表面の近くに空胞 (v) を形成している．×11,600

第5図：グルタミン酸ソーダ投与50日目．内節 ellipsoid は不鮮明な mitochondria (Mit) と滑面小胞体で満たされ，外境界膜の小空胞 (v) は数が少い．× 9,200

第6図：グルタミン酸ソーダ投与50日目．外網状層の Müller 細胞 (Mc) 基礎胞形質は電子密度が低く，不鮮明である．synaptic vesicle (Sv) の形態も不明瞭である．×19,600

第7図：ヨード酸ソーダ投与10日目．synaptic body の構成は明瞭で，vesicle (Sv) は大径化し，その数は僅かに増加している．×17,600

第8図：ヨード酸ソーダ投与30日目．synaptic body は明瞭で，vesicle (Sv) は大径化し，数は稍ゝ減少している．×32,000

第9図　：ヨード酸ソーダ投与50日目．Müller 細胞 (Mc) の基礎胞形質，グリコゲーン顆粒及び細糸は不鮮明であるが，synaptic body は明瞭で，vesicle (Sv) は大径化し，数は減少している．×36,000

第10図：グルタミン酸ソーダ投与10日目．synaptic body の構成は明瞭であるが，vesicle (Sv) は小径化し，数は稍ゝ増加している．×62,000

第11図：グルタミン酸ソーダ投与30日目．Müller 細胞及び synaptic body は明瞭であるが，vesicle (Sv) は小径化し，数は寧ろ減少している．×36,000

第12図：グルタミン酸ソーダ投与50日目．synaptic body の構成は不明瞭で，vesicle (Sv) の形態が崩れ，配列も乱れ，vesicle は著明に小径化し，数は甚だ減少している．×50,000

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討　論

米村　大蔵　Presynaptic vesicle を観察された presynaptic terminal は双極細胞あるいは水平細胞のどちらに接続していたか御教示下さい．

本田　孔士　Synapse の連絡に関して．

1) Bipolar cell は直接．

2) Horizontal cell は間接接合と聞いたような気がします．

水野　グルタミン酸の代謝物や誘導体について同様な実験を行つたことがあるか，たとえば，グルタチオンやGABA等を用いたら，変性の機序解明の参考になるのではないか．

小林　秀樹（答）米村先生に：今回，鏡検したものは，presynaptic body でありますが，その判別は非常に困難である．

水野先生に：今回の実験の二薬物の他には，アスパラギン酸ナトリウムについて，実験して居ります．尚，今後各種の薬物について，検討して行きたく思います．

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EFFECTS OF GAMMA-AMINOBUTYRIC ACID (GABA) AND SODIUM L-GLUTAMATE (GLUTAMATE) ON THE VISUAL SYSTEM AND EEG OF CHICKS*

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Summary—Immature chickens have a deficient blood–brain barrier and were therefore used to investigate the effects on brain and retinal electrical activity of GABA and glutamate which do not readily pass the mature barrier. These compounds occur normally in brain and are presumed by some to be synaptic transmitters. We observed gross behavior and recorded the electroencephalogram (EEG), electroretinogram (ERG) and responses evoked in the optic tectum by light flashes. Both compounds produced behavioral depression, EEG synchrony and depression of the optic evoked response but affected the ERG differently. GABA blocked both the *a*- and *b*-waves of the ERG and greatly increased the negative *b*- and *c*-waves as well the positive component of the "off" response. On the other hand, glutamate blocked the *a*-wave and enhanced the *b*-wave but had little effect on the "off" response. The evoked response was altered minimally during the period of marked alteration of the ERG.

INTRODUCTION

THE OCCURRENCE of GABA and glutamate in the vertebrate brain and the pharmacological effects of these on evoked and spontaneous electrical activity in the central nervous system has suggested that they may be chemical transmitters or modulators in brain function. KRNJEVIĆ (1965) supports the possibility that glutamate may be the principal excitatory transmitter in the cerebral cortex. The neural depressant properties of GABA have been described for both vertebrate and invertebrate preparations. ELLIOTT (1965) summarized the evidence for GABA as an inhibitory transmitter.

The studies reported here are part of a continuing series of investigations in which the chick, because of a demonstrated blood–brain barrier deficiency during the first four weeks of life, is used to advantage to study the effects of systemically administered compounds on vertebrate brain function and behavior. In a previous paper (KRAMER and SEIFTER, 1966) we described the effects of GABA on the electroencephalogram (EEG) and behavior of chicks. The present report is concerned with the effects of GABA and glutamate on potentials evoked in the optic tectum by light, retinal electrical activity (ERG) and brain electrical activity (EEG) in curarized chicks as indicators of the action of these compounds. The effects of glutamate on the EEG and gross behavior of unrestrained, unanesthetized chicks were also observed for comparison with our previously reported effects of GABA.

METHODS

Sex-linked hybrid cockerels supplied by Hall Brothers Hatchery, Wallingford, Conn. and ranging in age from 2 to 8 days were used. Ether anesthesia initiated in a chamber

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was continued during the operative procedure by means of an air–ether mixture blown into the intubated trachea. The lids and nictitating membrane of the eye were sutured open in order to be photically stimulated. After fixation of the preparation in a stereotaxic device, appropriate burr holes were made in the skull for electrode placement. The chicks were immobilized with intramuscular *d*-tubocurarine (0·6 mg) and artificially respired. Body temperature was monitored through a cloacal thermistor and regulated to 39·5°C by a Yellow Springs instrument which controlled a flow of heated air around the chick. Cardiovascular function was estimated by simultaneous visual and auditory monitoring of the electrocardiogram (ECG), the wave form and rate of which gave a satisfactory indication of the general state of the preparation.

Bipolar EEG electrodes were inserted epidurally over the ipsolateral cerebral hemisphere. Stainless steel coaxial electrodes were used to record the optic response from the the contralateral optic tectum. Bipolar recording increased the probability that the recorded response originated in the region of the electrode. The ERG was recorded through silver–silver chloride saline-wick electrodes with the indifferent lead placed on the muscles of the neck. The stimulus light source was programmed to turn on at 15 sec intervals for a duration of 2 sec which allowed separation of the "on" "off" complexes of the ERG. The stimulus was monitored by a photocell. The EEG, ERG, tectal evoked response and stimulus were recorded simultaneously on a Grass polygraph. In order to achieve greater base line stability the ERG was recorded a.c. with a low frequency cut off of half amplitude at 0·25 c/s and a high frequency cut off of half-amplitude at 60 c/s. For the evoked response the low frequency half-amplitude cut off was 0·6 c/s and the high frequency at 15 or 60 c/s.

All signals were processed through a TMC model 400B computer of average transients (C.A.T.). Computer-averaged plots of twenty-five responses were used to measure the amplitudes (voltages) of response components and to determine latencies of significant points in the signals relative to onset of the stimulus. All data of control and drug-altered responses were from the plots. The frequency response of the amplifiers seen by the C.A.T. was half-amplitude at 10,000 c/s. Samples of the polygraph records are presented to show the effects of GABA and glutamate on the EEG and tectal spontaneous activity.

After allowing 1 hr for elimination of ether and dark adaptation, three or four periods, each approximately 6 min and twenty-five responses, were recorded as control values. The drug was then injected intraperitoneally and its effects and dissipation were observed by averaging groups of twenty-five responses.

The effect of GABA on the evoked response only was studied in eight birds with doses ranging from 2 to 4 g/kg. The effects of GABA on the evoked response and ERG were studied in six birds with doses ranging from 1·75 to 5 g/kg. The effects of glutamate on the evoked response and ERG were observed in seven birds with a dose of 4 g/kg, and its effects on gross behavior and the EEG of unanesthetized unrestrained birds in eight.

RESULTS

The EEG of curarized, unanesthetized chicks was characterized mainly by irregular, large amplitude slow waves similar to those seen during natural sleep in untreated chicks, sometimes with periods of small amplitude fast activity carried on the slow waves. In contrast, the spontaneous tectal activity was usually characterized by smaller amplitude, higher frequency waves than those seen in the cerebral hemisphere, sometimes accompanied

by considerable small amplitude fast activity. Figure 1 illustrates a typical EEG (A), tectal evoked response (B) and ERG (C).

The response of the chick optic tectum to light flashes varied with experimental conditions. The wave form, duration and latencies of the response may be influenced by polarity of the electrodes, location of the electrodes in the tectum, and particularly intensity, rise time and duration of the stimulus. When recorded by a silver–silver chloride ball

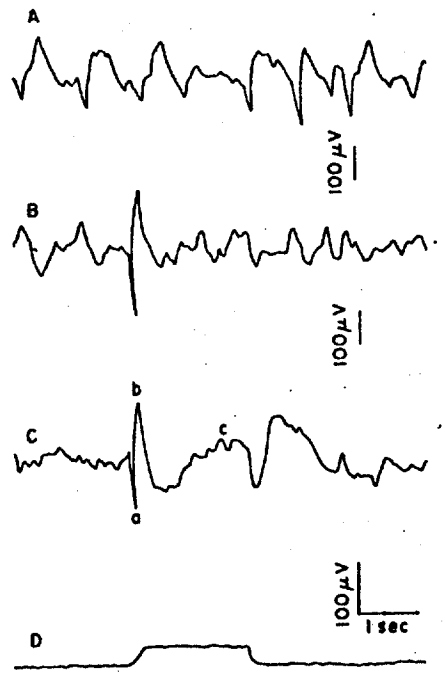

FIG. 1. Polygraph records of typical EEG (A), tectal evoked response (B), ERG (C), and the stimulus (D). In this and all the other figures, downward deflection in the evoked response is positive. When recorded from the tectal surface the wave form is the same with reversed polarity. On this basis, the initial component in the evoked response has been designated as surface negative. The ERG as displayed is recorded in the usual manner with corneal negativity shown as a downward deflection. The major components are marked *a*, *b*, and *c*. The negative deflection which follows the *b*-wave is the negative *b*-wave. The "off" response begins with cessation of the stimulus and consists of an initial negative component followed by a positive wave.

electrode on the tectal surface the response was biphasic with an initial surface negative fast component followed by a surface positive slow one. Simultaneous recording from concentric electrodes 1 mm below the tectal surface gave identical wave forms with reversed polarity. We therefore assigned polarity to the components with reference to the tectal surface. When stimulation was of higher intensity and shorter duration (10 μsec) than that used in the above experiments, the response was altered so that the initial "fast" wave and the "slow" wave had three minor peaks each.

The evoked response consisted of an initial surface negative "fast" wave of about 120 μV amplitude and 50 msec duration followed by a "slow" wave of opposite polarity of about 170 μV amplitude and 500 msec duration. The ERG had a prominent negative

a-wave and a positive *b*-wave, followed by a small negative overshoot, possibly an artifact of a.c. amplification. A *b*-wave negative overshoot has been reported for the guinea pig ERG (BOEHM *et al.*, 1944). GABA greatly increased this segment of the ERG and we refer to it with reservation as the negative *b*-wave. The negative *b*-wave was followed by a slow positive wave which we name the *c*-wave because it resembled this component of the d.c. amplified ERG. The "off" complex consisted of a negative wave followed by a longer lasting positive wave.

Effects on gross behavior and the EEG. In unrestrained chicks glutamate (4 g/kg) produced behavioral depression and an EEG characterized by irregular large amplitude slow waves indistinguishable from those seen in natural sleep (Fig. 2). These did not

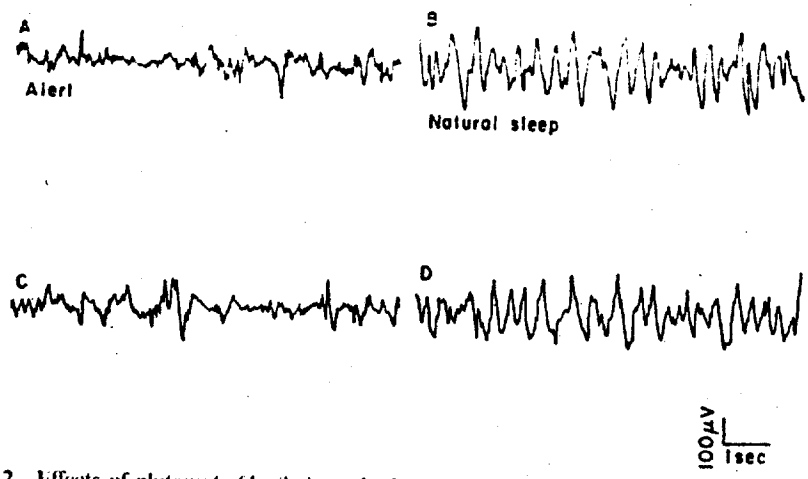

FIG. 2. Effects of glutamate (4 g/kg) on the EEG of a 2-day-old chick. Trace A, bird alert; Trace B, natural sleep. Trace C, bird standing with open eyes and marked wing droop 5 min after glutamate. Trace D, bird standing with eyes closed and wing droop 120 min after glutamate.

differ from the effects of GABA (KRAMER and SEIFTER, 1966). In addition, glutamate induced postural changes usually accompanied by marked wing droop several minutes after drug administration. These were present during both standing and sitting, sometimes standing with the caudal end raised and the head, with eyes closed, held parallel to the floor. This is unlike the head droop seen during roosting. The wing droop may be disassociated from depressed behavior and appeared during the early stages of drug action while the bird was behaviorally alert and had EEG activation. Neither GABA nor glutamate produced an observable effect in the EEG of curarized birds already dominated by large amplitude slow wave activity. Neither compound had an effect on tectal spontaneous activity (Figs. 3, 4, 5.)

Effects of GABA and glutamate on the tectal evoked response. GABA in dose of 3 g/kg or higher completely blocked the tectal response. Lower doses (1·75 g/kg) reduced both components of the evoked response by about 60% and full recovery occurred about 18 min after maximal depression. Figure 3 shows the effect of GABA (4 g/kg) on the tectal evoked response.

L-glutamate (4 g/kg) had essentially the same qualitative effect on the tectal evoked response as GABA. It produced depression of both components of the response (Fig. 5).

FIG. 3. Effect of GABA (4 g/kg) on the EEG (top trace), and the tectal evoked response (middle trace). Stimulus is shown on the bottom trace. Control (A), 18 min after GABA(B), 20 min after GABA (C), Recovery 150 min after GABA (D).

The "fast" wave, however, had an initial increase of 19% about 12 min after drug administration, and 51 min later reached maximal depression of 20%. The slow wave remained unchanged for the first 12 min after drug administration and 38 min later reached maximal depression of 27%. Larger doses completely blocked the response.

Effects of GABA and glutamate on the ERG. GABA changed most components of the ERG (Fig. 4). Data from a series of four experiments using 2 g/kg of GABA were averaged with the following results (Table 1). The initial effect was a reduction in both the *a*-wave and *b*-wave. The *a*-wave was considerably reduced 18 min after drug administration and was depressed 80% in 24 min. The *b*-wave measured from the base line was reduced 34% in 6 min and 60% in 12 min. Latency of the *a*-wave appeared unaffected by GABA. The latency to the beginning of the *b*-wave was shortened by 20%, and to the peak of the *b*-wave by 30% in 12 min. Ultimately the *a*- and *b*-waves were completely blocked. The most dramatic change occurred in the negative *b*-wave. This increased 5·5 fold in 12 min and reached a maximal amplitude of 10 times the control level in 24 min. Latency to the peak of the negative *b*-wave became increasingly shorter and was reduced by one half when the greatest increase in amplitude occurred. The point of origin of the increasing negative *b*-wave began to overlap the *a*- and *b*-waves so that during the period when the *a*- and *b*-waves were being blocked, their remnants were "riding" on the descending negative *b*-wave (Fig. 5, 2-B).

Some time after the *a*- and *b*-waves disappeared a new initial positive wave appeared (Fig. 4,2–D.E.) which superficially resembled the *b*-wave. The new wave reached a maximal amplitude in 36 min, at which time latency to its origin was 65% shorter than the control *b*-wave. On the basis of latency and other characteristics, the initial positive wave which appeared after *b*-wave blockade seemed to be a new component.

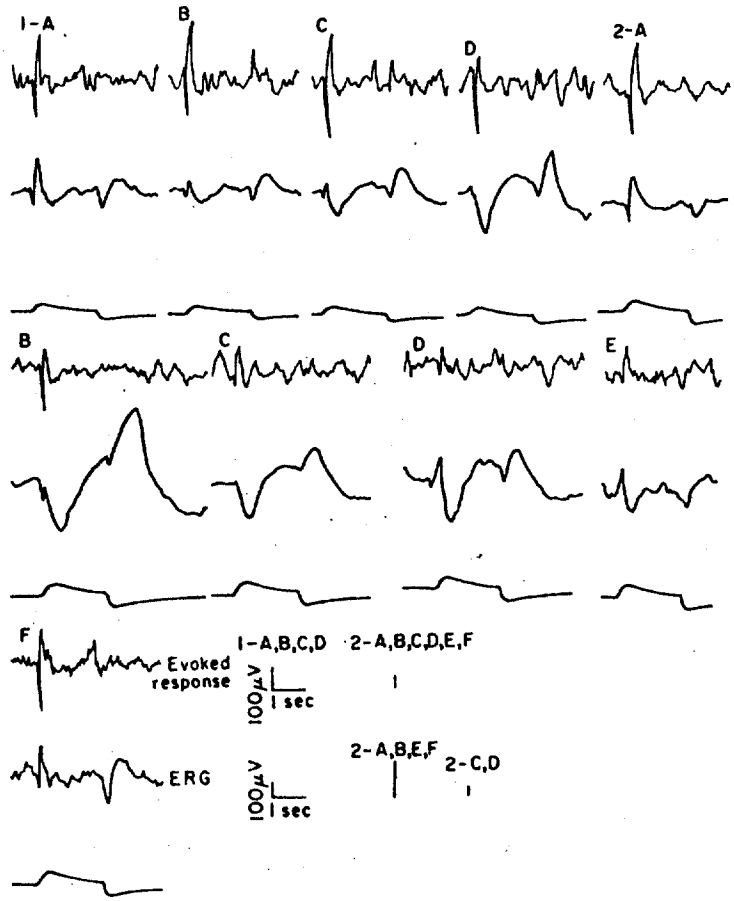

FIG. 4. Effects of GABA on the tectal evoked response (top trace), and the ERG (middle trace). The stimulus is shown on the bottom trace. Series 1 shows progressive changes produced by 4 g/kg. Control (A), 10 min after GABA (B), 11 min after GABA (C), 15 min after GABA (D). Series 2 shows the effects of 1·75 g/kg. Control (A), 10 min after GABA (B), 18 min after GABA (C), 25 min after GABA (D), partial recovery from GABA (E), and recovery 60 min after GABA (F).

The *c*-wave increased 22 fold in 12 min. The initial negative wave of the "off" response showed no change in amplitude with 2 g/kg of GABA. The positive wave of the "off" response increased 4 fold in amplitude in 24 min; it therefore reached maximal amplitude increase simultaneously with the negative *b*-wave. Recovery from drug effects occurred in reverse order. The negative *b*-wave, *c*-wave and "off" positive wave returned to near control values 54 min after drug administration. The *a*-wave was the last component to reappear.

Larger doses of GABA (3–5 g/kg) produced qualitatively similar but quantitatively different effects on the ERG. At maximal effect, the negative *b*-wave increased 25 fold in amplitude with a parallel decrease in latency to the peak. The *c*-wave increased 10 fold and the "off" positive wave 6 fold. The negative component of the "off" response increased only 2 fold.

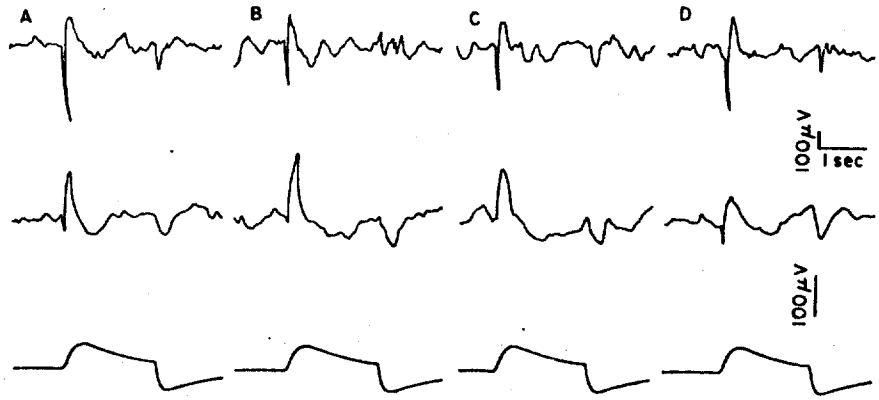

FIG. 5. Effects of glutamate (4 g/kg) on the evoked response (top trace) and the ERG (middle trace). The stimulus is shown on the bottom trace. Control (A), 50 min after drug (B), 59 min after drug (C), 100 min after drug (D).

TABLE 1. CHANGES IN THE ERG AND EVOKED RESPONSE INDUCED BY 2 g/kg GABA. ALL FIGURES REPRESENT PERCENT OF CHANGE FROM CONTROL LEVELS. DATA BASED ON AVERAGE OF FOUR EXPERIMENTS

Time after drug (min)	Evoked		ERG "on"				ERG "off"	
	fast	slow	a	b	neg b	c	neg	pos
6	0	0	0	−35	+20	+240	−12·6	+23
12	0	−15·6	− 6	−60	+560	+2580	−14	+245
18	− 5	−19	−64	−100	+860	+6410	*	*
24	−32	−52	−80	−100	+1000	*	*	*
30	−56	−64	−87	−100	+750	+2700	+14	+200
36	−56·4	−55	−96	−100	+650	+2900	+18	+200
42	−37·6	−52	−100	−14	+632	+2470	+10	+170
48	−42·7	−42	−100	−17	+692	+3270	−0·9	+179
54	−45·3	−49	−91·8	−28·6	+228	+1200	+4·8	+47·9
60	−34	−63	−82	−33·7	+236	+757	−2·9	+78

*Insufficient data

Figure 5 shows the effects of glutamate (4 gm/kg) on the ERG. The *a*-wave was depressed and often completely blocked. An average maximal depression of 90% occurred 50 min after drug administration. The *b*-wave increased and reached the maximum of 52% and the negative *b*-wave also increased maximally. The *c*-wave was unchanged by glutamate. The "off" response was essentially unchanged, although in one experiment increases occurred in both components. Latencies of the *a*- and *b*-waves were unchanged but latency to the peak of the negative *b*-wave was increased by 52%.

Relationship of drug effects on the ERG to those on evoked response. The ERG may reflect processes which involve generation and transmission of impulses in the retina, and because the arrival of visually-induced signals at the optic tectum is directly dependent on these, it is of interest to consider the simultaneous effects of GABA and glutamate on the ERG and evoked response.

The averaged results of four experiments with GABA (Table 1) show that marked alteration of the ERG, which includes reduction of the *a*-wave by 64% and complete block of the *b*-wave, occurred at a time when the evoked response was minimally reduced. Maximal depression of the evoked response occurred about 10 min after maximal changes in the ERG. The simultaneous effects of GABA on both the ERG and evoked response can be seen in Fig. 4.

Glutamate depressed both components of the evoked response before any significant change occurred in either the *a*- or *b*-waves of the ERG. However, maximal depression of the evoked response was correlated with blockade of the ERG *a*-wave and maximal increase in the *b*-wave, Recovery of the evoked response preceded return to approximate control values of the ERG *a*- and *b*-waves.

DISCUSSION

The EEG of the curarized, unanesthetized chick is often similar to that seen during normal sleep. Whether this results from direct action of *d*-tubocurarine on brain or from a reduction in proprioceptor activity through immobility, or both, was not determined. It is important that periodic administration of *d*-tubocurarine to maintain immobility did not affect either the evoked response or the ERG during control periods or during the changes induced by GABA or glutamate.

The similarity of behavioral depression and EEG sleep by glutamate to those described for GABA (KRAMER and SEIFTER, 1966) is not consistent with its excitatory action on single neurons (KRNJEVIC, 1965). It is consistent with the decreased glutamate content of focal epileptogenic lesions by local freezing of cat cerebral cortex and suppression of seizure discharged by glutamate administered i.v. (BERL *et al.*, 1961). Glutamate has also been implicated in the phenomenon of spreading depression (VAN HARREVELD, 1960).

The depressant behavioral response produced by glutamate in the chick differs from the sleep-like or roosting state produced by GABA and its nature remains to be clarified.

The effects of GABA on the tectal evoked response, spontaneous tectal activity, and the ERG in the chick was reported by SCHOLES and ROBERTS (1964). Our observations confirm the blockade of tectal evoked responses and the lack of effect on tectal spontaneous activity.

Our description of the effects of GABA on the ERG, however, differs from theirs. GABA completely blocked both the *a*-wave and *b*-wave of the ERG, markedly increased the negative *b*-wave and *c*-wave, and decreased latency to the peak of the negative *b*-wave. These changes occured quite rapidly, and transitional stages (Fig. 3) could be missed if drug effects were sampled infrequently. Consequently, SCHOLES and ROBERTS (1964) may have interpreted the large changes in the negative *b*-wave and *c*-wave to be increases in the *a*- and *b*-waves.

The disappearance of the *a*-wave and *b*-wave in our experiment is probably not due to masking by the marked increase in the negative *b*-wave, because the latter returned to approximately control levels long before the *a*-wave reappeared. On the other hand, the *b*-wave returned to control levels at least 10 minutes before any significant change in the negative *b*-wave.

The appearance of a positive wave superficially resembling the *b*-wave, after the *b*-wave has disappeared, is difficult to explain. Our differentiating it from the *b*-wave is based on its shorter latency and we have considered return to pre-drug *b*-wave latency values as indicating reappearance of the *b*-wave during recovery.

Because the generators of the components of the ERG are unknown, it is difficult to discuss the site of action of GABA and glutamate. The *a*-wave has been considered the leading edge of the late receptor potential (BROWN *et al.*, 1965). According to these authors, the late receptor potential occurs concomitant with the ERG but is not a generator potential in the sense that it leads to initiation of nerve impulses.

The non-specific blocking action of GABA on electrogenic membranes (by increasing conductance) may explain its actions on the retinal photoreceptors. The *b*-wave, whose origin has been attributed to the inner nuclear layer of the retina, particularly to the bipolar cells, may also be blocked by this action of GABA.

The generation of potentials of the magnitudes observed with GABA (1 mV for the negative *b*-wave) is difficult to explain. It seems most probable that the same retinal elements generate the large negative *b*-wave as well as the *a*- and *b*-waves because latency to the origin of the GABA-induced negative *b*-wave is about half the duration of the control *a*- and *b*-waves combined. It is unlikely that the blocking action of GABA unmasks the generator of the negative *b*-wave.

The large increase in the *c*-wave may reflect an effect of GABA on electrogenesis in the pigment epithelium. The origin of the *c*-wave has been attributed to this ocular tissue.

The chick should belong to those vertebrates which have an I-retina according to the classification of GRANIT (1947). This type of retina generates a prominent "off" effect. The chick ERG, however, does not show the initially positive *d*-wave typical of this group. The "off" response in the chick consists of an initial negative wave followed by a positive one. The ERG of the mature chicken (VON BRÜCKE and GARTEN, 1907) shows the same initial negative deflection. With the lowest dose used (2 g/kg), GABA greatly increased the positive component of the "off" effect but did not alter the initial negative component. The obliteration of the *a*-wave by glutamate may be due to increased amplitude of the *b*-wave masking the *a*-wave, but this is not likely because *b*-wave latency was not decreased or changed during the experiment. Since glutamate has both excitatory and inhibitory activity it is possible to account for both blockade of the *a*-wave and enhancement of the *b*-wave.

In a personal communication from Noell and Lansky cited by DE ROBERTIS (1964) it is stated that iontophoretic injection of glutamate into the inner synaptic retinal layer produces discharge of ganglion cells. This may indicate that depression of the tectal evoked response occurs at the tectal level.

Gluamate can be converted to GABA, and the time relationships suggest that some of the effects we observed with glutamate may have been due to this conversion. The peak effects produced by GABA on both the ERG and evoked response occurred considerably earlier than those produced by glutamate. On the other hand, aside from mutual blockade of the *a*-wave, the effects on the ERG were different and indicate that glutamate produces effects which are distinct from those of GABA.

Our experiments show that large changes in the ERG can occur without appreciably affecting the tectal evoked response. An *a*-wave depression of 64% and complete block of the *b*-wave existed at a time when the evoked response was minimally depressed. These findings support the evidence of others that neither the *a*-wave nor the *b*-wave reflect processes necessarily involved in the formation of optic nerve impulses. BROWN *et al.* (1965) cite evidence that on the basis of the late receptor potential (leading edge of the *a*-wave), the direction of extracellular current flow cannot function as a generator potential. The significance of the *b*-wave as a signal of impulse transmission and conduction in the

retina has also been questioned. In comments on a paper by RIGGS (1958) Lindsley presented evidence that the *b*-wave is not responsible for the optic nerve discharge. When both the *b*-wave and optic nerve potentials are depressed with iodoacetate (NOELL, 1952), return of the optic nerve potentials in some cases preceeds recovery of the ERG *b*-wave.

It is not possible from our experiments or those of SCHOLES and ROBERTS (1964) to determine precisely where blockade of the evoked response by GABA occurs. Their evidence indicates that it must occur in the retina, because they observed blockade of the nerve response recorded peripheral to the optic tectum. It seems probable, therefore, that the differential effects of GABA on spontaneous tectal activity and tectal evoked response can be explained by the possibility that GABA has no effect at the tectal level, although the possibility of a tectal effect of GABA cannot be excluded. We are presently conducting experiments to answer this question.

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FURTHER OBSERVATIONS ON THE TERATOGENIC NATURE OF INSULIN AND ITS MODIFICATION BY SUPPLEMENTARY TREATMENT

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It was shown previously that insulin treatment of developing chicken embryos produces morphological abnormalities. These defects are chiefly, perhaps exclusively, skeletal, but what parts of the skeleton are affected depends on the stage during which the embryos had been exposed to the hormone. Incidence and degree of malformation vary with dose of injected insulin, hereditary constitution of the embryos, season, and probably numerous other and minor factors. Very high doses of insulin are sometimes responsible for the simultaneous occurrence of abnormalities which with smaller amounts only occur after treatment in other developmental stages. The fact that all insulin-induced malformations appear to be phenocopies of known mutants adds greatly to the interest of analyzing the physiological events by which insulin produces its effects. The more important results of our experiments have been reviewed in an earlier publication (Landauer, '48a). Since then we have found that the teratogenic consequences of insulin injection can be averted, in part or entirely, by supplementing insulin treatment with certain other substances, such as nicotinamide and α-ketoglutaric acid (Landauer, '48b). The present report deals with an extension of these experiments.

Nicotinamide

The injection of insulin into the yolk sac of chicken eggs during the 4th or 5th day of development is followed by a high

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incidence of micromelia and beak defects. From the results of earlier experiments it appeared that the frequency of occurrence of micromelia could be much reduced by supplying nicotinamide along with insulin, but that such supplementation was less effective in regard to beak development. Survival of insulin-nicotinamide treated embryos was, however, less good in these experiments than in the group that had only been exposed to insulin, and, because of this, some doubt remained concerning the validity of the conclusions drawn from these data. Hence, we decided to repeat the experiments. At the same time we desired to obtain information on the following two questions. Does nicotinamide actually protect the embryos less well against insulin-induced beak defects than against abnormalities of the extremities? Secondly, will nicotinamide supplementation forestall the phenotypic effects that insulin has on hereditary polydactylism (Landauer, '48c)?

For our new experiments on the combined use of insulin (Iletin, Lilly) and nicotinamide (Niacinamide, Squibb) we used eggs from a mating of White Leghorn females to a Dorking male, the latter being homozygous for polydactylism. The results for incidence of micromelia and beak defects are given in table 1. Each of the three experimental groups included one lot in which insulin alone, in a dose of either two units (A and B) or one unit (C), was injected at 96 hours of development and another lot that served as untreated control. In addition, one lot of group A received two units of insulin and 5 mg nicotinamide at 96 hours, one of group B was given 10 mg of nicotinamide at 93 hours, followed by two units insulin at 96 hours, and one lot of group C was injected with 10 mg nicotinamide at 93 hours and one unit insulin at 96 hours. The results are unequivocal. When 5 mg nicotinamide were given simultaneously with two units insulin, the incidence of all types of malformations was much reduced; when the embryos were supplied with 10 mg of nicotinamide three hours prior to the injection of either one or two units of insulin, protection against the teratogenic ef-

fects of insulin was complete. In none of the three groups had nicotinamide a statistically significant effect on embryo mortality due to injection of insulin. In other tests, the results of which are not reproduced here, we found that higher doses of nicotinamide (20 mg) — similar in amount to those

TABLE 1

Experiments concerning the effect of nicotinamide on insulin-induced abnormalities of extremities, beak and eyes

F_1 embryos from Leghorn ♀♀ × Dorking ♂

GROUP	TREATMENT	MORTALITY PRIOR TO 18TH DAY	SURVIVORS OF 17TH DAY	MICROMELIA	BEAK ABNORMALITIES	EYE ABNORMALITIES
		%		%	%	%
A	Insulin 2 U. — 96 hours	26.8	60	48.3	33.3	6.7
	Insulin 2 U. + 5 mg nicotinamide — 96 hours	13.6	70	10.0	4.3	1.4
	Untreated	3.7	79	0	0	0
B	Insulin 2 U. — 96 hours	12.9	74	55.4	29.7	1.4
	Nicotinamide 10 mg — 93 hours, insulin 2 U. — 96 hours	19.5	66	1.5	0	0
	Untreated	10.0	72	1.4	0	0
C	Insulin 1 U. — 96 hours	24.4	96	16.7	9.4	2.1
	Nicotinamide 10 mg — 93 hours, insulin 1 U. — 96 hours	29.1	100	0	1.0	0
	Untreated	10.7	117	0	0	0

used in our earlier experiments — were definitely toxic whether used in conjunction with insulin or alone, but among 33 survivors only a few showed micromelia and none had beak defects.

It is clear from these results that the occurrence of those effects of insulin treatment (during the 4th or 5th day of incubation) which resemble the phenotypes of major gene

changes, viz. micromelia and beak defects, can be prevented by adequate and properly timed supplementation with nicotinamide. We have reported earlier that in polydactylous stocks insulin has modifier-like effects in regard to development of the toes. Data on the question whether or not these effects can be prevented by nicotinamide supplementation appear in table 2. These observations must be reviewed with reference to the different modes of expression of polydactylism. In the first place, it will be seen that, as reported earlier, insulin treatment greatly exaggerated the incidence of phenotypic lack of expression of the gene for polydactylism (column A). This insulin effect was not significantly altered when 5 mg nicotinamide were given together with two units insulin. When, however, 10 mg nicotinamide were injected three hours prior to either one or two units insulin the occurrence of non-polydactylous embryos was reduced very significantly ($\chi^2 = 9.90$ with $P = .002$ in the first instance and $\chi^2 = 11.71$ with $P = < .001$ in the second). As for the different modes of expression of polydactylism, it can be seen that under the influence of nicotinamide-supplementation there was a definite trend toward an increased incidence of the irregular types, such as heterodactylism and polyphalangism (E and F), in preference to a return to the bilaterally 5-toed condition which is typical for the control embryos. With optimal amounts of nicotinamide, however, the incidence of typical bilateral polydactylism was also augmented conspicuously (C). It is clear, therefore, that the changes which insulin produces in the expression of polydactylism can to some extent be prevented by nicotinamide-supplementation, but that the protective effect is lower than with reference to micromelia and beak abnormalities. This response difference will be discussed later.

In an earlier publication (Landauer, '48b) we have reported that a significant reduction in incidence of rumplessness was obtained when injection of two units insulin at 24 hours was supplemented with 18.9 mg nicotinamide, but that nicotinamide did not at this stage give the embryos as much

TABLE 2

Experiments concerning the effect of nicotinamide on insulin-induced modifications of polydactylism[1]

(This is the same material as in table 1)

F_1 embryos from Leghorn ♀♀ × Dorking ♂

TREATMENT	SURVIVORS OF 17TH DAY	A NON-POLY-DACTYLOUS	B BILATERAL POLY-DACTYLISM	C TYPICAL 5-TOED CONDITION ON BOTH FEET	D ATYPICAL BILATERAL POLY-DACTYLISM	E BILATERAL POLY-PHALANGISM	F HETERO-DACTYLISM
		%	%	%	%	%	%
Insulin 2 U. — 96 hours	60	58.3	33.3	18.3	15.0	1.7	6.7
Insulin 2 U. — 5 mg nicotinamide — 96 hours	70	55.7	28.6	17.1	11.5	4.3	11.4
Untreated	79	12.7	77.2	62.0	15.2	2.5	7.6
Insulin 2 U. — 96 hours	74	62.2	28.4	14.9	13.5	1.4	8.1
Nicotinamide 10 mg — 93 hours, insulin 2 U. — 96 hours	66	33.3	45.5	28.8	16.7	6.1	15.2
Untreated	72	11.1	76.4	62.5	13.9	4.2	8.3
Insulin 1 U. — 96 hours	96	40.6	40.6	25.0	15.6	5.2	13.5
Nicotinamide 10 mg — 93 hours, insulin 1 U. — 96 hours	100	20.0	46.0	33.0	13.0	11.0	23.0
Untreated	117	11.1	72.6	63.2	9.4	6.8	9.4

[1] Columns A, B, E and F contain the total of all observations; the data of B are broken down in C and D.

protection against insulin damage as it does at later stages (96 and 120 hours). Since the amount of nicotinamide used in these experiments proved to have considerable toxicity, we decided to repeat the experiment with a smaller amount of nicotinamide, viz. 5 mg. The results are summarized in table 3. The nicotinamide was injected three hours prior to insulin, simultaneously with it, or 3, 6 and 12 hours after insulin. Embryo mortality was, even with this lower dose of nicotinamide, much increased. When nicotinamide was given between three hours prior to and 6 hours following insulin treatment, the incidence of rumplessness was in each instance lower than with insulin alone. Corresponding to the smaller dose of nicotinamide, its effectiveness in forestalling rumplessness was reduced, and the differences were in no case great enough to be statistically significant. However, the χ^2 from the combination of probabilities for groups 2 to 5 as against group

TABLE 3

Experiments concerning the effect of nicotinamide on the occurrence of insulin-induced rumplessness in White Leghorn embryos

Dosage: 2 units insulin, 5 mg nicotinamide

GROUP	TREATMENT	FERTILE EGGS	MORTALITY FIRST 6 DAYS	SURVIVORS OF 17TH DAY	RUMPLESS (ALL TYPES)	ABNORMAL BEAK
			%		%	%
1	Insulin — 24 hours	209	30.5	121	26.4	0
2	Nicotinamide — 24 hours insulin — 27 hours	316	54.4	127	18.1	3.1
3	Insulin and nicotinamide 24 hours	347	62.8	119	21.0	5.9
4	Insulin — 24 hours nicotinamide — 27 hours	343	68.5	99	15.6	5.6
5	Insulin — 24 hours nicotinamide — 30 hours	359	54.5	138	15.2	3.6
6	Insulin — 24 hours nicotinamide — 36 hours	333	54.4	132	28.8	8.3

1 was highly significant ($\chi^2 = 19.930$, df = 8, P = .01). Nicotinamide given 12 hours later than insulin no longer affected the incidence of rumplessness. On the whole, these results confirm with a lower dosage (viz. 5 mg) our earlier conclusion that an amount of nicotinamide which completely or nearly completely forestalled the micromelia-inducing effects of two units insulin at 96 or 120 hours of incubation, reduced only slightly the incidence of rumplessness after two units insulin at 24 hours and that the toxicity of this amount of nicotinamide was much greater at the earlier period than at the later one.

Some observations on beak defects, however, do not seem to fit into the general interpretation of the protective action of nicotinamide. In experiments with sulfanilamide, Zwilling and DeBell ('50) found that nicotinamide-supplementation completely forestalled the appearance of micromelia. But the parrot-beak condition that generally accompanies sulfanilamide-induced micromelia was, in the presence of nicotinamide, frequently replaced by a shortening of the upper beak. This effect of nicotinamide-supplementation was found when both sulfanilamide and nicotinamide were given at the end of the 5th day of incubation, but also when sulfanilamide was injected at 120 hours and nicotinamide 24 or even 48 hours later. We had observed earlier (Landauer, '48b) that nicotinamide supplementation of insulin treatment at 24 hours was responsible for the appearance of a certain proportion of embryos with shortened upper beaks. Our present results bear out these observations. We had suggested earlier that "a shift of the insulin damage within embryos" had been produced by nicotinamide. This interpretation is no longer tenable since it was found that nicotinamide by itself will in certain developmental stages call forth abnormalities of the beak. This is true for 24-hour embryos, but does not hold at all or to only a very slight extent at 96 hours (table 4). The observations of Zwilling and DeBell, to which we referred, suggest, however, that responses similar to those at 24 hours may occur again after 5 to 7 days of development.

Glucose-1-phosphate, citric and oxalacetic acid

Experiments in which insulin treatment at 96 hours was combined with the injection of glucose-1-phosphate produced results of considerable interest (table 5). Glucose-1-phosphate by itself was non-toxic and without teratogenic effects. When the injection of two units of insulin was supplemented with 4 mg glucose-1-phosphate the post-operative mortality was higher than after insulin alone; there was no significant

TABLE 4

Effect of nicotinamide, injected at 24 or 96 hours of incubation, on rate of mortality and beak development. Eggs from White Leghorn fowl

		AGE AT INJECTION 24 HOURS DOSE 5 MG	AGE AT INJECTION 96 HOURS		SIGNIFICANCE OF DIFFERENCES BETWEEN INJECTIONS AT 24 AND 96 HOURS FOR DOSE OF 5 MG
			Dose 5 mg	Dose 10 mg	
Number of fertile eggs injected		292	128	123	
Mortality during first 6 days and from 96 hours to end of 2nd week, respectively	%	16.1	3.1	8.1	$\chi^2 = 13.949$ $P = <.001$
Survivors of 17th day		235	117	110	
Short upper beak	%	7.2	0.9	1.8	$\chi^2 = 5.336$ $P = <.02$
Other abnormalities		0	0	0	

change in the incidence of micromelia or syndactylism (the latter not shown in the table) among the embryos surviving the 17th day of development, but the frequency of beak defects was greatly reduced. The experiment was performed twice with similar results. It will be noted that the incidence of micromelia and of beak abnormalities was considerably lower in the second experiment than in the first. This was true whether insulin was used alone or in combination with glu-

TABLE 5

The effect of supplementation of insulin-treatment with glucose-1-phosphate, citric acid and oxalacetic acid at 96 hours of incubation. White Leghorn eggs

Dosage of insulin 2 units throughout

DATE	TREATMENT	NUMBER OF TREATED EMBRYOS	MORTALITY FROM 96 HOURS TO END OF 2ND WEEK	SURVIVORS OF 17TH DAY	MICROMELIA	ABNORMAL BEAK	OTHER ABNORMALITIES	SIGNIFICANCE OF DIFFERENCES IN INCIDENCE OF BEAK ABNORMALITIES
			%		%	%	%	
4/25	Insulin + 4 mg glucose-1-phosphate	146	18.6	61	78.1	9.4	6.3	$\chi^2 = 7.606$ P = <.01
	Insulin	157	23.6	112	75.0	26.8	4.5	
5/30	Insulin + 4 mg glucose-1-phosphate	307	36.8	189	51.3	16.1	2.6	$\chi^2 = 15.678$ P = <.0001
	Insulin	153	28.8	96	49.0	37.5	5.2	
	Insulin + 25 mg citric acid	162	43.2	90	51.1	12.2	8.9	$\chi^2 = 15.607$ P = <.0001
	Insulin + 7.15 mg oxalacetic acid	150	42.0	83	37.3	14.5	2.4	$\chi^2 = 12.142$ P = <.0001

cose-1-phosphate and is an expression of a seasonal trend, already noted in earlier experiments (Landauer, '45). The relative reduction in frequency of beak defects under the influence of glucose-1-phosphate supplementation was, however, of a similar order in the two experiments. The beak abnormalities which occurred were of 4 different kinds, viz., parrot beak, shortened upper beak, cleft palate and cross-beak. Parrot beak is the only one of these 4 types of defects which, in its milder forms of expression, might lead to mistaken classification. It was, therefore, decided to scrutinize the effect of glucose-1-phosphate by adding all specimens with parrot beak to the normal groups, thus limiting the statistical analysis to those forms of abnormality in which we were dealing with presence or absence. Even with this, unquestionably over-stringent, procedure it was clear that glucose-1-phosphate furnished protection against insulin-induced beak abnormalities ($\chi^2 = 10.645$; $P = .001$).

A similar lowering in the incidence of beak defects was observed when insulin-treatment was supplemented with fructose-6-phosphate (5 mg/egg), but our data are too scanty to be trusted without reserve. Supplementation of insulin-treatment at 96 hours with fructose-1,6-diphosphate, on the other hand, clearly did not modify any of the teratogenic effects of insulin.

At 24 hours, supplementation of insulin with glucose-1-phosphate (4 mg/egg), fructose-6-phosphate (5 mg/egg) or fructose-1,6-diphosphate (2.7 mg/egg) had no significant effect on subsequent incidence of rumplessness. In two tests with glucose-1-phosphate we observed, however, that embryo mortality was increased significantly and micromelia was found in 10 and 11%, respectively, of the survivors of the 17th day. There were no cases of micromelia among the embryos treated with insulin alone.

Results similar to those with glucose-1-phosphate were obtained when insulin-treatment at 96 hours was combined with the injection of oxalacetic or citric acid. Embryo mortality was increased by these supplements, the frequency with which

micromelia occurred was not affected, but the incidence of beak defects was reduced to a highly significant extent (table 5). When given alone, oxalacetic acid had appreciable toxicity for 96-hour embryos (mortality to end of second week 24.1%), but citric acid was harmless.

At the 24-hour stage, supplementation with oxalacetic acid did not significantly change the incidence of insulin-induced rumplessness. Citric acid, on the other hand, greatly reduced embryo mortality and incidence of rumplessness when added to insulin treatment at 24 hours (table 6). The results are

TABLE 6

The effect of citric acid on insulin-induced rumplessness. White Leghorn embryos. All injections at 24 hours

Dosage: insulin 2 units, citric acid 25 mg

TREATMENT	FERTILE EGGS	MORTALITY FIRST 6 DAYS	SURVIVORS OF 17TH DAY	RUMPLESS (ALL TYPES)	OTHER ABNORMALITIES
		%		%	%
Insulin	165	52.1	65	29.2	9.3
Insulin + citric acid	171	17.0	135	11.1	2.9
Citric acid	165	3.6	157	1.3	0

highly significant in both respects (χ^2 for embryo mortality = 46.035, P .0001; for incidence of rumplessness χ^2 = 10.165, P = .0005) and comparable to those obtained with lactic acid which will be discussed below. Isocitric acid (2.5 mg) given with insulin at 24 hours led to increased mortality and potentiation of the insulin effect (44.7% rumplessness, 10.5% beak abnormalities).

Pyruvic and lactic acid; sodium acetate

In testing for the alleviatory effect of other compounds on the teratogenic action of insulin we were particularly interested in relatively simple metabolic intermediates such as

pyruvic, lactic and acetic acid. Our observations with undiluted pyruvic acid (Matheson Co.) are summarized in tables 7 and 8. It can be seen that pyruvic acid, when given simultaneously with insulin, is very effective in forestalling the occurrence of rumplessness (table 7). The combined administration of these two substances at 24 hours of incubation reduced the incidence of rumplessness, in comparison to that after unsupplemented insulin treatment, from 23.1% to

TABLE 7

Experiments concerning the effect of pyruvic acid on insulin-induced rumplessness in White Leghorn embryos

Dosage: 2 units insulin, 0.05 cm³ undiluted pyruvic acid

GROUP	TREATMENT	FERTILE EGGS	MORTALITY FIRST 6 DAYS	SURVIVORS OF 17TH DAY	RUMPLESS
			%		%
1	Insulin — 24 hours	254	45.3	130	23.1
2	Insulin and pyruvic acid — 24 hours	256	18.8	174	5.2
3	Insulin — 24 hours pyruvic acid — 27 hours	243	39.1	124	16.9
4	Insulin — 24 hours pyruvic acid — 30 hours	257	40.1	134	17.2
5	Insulin — 24 hours pyruvic acid — 36 hours	245	41.6	124	19.4
6	Pyruvic acid — 24 hours	171	10.5	146	2.7

5.2% ($\chi^2 = 21.252$; $P < .0001$), and embryonic mortality during the first 6 days dropped from 45.3% to 18.8% ($\chi^2 = 41.206$; $P < .0001$). If pyruvic acid is, however, supplied three or more hours subsequent to the injection of insulin, it has little, if any, beneficial effect on either incidence of rumplessness or toxicity of the insulin. No similarly beneficial results followed phosphopyruvic-acid supplementation (2 mg/egg); in fact, the toxicity in combination with insulin was very high and the incidence of rumplessness increased.

TABLE 8

Experiments concerning the effect of pyruvic acid on insulin induced micromelia and beak defects in White Leghorn embryos

Dosage: 2 units insulin, 0.05 cm³ undiluted pyruvic acid

GROUP	TREATMENT	NUMBER OF TREATED EMBRYOS	MORTALITY TO END OF 2ND WEEK	SURVIVORS OF 17TH DAY	MICROMELIA	ABNORMAL BEAK	ABNORMAL EYES
			%		%	%	%
1	Pyruvic acid — 117 hours insulin — 120 hours	405	71.9	74	75.7	29.7	5.4
2	Insulin and pyruvic acid — 120 hours	425	69.6	77	71.4	11.7	2.6
3	Insulin — 120 hours	225	20.0	168	33.3	4.8	0.6
4	Insulin — 120 hours pyruvic acid — 123 hours	396	77.3	52	75.0	17.3	0
5	Pyruvic acid — 120 hours	137	83.2	23	0	0	0

The effect of pyruvic-acid supplementation is very different at the time when insulin treatment leads to the occurrence of micromelia and beak defects (table 8). Pyruvic acid, given simultaneously with insulin-injection or within three hours prior or subsequent to it, greatly increases, at this stage of development, embryo mortality as well as incidence of micromelia and beak defects. All differences in these respects between group 3 (insulin alone) and the other three groups are statistically highly significant, except for that relating to the incidence of beak abnormalities in groups 2 and 3. The toxicity

TABLE 9

Experiments concerning the effect of lactic acid on insulin-induced rumplessness in White Leghorn embryos

Dosage: 2 units insulin, 0.05 cm³ lactic acid (40%)

GROUP	TREATMENT	FERTILE EGGS	MORTALITY FIRST 6 DAYS	SURVIVORS OF 17TH DAY	RUMPLESS
			%		%
1	Insulin — 24 hours	336	42.0	181	24.3
2	Insulin and dl-lactic acid — 24 hours	334	20.4	235	12.8
3	Insulin and d-lactic acid — 24 hours	355	18.3	265	18.1
4	Insulin and dl-lactic acid — 24 hours	368	15.2	289	13.1

which was encountered in these experiments appears to be wholly due to the pyruvic acid. When pyruvic acid was injected by itself at 120 hours, mortality to the end of the second week amounted to 83.2% out of 137 treated embryos. No abnormalities of any kind were, however, found among the survivors. Phosphorpyruvic acid, given by itself, was harmless at this stage. In combination with insulin it increased embryo mortality without changing the incidence of abnormalities.

The results of experiments with lactic acid are shown in tables 9 and 10. When insulin treatment at 24 hours was

TABLE 10

Experiments concerning the effect of dl lactic acid on insulin induced micromelia and beak abnormalities in White Leghorn embryos

Dosage: 2 units insulin, 0.05 cm³ lactic acid (40%)

GROUP	TREATMENT	NUMBER OF TREATED EMBRYOS	MORTALITY FROM 96 HOURS TO END OF 2ND WEEK	SURVIVORS OF 17TH DAY	MICROMELIA	ABNORMAL BEAK	ABNORMAL EYES
			%		%	%	%
1	Lactic acid — 117 hours insulin — 120 hours	234	44.0	129	34.9	6.2	0
2	Insulin and lactic acid — 120 hours	221	59.7	78	41.0	12.8	1.3
3	Insulin — 120 hours	239	18.4	200	51.5	11.0	0.5
4	Insulin — 120 hours lactic acid — 123 hours	228	50.9	111	49.5	8.1	0.9
5	Lactic acid — 120 hours	148	54.7	67	0	0	0

supplemented by injection of racemic lactic acid, the incidence of rumplessness and embryo mortality were reduced to about one-half of that occurring after treatment with insulin alone. The differences are in both instances highly significant (between groups 1 and 2 χ^2 for embryo mortality was 36.451 with $P<.0001$, and for incidence of rumplessness $\chi^2=9.312$; $P<.01$). A repetition of the experiment with dl-lactic acid gave closely similar results (group 4), but with d-lactic acid (Pfanstiehl), the naturally occurring form, the results were less favorable than with the racemic acid.

The results of lactic-acid supplementation of insulin-treatment at 120 hours are shown in table 10. When racemic lactic acid was given three hours prior to insulin injection, the incidence of micromelia was reduced significantly ($\chi^2=8.708$, $P<.01$); there were also fewer beak abnormalities, but in this instance the difference was not statistically significant. Injection of lactic acid simultaneously with or three hours subsequent to insulin-treatment had no significant effects on incidence of either micromelia or beak abnormalities. It can be seen, on the other hand, that the combined treatment with lactic acid and insulin produced in all groups increased embryo mortality (in all instances $P<.0001$). This is accounted for by the high degree of toxicity which lactic acid alone has at this stage of development (group 5). As was true for pyruvic acid, lactic acid reduced the mortality from insulin treatment in early embryonic stages, but had a highly toxic effect in later ones.

Sodium acetate in a dose of 10 mg/egg was toxic, without producing abnormalities, but when injected together with insulin resulted in an exaggerated incidence of the teratologic effects of insulin as well as heightened embryo mortality. This was especially true at 24 hours, but to a lesser extent also at 96 hours.

Amino acids

We performed some experiments with l-tryptophane (1.2 mg/egg), dl-methionine hydrochloride (3 mg/egg), l-glutamic

acid hydrochloride (12.5 and 25 mg/egg) and dl-alanine (7.2 mg/egg). When administered by themselves and in the given amounts, none of these amino acids, with the possible exception of l-glutamic acid hydrochloride, was teratogenic at either 24 or 96 hours of development. Except for l-glutamic acid hydrochloride, no striking toxicity was observed after injection of these compounds. At 24 hours, l-glutamic acid hydrochloride was somewhat toxic and pronouncedly so at 96 hours. Methionine, tryptophane and alanine in combination with insulin led to increased embryo mortality and among the survivors the incidence of rumplessness was higher than after insulin alone. At 96 hours these three amino acids probably produced no significant change in embryo mortality, nor in the teratogenic effects of insulin. A more interesting situation obtains in regard to supplementation with l-glutamic acid hydrochloride. Injected alone, this compound showed some toxicity for 24-hour embryos (19.5 ± 3.45% mortality during first 6 days) and probably had a slight rumplessness-inducing effect (6.5 ± 2.46%). We made three tests at 24 hours in which the injection of two units insulin was supplemented by 12.5 mg/egg l-glutamic acid hydrochloride and two tests in which 25 mg/egg of this substance were given in addition to insulin. Since there was no apparent inconsistency between the results for the two different dosage levels, we are here reproducing only the totals for all 5 tests (table 11). It can be seen that l-glutamic acid hydrochloride supplementation reduced mortality as well as incidence of rumplessness. At 96 hours l-glutamic acid hydrochloride supplementation sharply increased embryo mortality, but did not change the teratogenic action of insulin.

Sodium succinate and 3-hydroxy-anthranilic acid

Some substances which are toxic and growth-retarding, but do not by themselves produce any teratological results, potentiate strikingly the incidence of insulin-induced micromelia and beak abnormalities when given together with in-

sulin at 96 or 120 hours of development. This is true, for instance, for adrenal cortex extract (Landauer, '47a). There are other substances which produce a similar potentiation of insulin action, but, when given alone and in the same amounts, neither affect viability nor interfere with normal development. Among compounds of the latter type are sodium succinate and 3-hydroxy-anthranilic acid. In experiments with 96-hour embryos 91.9% of all embryos survived the 17th day after the injection of 4 mg 3-hydroxy-anthranilic acid; among the 147 survivors the only abnormalities found were two cases of micromelia and one of cleft palate. After the injection of 8 mg

TABLE 11

Results of 4 tests with insulin alone and in combination with l-glutamic acid hydrochloride at 24 hours of development. Two units insulin all tests; l-glutamic acid hydrochloride 12.5 and 25 mg/egg, respectively, in different tests. White Leghorn eggs. Standard errors.

TREATMENT	FERTILE EGGS	MORTALITY DURING FIRST 6 DAYS	SURVIVORS OF 17TH DAY	RUMPLESSNESS
		%		%
Insulin	667	37.0 ± 1.91	391	24.3 ± 2.15
Insulin + l-glutamic acid hydrochloride	831	24.8 ± 1.50	600	13.3 ± 1.38
Difference		12.2 ± 2.43		11.0 ± 2.55

sodium succinate 88.8% of all embryos survived the 17th day and among the 143 survivors one rumpless chick was the only abnormality observed. When the same amounts of sodium succinate or 3-hydroxy-anthranilic acid were injected simultaneously with insulin, we obtained the results shown in table 12. For comparison we give the data for 4 tests with insulin alone, done during the same period. It can be seen that the incidence of all malformations was greatly exaggerated by the presence of either sodium succinate or 3-hydroxy-anthranilic acid. This potentiating effect was relatively the greater, the lower was the frequency with which a particular type of abnormality occurred under the influence of unsupplemented

TABLE 12

Data concerning the potentiating effect of sodium succinate and 3-hydroxy-anthranilic acid on the teratogenic action of 2 units insulin at 96 hours.

White Leghorn eggs

TREATMENT		SURVIVORS OF 17TH DAY	MICROMELIA	SYNDACTYLISM	BEAK ABNORMALITES
			%	%	%
Insulin + 8 mg sodium succinate 5/23/50		95	84.2 ± 3.75	18.9 ± 4.02	34.7 ± 5.17
Insulin + 4 mg 3-hydroxy-anthranilic acid 6/20/50		65	89.2 ± 3.86	36.9 ± 6.0	69.2 ± 5.75
Insulin (4 tests) 5/23 to 6/27 1950	Mean	425[1]	60.0 ± 2.38	4.5 ± 1.01	26.1 ± 2.13
	Range	96–123	49.0–66.3	2.4–6.3	12.2–37.5

[1] Total of the 4 experiments.

insulin injection. For combined treatment with insulin and 3-hydroxy-anthranilic acid the degree of potentiation in terms of the mean incidence in the unsupplemented insulin control groups is demonstrated by the following figures.

	INCIDENCE AFTER INSULIN ALONE	POTENTIATION DUE TO 3-HYDROXY-ANTHRANILIC ACID
	%	%
Micromelia	60.0	48.7
Beak defects	26.1	165.1
Syndactylism	4.5	720.0

Syndactylism shows the most extreme effect. Syndactylism, not heretofore reported as an insulin-induced abnormality and to be discussed in more detail subsequently, was found in about 4.5% of Leghorn embryos which had been treated with two units of insulin at 96 hours. When, in addition to insulin, 8 mg of sodium succinate or 4 mg of 3-hydroxy-anthranilic acid were injected, the incidence of syndactylism rose to 18.9% and 36.9%, respectively.

Results with sodium succinate at 24 hours were very similar to those at 96 hours. Given by itself, it was non-toxic and non-teratogenic; added to insulin treatment, it greatly increased embryo mortality and incidence of rumplessness. The effect of 3-hydroxy-anthranilic acid at 24 hours was more complex. When this compound was injected by itself (4 mg/egg), it showed some toxicity (mortality during first 6 days $29.5 \pm 3.5\%$) and among the survivors of the 17th day we found 11.9% rumpless embryos. In a preliminary test with the same dosage the frequency of rumplessness had been 10% and for the combined data we have an incidence of $11.4 \pm 2.59\%$. It is evident that 3-hydroxy-anthranilic acid is by itself relatively potent in inducing rumplessness. Combined injection of insulin and 3-hydroxy-anthranilic acid resulted in very high mortality (84.4% during the first 6 days), but among the small number of survivors the incidence of rumplessness was not remarkably high (18.2%), possibly on account of differential mortality. On the other hand, this treatment led to the occurrence of some cases of micromelia, cleft palate and syndactylism, none of which were at this developmental stage produced by two units of insulin alone.

DISCUSSION

The major results of our experiments are as follows. A repetition and extension of earlier experiments with nicotinamide showed anew that, if proper dosage levels were used and the amount of administered insulin was not too great, supplementation with this vitamin gave complete protection against the usual teratogenic effects of insulin treatment at 96 or 120 hours of incubation. In spite of the fact, however, that supplementary nicotinamide prevented the occurrence of micromelia and beak defects[1] among the survivors to late stages, the post-operative mortality was not reduced. To a certain extent nicotinamide also protected genetically polydactylous embryos against the modifier-like effects that in-

[1] These malformations of the beak have nothing in common with the post-mortem beak abnormalities reported by Blattner and Williamson ('50).

sulin has on the expression of this mutation. Lactic acid, when given three hours prior to insulin treatment, reduced the incidence of micromelia, but had little influence on the frequency of beak abnormalities. Lactic acid had no protective value when administered simultaneously with or subsequent to insulin. In all these situations the combined use of insulin and lactic acid was, however, highly toxic. In contradistinction to the effects of lactic acid, supplementation of insulin-treatment with citric acid (when given simultaneously) did not change the incidence of micromelia, but furnished considerable protection against the occurrence of beak defects. Results similar to those with citric acid were obtained with glucose-1-phosphate, oxalacetic acid and probably fructose-6-phosphate.

In accord with earlier observations we found that during the rumplessness-inducing period of insulin (24 hours of incubation) nicotinamide gave some protection to embryos, but that its effectiveness was much lower than at the later stage and that, in addition, it was definitely toxic at this earlier time. Supplementary pyruvic acid, on the other hand, sharply reduced the incidence of rumplessness and greatly lowered the toxicity which follows treatment with insulin alone. Among many substances tested, pyruvic acid was most effective in this respect. Results of a similar nature, but at a lower level of efficiency, followed supplementation with lactic and citric acid; l-glutamic acid hydrochloride gave a slight, but significant, amount of protection. From present and earlier evidence we may conclude that the sequence of protection against insulin-induced rumplessness, beginning with the most effective compound, is about as follows: pyruvic acid > lactic acid = citric acid > nicotinamide > α-ketoglutaric acid ≥ l-glutamic acid hydrochloride.

It has been shown in previous communications that the production of micromelia and beak defects can be achieved by treatment with eserine, sulfanilamide or insulin and that in all three instances supplementary nicotinamide protected embryos against the teratogenic action of the three compounds

(Landauer, '49; Zwilling and DeBell, '50). We could further demonstrate that the rumplessness-inducing action of sodium cacodylate and insulin can similarly and to a large extent be forestalled by added pyruvic acid. It seems justified to draw three conclusions from this evidence, viz., (1) that, as pointed out by Ancel ('45b, '50), certain chemical compounds have more or less specific teratogenic potentialities; (2) that insulin has several different potentialities depending on developmental stage; and (3) that, irrespective of the teratogenic agent employed, the same supplements were effective in forestalling the occurrence of a particular malformation or in reducing its incidence.

There is additional evidence in support of these conclusions. The two substances that in Ancel's ('45a, '50) experiments most regularly produced micromelia and beak defects, viz., eserine and sulfanilamide, were also the ones that led most frequently to the occurrence of syndactylism. We had not previously reported on the production of syndactylism by insulin, but our present observations show that during the stages when insulin treatment leads to the occurrence of micromelia and malformations of the beak, it is also responsible for the appearance of a certain incidence of syndactylism. Nicotinamide protects the embryo against this abnormality as well as against the other symptoms of insulin-treatment typical for the 96- or 120-hour stages. Syndactylism thus represents another malformation that can be produced by three different and unrelated chemical compounds and that in all instances can be forestalled by the same supplement. It is of particular interest to note that insulin-induced syndactylism always involves the 3rd and 4th toes, as does hereditary syndactylism. This is additional evidence for our earlier conclusion that the majority, perhaps all, of the insulin-induced abnormalities are of the nature of skeletal phenocopies. Hereditary syndactylism was believed to be inseparable from brachydactylism and feathering of the feet (Danforth, '19; Jaap, '39), but no instances of such association occurred in our material and Warren ('50) has recently

described a hereditary form of syndactylism, unaccompanied by these traits.

Among the beak defects produced by insulin one can distinguish 4 different types, viz., parrot beak (associated with a moderate shortening of the lower beak), short upper beak, cross-beak and cleft palate. The proportions in which these defects occur vary considerably from experiment to experiment, probably parallel with minor differences in the developmental stages obtaining at the time of treatment. All 4 types of beak defects are prevented by supplementary nicotinamide. We had illustrated a case of cleft palate and facial coloboma in an earlier publication (Landauer, '47b, fig. 4) but did not then describe it in detail. Ancel could produce cleft palate with trypaflavin, but with none of the other substances tested by him, in particular not with eserine and sulfanilamide, the two compounds leading to a high incidence of micromelia and parrot beak. This is, therefore, another instance in which insulin has the greater teratogenic latitude and in which it has been shown that nicotinamide-preventable abnormalities may be separately produced by unrelated chemical compounds. It cannot be doubted that these various substances, producing the same malformations and being made non-teratogenic by the same supplements, create their effects via identical or closely similar pathways.[2]

Throughout our observations it has become evident, however, and we shall have further occasion to discuss it, that, in spite of these similarities in the teratogenic action of groups of substances, there are many minor, but significant, dissimilarities in their effects. We shall here call attention to two examples concerning the position in time of the maximum susceptible period for certain compounds. It was shown earlier that the incidence of rumplessness after insulin treat-

[2] In mammalian embryos cleft palate has been produced experimentally in rats with trypan blue (Gillman, Gilbert and Gillman, '48), maternal riboflavin deficiency (Warkany, Nelson and Schaffenberger, '43), and maternal folic acid deficiency (Evans, Nelson and Asling, '51), in mice as a consequence of anoxia (Ingalls, Curley and Prindle, '50). Some of these techniques may in their biochemical effects be related to those described for chicken embryos.

ment increases from 0 hours (unincubated eggs) to a maximum early in the second day of incubation (Landauer and Bliss, '46). In contradistinction, sodium cacodylate has a significantly higher rumplessness-inducing effect at 0 hours than later. It is also of interest that following treatment with sodium cacodylate the protective value of nicotinamide seems to decrease during the same period (0–24 hours). A difference similar to that between sodium cacodylate and insulin, in regard to the production of rumplessness, exists between sulfanilamide and insulin with reference to the origin of micromelia. The incidence of micromelia after insulin-treatment rises to a maximum at approximately 120 hours of incubation (Landauer and Bliss, '46), whereas sulfanilamide-induced micromelia reaches its peak frequency at about 48 hours with a steep decline thereafter, the incidence following injection at 120 days being only about one-quarter that at 48 hours (Zwilling and DeBell, '50). In spite of these great differences in the stages of maximum effectiveness, nicotinamide completely forestalls the occurrence of micromelia in both instances. It is difficult to escape the conclusion that these time dissimilarities of chemical action are caused by effects on different, if closely related, links of the same metabolic chain, the links in question presumably having varying biochemical importance at one time or another, but damage to the chain being in either event repairable by identical supplements.

Of apparently quite another order are the events that, at different developmental stages, produce distinctive and dissimilar abnormalities, such as rumplessness or micromelia. Attention has already been called to the fact that, according to Ancel's observations, some chemicals have "specific" teratogenic effects. It does not reduce the importance of this finding that in those instances where well-defined stage-response differences exist, as with insulin, the stage "specificity" can to some extent be overcome by an increased dose of the teratogenic agent or by supplementation with substances that by themselves are non-teratogenic. For, even

with such substances as insulin, it is clear that the response differences of the embryo in different developmental stages are not merely the effect of an interference with identical physiological processes in altered morphological situations.

There is evidence of a varied nature for the conclusion that the origins of these stage and type-specific effects are separated by important changes in biochemical functions. To start with, it has been seen that striking differences exist in the protective efficiency of various supplements, such as pyruvic acid being best in forestalling rumplessness, irrespective of the chemical character of the teratogenic agent, and nicotinamide being the most successful protective substance against micromelia and beak defects, again without regard to the chemical constitution of the teratogenic compound. The nature of these differences will be further discussed below. Secondly, altered biochemical situations are evident from the toxicity relationships of various supplements. It has been shown, for instance, that pyruvic acid, while reducing insulin toxicity (as well as its teratogenic effects) during the rumplessness-inducing stages and being quite harmless when given alone, becomes extremely toxic (and loses its protective properties) probably prior to the establishment of circulation and certainly by the time when insulin and other chemicals lead to micromelia. Similar situations exist for lactic, citric and l-glutamic acid. Conversely, substances such as nicotinamide and glucose-1-phosphate, are definitely toxic during the early embryonic stages when they have little or no protective value as far as the origin of rumplessness goes, but are non-toxic later when they serve to reduce or prevent the production of micromelia and/or beak abnormalities. All these statements can, of course, be only made with assurance for the dosages used in our experiments. Still other evidence of a similar kind comes from the distinctive manner in which various substances potentiate the teratogenic insulin effects at one stage, but not at another. Thus adrenal cortex extract has no influence on the manner of occurrence of rumplessness after insulin, but forcefully potentiates incidence and ex-

pression of the abnormalities induced by insulin during the 4th or 5th day (Landauer, '47a); contrariwise, phosphopyruvic acid (2 mg/egg) and glutamine (1.8 mg/egg) greatly exaggerate the effects of insulin during the rumplessness-inducing period (unpublished data), but do not produce similar results when added to insulin during the micromelia-inducing stages. All these observations point to the existence of important physiological dissimilarities in the origin of abnormalities during the two developmental periods.

Even at one and the same stage one finds response differences to supplementation which any attempt at interpretation must take into account. Insulin treatment at 96 or 120 hours produces micromelia, beak defects and syndactylism, the incidence of the three types of defects decreasing in the given order. These variations in frequency of occurrence may be determined by developmental differences between the several primordia at the time of treatment or by dissimilarities in their quantitative dependence on the processes that are disturbed by insulin treatment. There are indications, however, that even in these situations qualitative variations in local functions may play an important role. This is suggested by our evidence for differential protection and differential potentiation following treatment with insulin and various supplements. Instances of differential protection (in experiments at 96 hours of incubation) include our observation that lactic acid supplementation reduced the incidence of insulin-induced micromelia, but not that of beak defects, or, that citric acid, glucose-1-phosphate and yet other substances protect against insulin-beak defects, but not against micromelia. Differential potentiation is well illustrated by the fact that supplementation of insulin treatment at 96 hours with 3-hydroxy-anthranilic acid produced relatively the greater potentiation the lower was the incidence of a particular abnormality after insulin alone (order of potentiation: syndactylism > beak defects > micromelia). Sodium succinate supplementation gave similar results. It would be difficult to interpret these observations in any fashion other than the occurrence of dif-

ferential biochemical effects of insulin treatment on various organs and parts. It is quite possible that the primary action of insulin is the same in all instances, but that the results flowing from it vary according to the metabolic importance of the affected substance at the time of treatment and that the effectiveness or toxicity of supplements is in part determined by changing substrate requirements.

The extreme complexity of the teratogenic mechanisms that are responsible for our results is sufficiently clear. At the present stage of almost complete ignorance about the biochemical processes involved in morphogenesis and differentiation one cannot expect to define more than the most general outlines of the events that took place under the conditions of our experiments. It cannot be doubted, at any rate, that the principal, if not only, primary disturbance which, in our material, leads to abnormal development is an interference with embryonic carbohydrate metabolism. This is evident from the nature of the teratogenic substances, from the type of compounds which have preventative effects when used as supplements, and from the kinds of substances acting as potentiators of the teratogenic agents. Additional evidence from experiments with inhibitors of glycolysis will be presented below. This general conclusion is, of course, in good agreement with what is known about the importance of carbohydrate utilization in early development.

Our results also demonstrate clearly, however, that between our "early" (0 or 24 hours) and "late" (96 or 120 hours) stages of experimentation important differences exist in the manner in which the embryonic carbohydrate metabolism is interfered with by our procedures. Before proceeding with a discussion of these differences, it will be well to present the results of experiments with various inhibitors of anaerobic glycolysis. We tested the effect of sodium malonate, sodium fluoride, glyceraldehyde, phlorizin and monoiodoacetic acid on development of the chicken embryo at 0, 30 and 96 hours. The results are shown in table 13. This table indicates only the incidence of rumplessness at 0 and 30 hours and of

micromelia and beak abnormalities at 96 hours; no other defects occurred in significant numbers. During the two early stages phlorizin and monoiodoacetic acid produced rumplessness in highly significant frequencies: both were more effective at 30 hours than at 0 hours. Among a large number of tested substances, monoiodoacetic acid ranks with insulin and Ancel's arsenic compounds (sodium cacodylate and so-

TABLE 13

The effects of inhibitors of anaerobic glycolysis at 0, 30 and 96 hours of development. White Leghorn embryos

TREATMENT	AT 0 HOURS		AT 30 HOURS		AT 96 HOURS	
	Number	Rumplessness	Number	Rumplessness	Number	Micromelia and beak defects
		%		%		%
Sodium malonate (10 mg/egg)	57	3.5	[1]	[1]	129	0.8
Sodium fluoride (40 γ/egg)	101	0	131	3.8	152	0
Glyceraldehyde (63 γ/egg)	101	4.0	134	3.0	146	0
Phlorizin (250 γ/egg)	146	6.2	139	8.6	148	0
Monoiodoacetic acid (0.6 mg/egg)	96	6.3	93	17.2	120	0

[1] Not tested.

dium methyl arsonate) as one of the most potent agents for the production of rumplessness. None of the 5 inhibitors had significant teratogenic effects at 96 hours; this was even true when twice the amounts indicated in the table were used and in spite of the fact that some of the inhibitors, e.g. monoiodoacetic acid, were definitely toxic.

In view of these observations as well as of the evidence presented previously there can be little doubt that in our experiments the production of rumplessness between 0 and

30 hours of incubation had its origin in a disruption of anaerobic glycolysis. It is more hazardous to speculate on the point of interference within the metabolic chain, but some interesting facts should be pointed out. To start with, it is known that the pyruvate oxidase system (pyruvate dehydrogenase) is especially sensitive to arsenical poisoning. This has been shown for trivalent organic arsenicals (Barron and Singer, '43) and for arsenite (Sinclair, quoted from Bernheim, '48; Cohen-Bazire et al., '48), and our evidence (Landauer, '49) suggests that the same holds for *organic* arsenic compounds of pentavalent origin. On the assumption that the arsenic compounds used by Ancel and ourselves interfere with steps between phosphopyruvate and lactate and thereby produce rumplessness, it would become understandable that pyruvate and lactate are effective in preventing or reducing the occurrence of rumplessness and that phosphopyruvic acid, given in addition to one of the teratogenic agents (insulin), not only is very toxic, but greatly increases the incidence of rumplessness.[3] It seems reasonable to assume that insulin treatment blocks the coenzyme supply and that pyruvic acid during these early and predominantly anaerobic stages of development aids in the reoxidation of the pyridine nucleotides, thereby making them again available to the embryo. The fact that monoiodoacetic acid, among the inhibitors tested, stands out for its effectiveness in producing rumplessness also fits well into this interpretation. Finally, Spratt ('50) has shown in explantation experiments that the damaging effects of monoiodoacetic acid can be prevented by substituting pyruvate or lactate for the glucose of the culture medium. It must be admitted, however, that many of the facts relating to the experimental production of rumplessness could also be explained on the basis of interference with phosphorylating mechanisms. No final answer can at present be given.

[3] The combination of two units insulin and 2 mg phosphopyruvic acid injected at 24 hours produced a mortality of 83.5% during the first 6 days. Among the survivors of the 17th day 42% were rumpless. At 96 hours phosphopyruvic acid did not have similarly toxic or potentiating effects. Reduced glutathione did not protect against sodium cacodylate-induced rumplessness.

The stages during which rumplessness is most readily produced by teratogenic substances occur prior to the existence of circulation, and the action of these substances is presumably directly on cellular functions of the embryo itself. At the later period, however, when the embryo is likely to respond with micromelia and beak abnormalities, the circulatory system is well established. It is true that even at this stage the endocrine regulation of carbohydrate utilization has not yet come into being, the embryos in this respect still resembling the hypophysectomized and pancreatectomized adult mammal, but the yolk sac membrane has begun to function as "transitory liver." The investigations of Zwilling ('51b) have shown that one of the agents inducing micromelia and beak abnormalities, viz. insulin, has profound effects on the carbohydrate storage functions of the yolk sac membrane, thereby producing hypoglycemia and carbohydrate starvation of the embryo (Zwilling, '48, '51a b). Whether or not the disturbances of yolk sac membrane functions are the only primary consequence of insulin treatment at this stage, carbohydrate deficiency and malformations of the embryos being a consequence, remains to be determined. It seems at present more likely that the teratogenic agents responsible for micromelia and beak abnormalities have direct, as well as indirect, effects on the embryo since a similar syndrome of defects (and one also preventable by nicotinamide supplementation) follows sulfanilamide treatment in the absence of hypoglycemia (Zwilling and DeBell, '50).[4] Moreover, the same syndrome or similar ones can be produced by treatment with eserine sulfate or by deficiencies of the maternal diet in biotin, riboflavin and manganese, none of which conditions are known or likely to be accompanied by changes in the blood sugar level.

Taking into account the great stage-dependent differences in effect which supplementary substances, such as nicotinamide and pyruvic acid, produce in conjunction with insulin,

[4] It is interesting in this connection that according to Mendes Alves ('46) sulfanilamide and other sulfa compounds reduce the hypoglycemic effects of insulin and that this anti-insulin action is removed by nicotinamide.

and in view of the fact that inhibitors of anaerobic glycolysis which are teratogenic in early development become non-teratogenic later on, it must be concluded that during the transition from the 24-hour stage to that at 96 hours important changes have occurred in the normal metabolic activities of chicken embryos. It may be suspected that the altered responses are related to the increasing importance of aerobic respiration, as indicated *inter alia* by the sudden rise in cytochrome oxidase after the third day of development (Albaum and Worley, '42; Albaum, Novikoff and Ogur, '46). Establishment or increasing importance of the tricarboxylic acid cycle may be a factor.

In view of the high efficiency with which nicotinamide protects, between 96 and 120 hours of embryonic development, against the teratogenic effects of insulin, there can be little doubt that during this period insulin interferes with codehydrogenase activity.[5] This is, in any event, the only established function of nicotinamide (via the pyridine nucleotides). It seems likely that, as in the earlier rumplessness-producing stages, insulin temporarily reduced or abolished existing coenzyme supplies, possibly by competing for the apoenzyme, but that at this later stage with its different respiratory activities nicotinamide (rather than pyruvic acid) is needed as a supplement in order to insure the availability of adequate amounts of coenzyme. Most of our observations with various supplements, whether beneficial or harmful, can be reconciled with this view. In particular does it become understandable that nicotinamide, in excessive amounts (equal amounts being more excessive in early stages), is likely to be toxic and teratogenic by acting as an inhibitor of its own coenzyme.

Certain pathways which might be suspected to play a role in the origin of our malformations are ruled out by available evidence. Inhibition of succinic dehydrogenase activity seems excluded since several inhibitors of this system (sodium

[5] Measurements are in progress.

malonate, triphenyl tetrazolium chloride and oxalacetic acid) have no teratogenic effects on 96-hour chicken embryos, even when given in toxic amounts.

Since eserine is one of the substances producing micromelia and beak defects, it might be asked if the origin of these abnormalities is related to anticholinergic action. This is very unlikely for two reasons. The anticholinergic consequences of eserine presumably occur after much smaller amounts of the drug than Ancel and ourselves found effective for the production of abnormalities. Furthermore, unpublished experiments with diisopropylfluorophosphate (0.2 cm^3/egg of 0.1% solution) at 96 hours gave no indication that this anticholinergic substance is teratogenic. It has been shown, on the other hand, that eserine sulfate, in such concentrations as are needed for the production of micromelia, leads to interference with processes of dehydrogenation (Brooks, Rensmeier and Gerard, '49), and it is in better accord with all our other evidence to assume that a mechanism of this kind is responsible for the micromelia-inducing action of eserine.

It is certain that the ultimate results of those biochemical changes which are impressed upon embryos by teratogenic substances may be modified in various ways by conditions existing in the embryos themselves, but we have little specific information about the manner in which these modifications are produced. One such modifying agency which has been noted again and again in our work is related to the age of the pullets from which eggs had been obtained. It finds expression in a gradual decline in response of embryos to insulin from early spring to mid-summer. This has been found true with regard to the incidence of rumplessness as well as of micromelia and beak defects. It seems likely that this change of response is produced by a regular trend of some kind in the composition of eggs, such as has been recorded by Csonka ('50) for nitrogen, methionine and cystine content.

Another source of variation in response to teratogenic substances, relating more to manner and degree of expression

than to incidence, is probably to be found in the exact developmental (and therefore presumably biochemical) condition of a particular organ primordium at the moment at which a teratogenic substance takes effect. Such relatively minor differences in developmental age presumably decide in which of several alternative morphological patterns rumplessness arises after insulin treatment (Moseley, '47) or in which of several possible directions beak development deviates from the norm. The lesser effectiveness of nicotinamide in preventing insulin-induced changes of expression of polydactylism as compared with its protective value against micromelia may be related to the nearness of irreversible determination of the toe primordia at the time of treatment. However, at this same time, i.e. 96 hours of development, it is still possible to produce in normal-toed stock a differential rise in the incidence of insulin-induced syndactylism by giving 3-hydroxy-anthranilic acid in addition to insulin. The developmental origin of syndactylism presumably occurs very soon after that of polydactylism. It seems, therefore, that for changes in the pattern of toe development the time of maximum sensitivity falls near the moment of irreversible differentiation.

A different situation obtains in regard to growing bones. Insulin treatment produces micromelia and, if less frequently, beak defects of chicken embryos as late as 168 hours of development (Landauer, '47) and probably still later. The long duration of susceptibility of the growing long bones to insulin is presumably accounted for by the special importance of glycogen storage in cartilage (for review see Follis and Berthrong, '49) and the existence of a phosphorylating system associating glycogenolysis with calcification (Gutman and Gutman, '41; Gutman, Warrick and Gutman, '42). Reduced glycogen stores and/or impaired glycogenolytic activity probably slow down calcification and, indirectly, many other processes of growing bones. Interference of this kind can be expected from the time calcification begins (or perhaps even earlier) to the end of cartilage replacement by bone. Hence

the protracted effectiveness of insulin in calling forth micromelia and allied defects. It remains to be explained, however, what causes the differential response of various skeletal parts, e.g. why, irrespective of the time of treatment (and also in all the mutant forms), the long bones of the leg are more affected by abnormal conditions than are those of the wing.

Many other details of our observations must remain unexplained for the time being. It is not clear, for instance, why l-glutamic acid hydrochloride affords a significant, if low, degree of protection against rumplessness after insulin-treatment, but tends to produce a certain incidence of rumplessness, when given by itself.

It is a common experience that substances of very different chemical constitution have similar pharmacological effects, but only in exceptional cases has their common pathway been discovered. This has often led students to the premature conclusion that common pathways do not exist. In the analysis of biological problems such situations have frequently been disposed of with the inference that particular responses are of an "unspecific" nature, a concept to which many shades of meaning became attached.

The idea that an "unspecific" retardation or arrest of development is the cause of malformations has a long history in which appear the names of Harvey, Haller, C. F. Wolf, Etienne and Geoffroy Saint-Hilaire, and Dareste. Its best known modern proponent, C. R. Stockard ('21) put it in the following words: "First, all types of monsters, double as well as single, may be caused by one and the same treatment; second, any one type of monster . . . may be produced by a great number of different experimental treatments; third, all effective treatments tend primarily to lower the rate of development, and, fourth, the type of monster induced depends upon the particular developmental moment or moments during which the developmental rate was reduced."

The work of Ancel and our own experiments have shown conclusively that treatment of chicken embryos at one and the same developmental stage will lead to different results

depending on the particular chemical agent to which the embryo was exposed, e.g. sodium cacodylate will give rise to rumplessness in 24-hour embryos, but not to micromelia at 96 hours, eserine sulfate or sulfanilamide will induce micromelia at 96 hours, but not rumplessness at 24 hours. It is a fact that one and the same type of abnormality frequently can be produced by several, and often chemically unrelated, substances, but wherever tests with supplementation have been applied, it could be shown that specific derangements of metabolism caused particular defects. This, of course, does not preclude the possibility that one and the same malformation may result from more than one type of biochemical interference. Nor is it justifiable to point, in support of Stockard's generalization, to our observation that one and the same compound, e.g. insulin, may produce several types of malformations depending on the developmental stage of treatment. For, our experiments with metabolic supplements make it clear that this versatility of insulin is due to a multiplicity of reactions rather than a lack of specificity.

If Stockard's third principle was meant to imply that *any* lowering of developmental rate at a susceptible period will *ipso facto* be responsible for the subsequent occurrence of malformations, it is demonstrably untrue as is shown by many cooling experiments with developing chicken embryos. But even where, following treatment with teratogenic substances, the emergence of morphological defects is associated with growth retardation, a causal relationship should not be assumed without proof. In the case of sulfanilamide, for instance, Zwilling and DeBell ('50) have shown that growth retardation and micromelia are independent and separable effects.

It is true that many of the teratogenic substances used by us are known to have inhibitory effects on mitosis. This has been shown, e.g., for insulin (Bullough, '49; Bullough and Eisa, '50), sulfanilamide (Fisher, Henry and Low, '44), arsenic compounds (King and Ludford, '50) and monoiodoace-

tic acid. Not only was it possible, however, to separate, in the case of sulfanilamide, growth retardation and teratogenesis, but many of our malformed embryos and chicks had normal body weight. Since the publication of Stockard's paper the origin of many abnormalities has been explained on the basis of interference with mitotic activity and embryo growth (e.g. Bodenstein and Abdel-Malek, '49), but in many of these instances closer scrutiny may yet trace the causal sequence to metabolic upsets within particular primordia.

Finally, the 4th of Stockard's principles can only be accepted with reservations. We could demonstrate, for instance, that the latitude of susceptible periods, in regard to a certain defect, varies with the dose of the particular teratogenic substance or that for different teratogenic agents the maximum period of susceptibility differs with respect to one and the same abnormality.

All our observations point to the conclusion that, as development proceeds, there are shifting substrate requirements and changing enzyme activities in different parts of the embryo and that the resulting internal competition is responsible for the occurrence of critical periods and their attendant dangers.

The functioning of particular enzyme systems and the availability of and needs for particular substrates presumably are inextricably interwoven during development and dependent on each other. But the immediate cause of the failure of certain organ primordia to develop normally after treatment with teratogenic agents must be looked for in a lack or insufficient supply of necessary substrate and it may be concluded from our observations that selective starvation plays an important role in the origin of malformations. The patter of abnormal development varies with the stage attained at the time of treatment, but differs considerably from that observed on explanted blastoderms (Spratt, '50). The selective susceptibility of skeleton-forming material is one of the outstanding features of our observations.

SUMMARY

Our observations can be summarized as follows.

With proper dosage and timing supplementary nicotinamide protected against the abnormalities (micromelia and beak defects) produced by insulin-injection at 96 or 120 hours of development.

Nicotinamide decreased to some extent the modifying action of insulin on the expression of hereditary polydactylism.

The incidence of rumplessness after insulin at 24 hours was only slightly reduced by supplementary nicotinamide.

At 24 hours, but not at 96 or 120 hours, embryo mortality after insulin-treatment was much increased by supplementary nicotinamide. At the earlier developmental stage nicotinamide had considerable toxicity when given alone and was responsible for the occurrence of beak abnormalities.

The incidence of beak defects after insulin at 96 hours could be selectively reduced by supplementary glucose-1-phosphate, citric acid, oxalacetic acid and, probably, fructose-6-phosphate. There was no similar lowering in abnormalities of the extremities (micromelia, syndactylism).

Pyruvic acid, when given as a supplement to insulin-treatment at 24 hours, greatly reduced the incidence of rumplessness and lowered embryo mortality. The same was not true for phosphopyruvic acid. Results similar to those with pyruvic-acid supplementation, if less spectacular, followed supplementation with lactic and citric acid. Isocitric acid was highly toxic and increased the incidence of rumplessness.

In later developmental stages pyruvic acid was very toxic and in combination with insulin potentiated the teratogenic effects of the latter. Lactic acid also became toxic at later stages, but, when given three hours prior to insulin, it still had some protective value.

The l-glutamic acid hydrochloride, when injected by itself at 24 hours, had a low rumplessness-inducing effect, but it reduced the incidence of rumplessness after insulin treatment when used as a supplement.

Sodium succinate and 3-hydroxy-anthranilic acid potentiated the effect of insulin at 96 hours. This potentiation was the greater the lower was the incidence of a particular abnormality after insulin alone. Given by themselves both compounds were harmless.

Malformations produced by insulin-treatment at 96 or 120 hours, and not heretofore reported, are syndactylism and cleft palate, the latter associated with coloboma of the face.

Tests with inhibitors of anaerobic glycolysis (sodium malonate, sodium fluoride, glyceraldehyde, phlorizin, monoiodoacetic acid) showed that phlorizin and especially monoiodoacetic acid, injected at 24 hours of incubation, produced a considerable incidence of rumplessness. None of these inhibitors were teratogenic at 96 hours.

Our results demonstrate that certain substances provide protection against those teratogenic compounds which in 24-hour embryos tend to induce rumplessness, that other substances forestall, completely or in part, the production by chemical interference at the 96- or 120-hour stage of micromelia, syndactylism and beak defects, that some supplements furnish differential protection (e.g. against beak defects, but not against micromelia), and that other supplements produce differential potentiation of the insulin effects (e.g. raising the incidence of syndactylism much more than that of beak abnormalities). It follows that the metabolic situations upon which act the teratogenic agents as well as the substances used as supplements, vary not only between different developmental stages, but also between different organ primordia of one and the same stage.

It seems likely that the teratogenic effects of insulin are produced by rendering coenzyme unavailable to the embryo. During the early and predominantly anaerobic stages of development pyruvic acid, by aiding reoxidation of the pyridine nucleotides, presumably serves to prevent insulin damage. During later stages, and on account of the changed respiratory conditions of the embryo, nicotinamide supplementation is required to offset the blocking effect of insulin on the co-

enzymes. Chemicals which resemble insulin in their teratogenic effects probably do so by interference with the same or closely related metabolic steps. Supplements to insulin treatment seem to be beneficial or noxious according to whether they aid or hinder in relieving the insulin determined scarcity of coenzyme.

The long duration of susceptibility to insulin treatment of the long bones and other skeletal parts can be explained on the basis of the special significance of glycogenolysis in bone calcification.

The principles of "unspecific" retardation of embryo growth, as enunciated by Stockard and others, cannot explain the origin of the teratogenic processes here described.

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LEMKEY-JOHNSTON, N., and W.A. REYNOLDS, Illinois State Pediatric Institute and Department of Anatomy, University of Illinois, Chicago, Illinois. Incidence and extent of brain lesions in mice following ingestion of monosodium glutamate (MSG).

Administration of MSG to neonatal mice has been reported to result in discrete lesions of the arcuate nucleus and in obesity, female sterility and skeletal stunting (J. Olney, Science, 1969). We have studied the incidence and extent of MSG-induced lesions in neonatal mice in an attempt to understand the underlying factors. Experiments were performed to determine the progress of the lesion in hypothalamus, thalamus and midbrain after a 4 mg/gm oral dose. A mild edema and necrosis of arcuate neurons could be found at 20 min. on the light microscope level. By 30 min., a substantial lesion involved both preoptic and arcuate neurons. At 60 min., damage extended to habenular nuclei and epithalamic regions. By 2 hr., in some animals, extensive lesions of tectum occurred. At 24 hr., arcuate nucleus showed neuronal depletion and fields filled with phagocytic cells. Electron microscopic observations confirmed that at 30 min. the primary lesioned elements were neurons, upon which immature synapses could be found.

To ascertain the maximal extent of brain areas encompassed by MSG-induced lesions, mice were given 4 mg/gm of MSG and sacrificed after 3 hr. Examination of serial sagittal sections revealed that in addition to the usual arcuate-preoptic lesions, external layers of cerebral cortex, portions of olfactory bulb, claustrum, caudate-putamen, dorsal lateral regions of thalamus, hippocampus, habenular and vestibular nuclei, and external layers of both superior and inferior colliculi were damaged. Most striking was the close proximity of the observed lesions to cerebrospinal fluid (CSF). Further, the lesion was encountered as a diffuse band in various regions rather than being delimited to well-circumscribed nuclei.

In conclusion, we would say that lesions resulting from ingestion of lower doses (1mg/gm) of MSG result in neuronal damage to only selected regions of brain which appear most vulnerable, viz., the arcuate-preoptic regions. At 2-4 mg/gm doses, the lesion may spread to many other regions of the brain, all of which are adjacent to circulating CSF.

Monosodium Glutamate and the Restaurant Industry

PAUL LOGAN, Colonel, QMC, ret'd
The National Restaurant Association

The widespread acceptance of monosodium glutamate by restaurants is a tribute to the product, its producers, and the restaurant industry. The product itself has lived up to the remarkable claims made about it. The producers have done an outstanding job in making its unique advantages known, and it is particularly pleasing to me to see that the advantages gained through its use are recognized by such a large number of restaurant operators. Today, there is no longer any question of how many can be persuaded to use monosodium glutamate. It's only a question of how extensively it will be used in each operation.

I am convinced that the main reason for this phenomenal success story has been due to a greater appreciation of the importance of flavor to commercial restaurants. In my opinion, nothing else has as much influence on success or failure in the restaurant business today. The producers of monosodium glutamate have wisely recognized this in their promotional efforts and used it to advantage.

You will always find, I'm sure, that in any business the easiest people to reach are the leaders, simply because they are constantly on the lookout for competitive advantages. They are the ones who realize the real value of the intangible and unseen advantages in building a healthy business. For this reason, our most successful restaurant operators are the ones who use monosodium glutamate regularly and with the best effect.

The elusive element of flavor is actually the best merchandising factor that anyone in the restaurant business can use to build

volume and hold patrons. Monosodium glutamate effectively builds and holds natural flavor which makes it important to every operator. Due to the aggressive competition among restaurants, the public has become quite selective and particular. They want good food and pleasant service at reasonable prices in clean and well-appointed surroundings. I believe that monosodium glutamate has made it easier for food service people to produce tastier food at minimum expense.

Experience has shown that restaurant patrons frequently ask for the recipe of a dish which is pleasing to them. It is always readily given and, in this manner, new seasonings and new cooking procedures are introduced to homemakers.

It is estimated that 70,000,000 meals are served by public feeding establishments every day. Of every dollar spent for food, 25 cents is spent outside the home. Food producers and processors have come to a realization of the tremendous potential market offered by the public feeding industry. In the consumer market there are over 40,000,000 individuals to reach by sales effort, while in the institutional field there are only a little over 400,000.

The institutional market is divided into four basic types of quantity-feeding operations. These are commercial, semicommercial, charitable, and government. Over 70% of all food purchases fall into the commercial group of operations, and 52% of total food purchases are made by restaurants alone. Another interesting fact is that of the total number of restaurants, only 30,000, or less than 20%, accounted last year for 57% of the business done by the entire group. Not only does this separate the men from the boys, but it certainly puts the spotlight on the importance of reaching the right people.

These facts have caused many food manufacturers to develop special departments, production, packaging, and selling techniques to take advantage of this great potential and serve the needs of the institutional food industry, but in my opinion, there is still a long way for manufacturers and suppliers to go before they are really in tune with this industry.

A restaurant is a quantity-food operation whose only reason for being in business is that of serving food. In type of service, it can vary from a stand-up, grab-it-on-the-run counter to the red-plush, timeless atmosphere of the specialty place. Without exception, however, a restaurant is an actual food processing plant with an extremely short cycle for its products. From the time of purchase, especially in the case of fresh foods, to processing and then to consumption is only a matter of a few hours. Even in this short time, several important factors must be considered, the most important being flavor-loss. In my opinion, flavor is really

all a restaurant has to sell. The rest is given away! By this I mean that it is flavor appeal which makes one restaurant more popular than another in most instances.

The restaurant industry is a complex industry. Good restaurant management must include very thorough knowledge of food costs, cookery, and menu preparation; of public relations in receiving, serving, and satisfying patrons; of personnel management in training and making the best use of employees; of merchandising in promoting greater sales by various advertising media. His accounting system must be such as to assure accurate daily inventory control, accurate portion control, elimination of loss through waste, and proper balance of costs in the twelve to fifteen items of overhead which are always present. Above all, the operator must know how to produce the type of food most desired by his class and type of patrons.

The commercial food service industry is growing by leaps and bounds, but the competition is keen. Furthermore, I've noticed that this competitive atmosphere is developing among suppliers as well. This healthy condition has brought about some wonderful improvements in operation and service on the part of the restaurant in order to win public favor and the supplier to win favor with restaurants. Monosodium glutamate has established a definite role in the food production plans of both groups.

Very few people in the entire food service field were aware of the existence of monosodium glutamate eight years ago. Today, the entire restaurant industry is aware of its existence and of advantages gained through its use. It is generally regarded as a necessary supplement which can be used to bring out and hold the full flavor of a particular dish.

In addition to the knowledge gathered through years of research, a great deal has been learned concerning the application of monosodium glutamate in the restaurant industry by the operators themselves. In the fall of 1948, the Food Testing Committee of the National Restaurant Association made blindfold tests of six frequently used dishes with and without glutamate. On this occasion the preference for monosodium glutamate in some of the tested foods ran as high as 90%, while in others its use was disapproved. Another experiment which interested me greatly was where the patrons of a certain restaurant were asked to register their preference for one of two portions of soup and vegetables without knowing that one the portions had been prepared with monosodium glutamate. This time, I believe, after making this taste test on more than 180 restaurant patrons, 75%, or three out of four, preferred the portions with monosodium glutamate "because they were tastier."

A recent pilot survey has indicated that as many as three out of four of all good restaurant operators are using monosodium glutamate. Many problems lie ahead in promoting greater and more efficient use of monosodium glutamate. Operators, production managers, and cooks need to be trained in its proper use.

The International Minerals and Chemical Corporation has done a great deal to build a steady demand for monosodium glutamate through its promotions and services. The excellent series of basic quantity recipes, the Costolator, and the other pieces of information so attractively produced are appreciated services. In its advertising, I believe it has furthered the cause of monosodium glutamate a great deal by associating the product with basic restaurant problems, fairly and logically.

In summary, it can be stated that the introduction of pure monosodium glutamate to the public feeding industry afforded the means of enhancing food flavor in a manner not previously known. The increasing use throughout the entire restaurant industry of monosodium glutamate attests the importance which the restaurant operator places upon food flavor. Most of the restaurant operators are already fully cognizant of the advantages to be gained through the use of monosodium glutamate, but there still remains an enormous untapped market in the public feeding field. Many food processors, in the last three years, have come to a realization of the great potential for sales volume offered by the institutional market, and have organized a special department to handle the different sales and advertising techniques required. Undoubtedly, most of the important food producers are fully aware of the advantages to be found through the use of monosodium glutamate. It is, therefore, possible to forecast that within the food processing industry which supplies the operators of public feeding establishments and also within the latter group there will be a steady and permanent increase in the total volume of monosodium glutamate used.

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Monosodium Glutamate: Specific Brain Lesion Questioned

Olney and Sharpe (*1*) administered, by subcutaneous injection, a single dose of monosodium glutamate (15 mmole per kilogram of body weight) to one newborn premature rhesus monkey and concluded that the lesion in the periventricular arcuate region of the hypothalamus was specifically induced by glutamate.

They stated "We have demonstrated susceptibility of a primate species to the mechanism of the glutamate effect" and "Presumably an elevated blood concentration of glutamic acid is an important requisite to the lesion formation." However, they did not supply data on concentrations of glutamic acid in the blood, nor did they supply control data for their experiment. A minimum requisite would have been the use of inorganic sodium salts administered in a way similar to that used for monosodium glutamate, the use of sodium salts of several other amino acids, and the administration of glutamic acid as the free acid. Perhaps most critical is the fact that they used only one animal.

An explanation as plausible as that presented is that the findings reflect a nonspecific solute effect. Finberg *et al.* (*2*) have shown that an intravenous or intraperitoneal dose of many univalent completely ionized salts (15 meq per kilogram of body weight) given to an infant cat reduces the cerebral spinal fluid pressure to zero in 180 minutes (the time interval used by Olney and Sharpe). Under these circumstances, there is always dilatation of capillaries and small vessels and occasionally, at this dosage, tearing of vessels in the Virchow-Robin space, with consequent hemorrhage. The specific lesion presumably varies with the conformation of the intracranial contents and skull of the animal model used.

The implication of a risk of brain damage as a result of human infants' consuming infant food products is unsupportable. Infant food products on the market, until recently, contained between 150 and 180 mg of monosodium glutamate per jar. A few contained as much as 600 mg per jar. A 5-kg infant would have had to consume, at one sitting, 20 jars of an infant food product containing 600 mg per jar to obtain the amount of monosodium glutamate used by Olney and Sharpe. The controversy surrounding monosodium glutamate in infant food products has aroused fear on the part of mothers that their infants would become mentally retarded as a result of their having been fed commonly available infant food products. There obviously was no such risk and no basis in fact that this could have occurred.

The issue of whether or not monosodium glutamate should be in infant food products is a different one. The amino acid serves no useful nutritional purpose either as a flavoring agent or as a supplement to an infant diet already high in glutamate as a result of milk and wheat content. Furthermore, the addition of glutamate probably increases the price of the product, and under these circumstances inclusion cannot be condoned. However, this issue is completely different from the proposition that a specific neurological lesion occurs in infants receiving these foods, and publishing information based on an experiment in a single animal with no controls is only confounding.

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Present Use of Glutamate by the Marine Corps

W. R. Lucius, Lt. Col., U. S. Marine Corps

LOOKING at this distinguished group, containing many of the learned people in the glutamate industry, I realize that I shall have to deal in generalities rather than technicalities. The last 23 years of my life have been spent as a member of the Marine Corps. First of all, in order to lead up to the use of monosodium glutamate, I think it fitting that I discuss the Marine Corps and its present setup as regards food service.

As many of you know, the Marine Corps is a separate Service, as is the Army, Navy, and Air Force. We have our own Supply Department similar to the Quartermaster Corps of the Army and the Bureau of Supplies and Accounts in the Navy. Our Supply Department is headed by a Quartermaster General with the rank of Major General. We have a separate Subsistence Supply Section, which, like the Army, encompasses food service, audit, commissary stores, and the procurement and distribution of subsistence stores.

Working out of our Subsistence Section at Headquarters, Marine Corps, are three food service teams: the East Coast Food Service Demonstration Team covers all of the Marine activities on the East Coast of the United States, Cuba, and Newfoundland; the West Coast Food Service Demonstration Team covers all Marine installations west of the Mississippi, Hawaii, and Alaska; a Steward's Instruction and Demonstration Team covers all of the Marine Officer (closed) messes in the United States and Hawaii. I will come back to these teams and the part they play in our instruction procedures a little later in this talk.

History of monosodium glutamate in the Marine Corps. An article on monosodium glutamate by Colonel Paul Logan, which appeared in the News Bulletin, National Research Association, June, 1949, stated: "Within the past few months, a comparatively new substance in the American food market has found its way into our restaurant kitchens. This new substance is monosodium glutamate, and it is bringing a new concept into the art of food seasoning."

This article created considerable interest among personnel responsible for administering the Marine Corps Food Service Pro-

gram. Subsequent to the publication of the aforementioned article, representatives of the food industry contacted the Food Service Section, Headquarters, Marine Corps, and requested permission to conduct a demonstration of this product in a Marine Corps mess. Headquarters, Marine Corps, decided that the proper place to hold such a demonstration was the Marine Corps Food Service Schools located at Camp Lejeune, North Carolina. Accordingly, a demonstration was conducted at the school by representatives of International Minerals and Chemical Corporation on 6 January 1950. As the result of this demonstration, the report that follows was submitted to Headquarters, Marine Corps, by the Commanding Officer of the Marine Corps Food Service Schools.

Report of demonstration conducted using monosodium glutamate in the preparation of foods. As a result of a demonstration conducted by Mr. Harry E. Cook and Mr. Elliot, representatives of International Minerals and Chemical Corporation, a report was prepared which I shall now draw upon.

On 6 January 1950, Mr. Cook and Mr. Elliot, representatives of International Minerals and Chemical Corporation, demonstrated the use of monosodium glutamate in the preparation of food. Present at the above-mentioned demonstration were the staff and students of the Food Service School. Mr. Cook first gave a 30-minute lecture on the chemical analysis and proper usages of monosodium glutamate, after which a group taste test was conducted. In conducting the taste test the following foods were prepared using standard recipes from *The Navy Cook Book:* Cream of Mushroom Soup, Wax Beans, Fried Cabbage, Buttered Carrots, Creamed Cauliflower, Mashed Potatoes, and Pan Gravy. At that time, two samples of each of the above foods were taken. To one of the samples in each case was added a small amount of monosodium glutamate, while to the remaining sample nothing was added. The individuals participating in the taste test, not being aware of which sample contained the added ingredient, were requested to taste both samples of each food and indicate which sample they preferred, with the following results:

Item	With MSG	W/O MSG	No Preference
Mushroom Soup	17	8	
Wax Beans	16	5	4
Fried Cabbage	15	7	2
Buttered Carrots	14	9	3
Creamed Cauliflower	16	10	
Mashed Potatoes	8	12	5
Pan Gravy	20	5	

Further Marine Corps tests. From this simple test, the quartermaster General of the Marine Corps, at that time Major General W. P. T. Hill, decided that this surprisingly effective product merited further attention from the Marine Corps. Additional tests under General Hill's direction were conducted at the Food Service schools. The results of these tests definitely proved that monosodium glutamate as a product had a definite place in the Food Service system of the Marine Corps. With this background information, members of the Food Service Demonstration Teams, of which I spoke, were instructed in the use of monosodium glutamate. They, in turn, proceeded to disseminate this information throughout the Corps. Approval was spontaneous and universal at each and every mess visited.

In order to understand the workings of these teams, I might go into a little more detail in clarification of the aforementioned statement. Food Service Demonstration Teams were first formed in the Marine Corps in 1948 in order to correct poor techniques and improve troop acceptance of food prepared at Marine Corps messes. Loss of personnel trained in Food Service procedures was the source of the difficulty. These personnel left the Marine Corps after the war, due to the comparative slowup of training activities in this field and doubtless for personal reasons. These teams, comprised of an officer qualified in all phases of Food Service management, three master sergeants, a butcher, a baker, and a mess management chief, each with many years' experience in Food Service behind them, are highly successful in improving the messes. They actually go into every post or station in the Marine Corps and move into the mess in question. They spend approximately two weeks working with the personnel of that mess or actually showing them the correct techniques involved—as in the instance of monosodium glutamate. These men spend the whole workday in this mess. Naturally, this was not an easy task. The teams themselves had to be taught. However, dividends were immediately paid in the noticeable improvement of messes Marine Corps-wide.

As stated previously, these teams, late in 1951, commenced demonstration of the use of monosodium glutamate throughout the Corps, actually bringing samples of the product with them. As I said before, much favorable comment was received regarding the improved quality of the food immediately after the team arrived. This, we felt, could justly be traced to the use of monosodium glutamate, inasmuch as these teams had been in operation for some few years. Comments generally had been most favorable from the beginning, but never reached the laudatory stage as was evidenced after the innovation of monosodium glutamate.

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It might be stated that while these comments appear to be completely in the Marine Corps' favor, the use of this product and the acceptance of its use by the old line cooks made it an uphill effort in order to assure its use. When the merits of this product definitely gained recognition, the Marine Corps' *Recipe Manual*, which contains over 590 recipes, was promulgated, including monosodium glutamate in various recipes. In the Marine Corps' *Master Menu* we publish, from time to time, hints regarding the use of seasoning. We have also stressed the use of monosodium glutamate in this publication.

I might go on and say a few words about the use of the *Master Menu* in the Marine Corps. The *Master Menu* is published monthly at Headquarters, Marine Corps' level. It is prepared to provide an adequate diet each and every day. It is varied, as can be seen by one day's menu from it:

TUESDAY, 7 JUNE 1955

Breakfast	*Dinner*	*Supper*
Chilled Cantaloupe	Pot Roast Beef	Vegetable Soup
Assorted Dry Cereal	Bread Dressing	Crackers
Chilled Fresh Milk	Stock Gravy	Spareribs & Sauerkraut
Egg Omelet W/ Spanish Sauce	Mashed Potatoes	Parsley Buttered Potatoes
Hashed Browned Potatoes	Corn on the Cob	Buttered Green Lima Beans
Coffee Cake	Sliced Tomatoes	Golden Glow Salad
Hot Toast	Radishes & Green Onions	Ice Cream
Butter/Jam/Coffee	Strawberry Shortcake	Bread/Butter/Coffee
	Assorted Bread (White/Rye)	
	Butter/Beverage	

The *Master Menu* has been invaluable in citing the use of specific items which are in long supply, Marine Corps-wide. We received most favorable comments from the Hoover Commission regarding these procedures. Should any of you desire copies of the *Master Menu*, it will be available for the asking from Headquarters, U. S. Marine Corps, Code CSI.

We have made considerable progress, Marine Corps-wide in the last few years on many aspects of subsistence concern. Commissary store sales have increased from three million dollars per year to eleven million dollars per year. We have stratified inventories comparable to Marine Corps' strength in order that existing stocks may be turned over as rapidly as possible. We have set up a Steward's Instruction and Demonstration Team in order that the officers' messes, too, may have the benefits previously afforded only enlisted messes. We have set up our accounting systems in accordance with good business practices. All of our Marine Corps' commissary stores except one are of the self-service type and are comparable to like civilian operations.

Finally, I believe, we have accomplished something which I consider most important of all. We have made contact with industry. Every day representatives of industry visit me in my office. We are members of the National Security Industrial Association participating with industry in the betterment of equipment and recipes. Last, but certainly not least, I am with you today representing the Marine Corps. This is the first occasion that a Marine has ever appeared before this group to relate his experiences in the use of this industrial product that has contributed so much to subsistence improvement.

Use of Glutamate in Pre-cooked Frozen Foods

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Results of studies conducted at the Institute and other research organizations on fresh frozen foods have indicated that the addition of MSG to precooked frozen meals improves the quality of precooked frozen products by:

a. Preventing the development of the "warmed-over" off flavor on storage.

b. Retarding or preventing the development of off flavors on storage.

c. Retaining the color and freshness of the product so treated.

Limited investigations have been made at the Institute to determine whether the addition of MSG will improve the acceptability and stability of precooked frozen meals on long range storage at 0° F. Several studies are still in progress. The results obtained to date indicate that the addition of MSG improves the acceptability and stability of precooked frozen foods, but then data are not conclusive since the studies have been limited to only a few precooked frozen products with only one level of MSG. The experiment did not include a study of the monotony factor, which might conceivably be a problem if all the meals contained MSG.

The completed part of the study, which indicated that the addition of MSG even at a low level improved the acceptability and stability of the product, was conducted at the Institute on the precooked frozen veal breakfast patty. The breakfast veal pat

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THE CONTENT OF FREE L-GLUTAMIC ACID IN VARIOUS FOODS

by

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1. Introduction

It has been a matter of public knowledge that free glutamic acid plays a major role in the taste of foodstuffs since the studies of Dr. Kikunae Ikeda on the taste components in kelp which he conducted in 1908.[1] The question of the correlation between the free glutamic acid content and the taste of natural foods is an extremely interesting one.

There is considerable literature on animal and plant tissues and glutamic acid in foods, the principal ones being those listed in the bibliography.[2-2]

It is believed that there are differences in the free glutamic acid content of animal and plants and natural foods as a function of the physiological state of the living body, the condition under which the samples are taken, the method of preparing the samples and the method of determining the quantity of glutamic acid.

The writers performed the following experiments for the purpose of determining the correlation between the taste of foodstuffs and the free glutamic acid content in foods in the actual state in which they are offered for consumption. Specifically, natural foods such as seafood meat, fresh vegetables and fruits, potatoes and mushrooms which had not been processed and such foodstuffs as soup stock kelp, and dried bonito, which are used for the preparation of soup stock were selected. Microbiological quantitative analysis methods were used for the measurement of the free glutamic acid content.

As a result of these measurements, it was determined that there is a certain correlation between the taste of foodstuffs and the free L-glutamic acid content. A report is rendered on this below.

(Experiments)

Test Samples.

It is believed to be only natural that there will be differences in the free glutamic acid content of natural foods as a function of the type of food, maturity, size, and differences in freshness. Particularly in the case of fish, there are differences in the extent of autolysis as a function of processing subsequent to catching and the passage of time. Accordingly, it is believed that there will also be variations in the free glutamic acid content.

For the purposes of the present experiments, the samples were collected under conditions as close as possible to the actual preparation of foods, using commercial foods, as a means of making a determination of the general trends in actual foods.

The foodstuffs used as samples in the experiments were a total of 53 types as shown in Tables 1, 2 and 3 including 20 types of fish, five types of meat, 12 types of vegetables, eight types of mushrooms, six types of soup stock and two other types of food.

The free glutamic acid content and the water content of each sample were measured and additional measurements were made of the pH of the fresh samples.

Preparation of the Samples.

For the measurement of the pH and water content of the seafood and soup stock, the samples were homogenized in a mortar mixer in a homogenizer.

For the purposes of the determination of free glutamic acid, the samples were weighed out in fixed quantities, hot water added in approximately twice the quantity of the sample taken to moisten it, then the supernatant taken subsequent to centrifuging. The residue was similarly extracted. These operations were repeated 8--10 times to extract the free glutamic acid extracted. The educt from each of these operations was collected, the pH adjusted to 6.0 and constant volumes of the educt used as samples. These samples were also sealed in ampules, sterilized under pressure at 110°C for 10 minutes, then held at normal temperature and used as required for the determination of glutamic acid.

As is commonly known, the highly active glutamic acid decarbonation enzyme is present in the tissue of higher order plants. Therefore, the fresh vegetable and potato samples were cut, weighed and moistened for a constant period of time in hot water in the vicinity of 100°C to prevent the breakdown of the glutamic acid. Subsequent to this treatment, the above described sampling technique was used.

For the purposes of the pH and water content measurements, the samples were ground up and homogenized in a mixer.

The mushroom samples were prepared in the same way as the vegetable samples.

In the case of all of the samples, the portions tested were from the so-called edible parts in order to most nearly approximate actual preparation conditions. The details are given in the Remarks column in Tables 1, 2 and 3.

Microbiological Quantitative Analysis.

The determination of glutamic acid was by the microbiological quantitative method as given in Akahori and Mishima, ed. Protein Chemistry.[13]

The bacillus used was Lactobacillus arabinosus.[17-5] Every one week to 10 days the bacilli were cultivated in a holding medium (an agar medium) containing 10% bread yeast, 1% dextrose, 0.5% polypeptone and 0.5% anhydrous sodium acetate, then stored in an icebox for use.

All of the components for the basic medium (the amino acids, the vitamins, the organic salts, and the inorganic salts, etc.) were all stored in an icebox in the form of solutions of a constant concentration.

For the purposes of the determination, each one of the above components was taken in a fixed quantity and used to prepare an analytical culture (pH 6.0) containing no glutamic acid.

Into a test tube was placed 1 cc of this medium and either 1 cc of standard glutamic acid or 1 cc of the sample solution. The concentration for the standard curve was in 14 stages between the limits of 1 and 20 r/cc while 3--5 stages were used for the samples.

After pouring, the samples were sterilized under pressure by heating at 110°C for 10 minutes. Inoculation was with bacteria that had been previously cultured (the agar taken from the holding culture and inorganic salt added). Inoculation was into 2 cc and culturing was for 16--20 hours at 37°C.

Prior to the above operations, the culture was separated by centrifuging, the supernatant poured off, the bacteria washed with a sterilized 0.9% aqueous solution of table salt. After repeating this operation twice, the

Table 1. The Free Glutamic Acid Content of Foods.

Sample	Water content	Free glutamic acid content: Within the sample		Free glutamic acid content: Converted to a dry state		pH	Notes
		Glutamic acid	Amino-N	Glutamic acid	Amino-N		
Seafood	%	mg%	mg%	mg%	mg%		
Fish							
Sardine	63.8	280	27	880	85	6.15	Head, tail and bones removed
Carp	75.0	12	1.1	48	4.4	6.33	Sliced raw
Prussian carp (Cyprinus auratus)	76.5	13	1.2	55	5.1	6.61	(Including the tail)
Eel	62.2	10	0.95	27	2.6	6.53	Head, tail, intestines and bones removed
Mackerel	53.8	36	3.4	78	7.4	6.12	Head, tail and bones removed
Horse mackerel	74.6	19	1.8	75	7.1	6.37	Head, intestines, bones, tail and scales removed
Tuna (Kihada)	77.3	8.9	0.85	39	3.7	5.95	A quantity equivalent to a whole fish taken from the anterior and middle section of the posterior third.
Tuna (Bincho)	73.6	5.3	0.50	20	1.9	6.30	"
Sea bream	78.1	9.3	0.89	42	4.0	6.47	Sliced raw (including the skin)

Table 1 (cont'd)

Sample	Water content	Free glutamic acid content: Within the sample		Free glutamic acid content: Converted to a dry state		pH	Notes
		Glutamic acid	Amino-N	Glutamic acid	Amino-N		
Blowfish	80.3	6.8	0.65	35	3.3	5.87	Flesh
Turbot	78.1	38	3.6	174	16.4	6.45	Head, tail, bones, intestines and skin removed
Crustaceans							
Prawn	75.1	43	4.1	173	16.5	7.05	Vein removed
	76.1	51	4.9	210	21	7.17	
Shellfish							
Clams	88.7	41	3.9	360	35	6.51	Shucked
Ark-shell	85.6	151	14.4	1050	100	6.63	"
Corbicula	89.3	23	2.2	214	20.4	6.83	"
Short neck clam	86.1	136	12.9	980	93	6.51	"
Abalone	80.3	72	6.9	370	35	6.00	"
Oyster	81.0	137	13.0	720	69	6.17	"
Cephalopods							
Sagittated calamary	77.5	146	13.9	650	62	6.03	Entrails removed

*Allowed to stand in a refrigerator for one night after killing.

Table 2. The Free Glutamic Acid Content of Food

Sample	Water content	Free glutamic acid content: Within the sample		Free glutamic acid content: Converted to a dry state		pH	Remarks
		Glutamic acid	Amino-N	Glutamic acid	Amino-N		
Meat	%	mg%	mg%	mg%	mg%		
Beef tenderloin	75.9	33	3.1	140	13.1	5.55	1 head
Beef round	75.5	11	1.0	45	4.3	5.47	
Pork tenderloin	72.0	23	2.2	82	7.8	5.85	1 head
Chicken meat	75.6	44	4.2	180	17.1	5.97	1 fowl
Chicken bone	48.6	40	3.8	78	7.4	7.82	1 fowl
Vegetables							
Fruits and vegetables							
Eggplant	93.7	16	1.5	250	24	5.60	Stem removed
Tomato (unripe)	95.0	58	5.5	1160	110	3.89	"
(Ripe)	93.9	140	13.3	2280	217	4.10	"
Cucumber	95.4	23	2.2	500	48	5.85	Both ends removed
White muskmelon	96.1	16	1.5	410	39	5.78	Both ends and the seeds removed
Squash	92.9	47	4.5	660	63	5.83	Stem and seeds removed
Green soybean	71.1	19	1.8	66	6.3	6.87	Husk and membrane removed

Table 2 (cont'd)

Sample	Water content	Free glutamic acid content: Within the sample		Free glutamic acid content: Converted to a dry state		pH	Remarks
		Glutamic acid	Amino-N	Glutamic acid	Amino-N		
Leafy vegetables							
Cabbage	93.3	37	3.5	550	52	6.14	Stem removed
Onion	92.1	18	1.7	228	22	5.13	Both ends and membranes removed
Spinach	91.5	39	3.7	460	44	6.11	Root removed
Edible roots							
Bermuda onion	91.7	21	2.0	250	24	5.17	Skin and root removed
Carrots	89.1	33	3.1	300	29	5.88	
Potatoes							
Sweet potato (Kanetoki)	66.4	60	5.7	179	17.0	6.22	
(Taihaku)	66.2	51	4.9	151	14.5	5.80	

sample was diluted and suspended in five times the quantity (10 cc) of a 0.9% reduced bacteria content, table salt solution. After sterile inoculation of each of the test tubes with one drop of the inoculum, using a pipet, the sample was cultured for 72 hours at 37°C.

The bacteria were killed by heating the test tubes in boiling water for 5 minutes, after culturing. The quantity of acid formed was measured by titrating with 1/20 N caustic soda. The analysis of the samples was then completed by constructing a standard curve.

(Results and Discussion)

Tables 1, 2 and 3 show the measured water content and pH as well as the free glutamic acid content.

To examine the pH of these foods, that of the tomato is relatively low while that of the shrimp and the chicken bone is fairly high. The other foods show a pH generally within constant limits.

Concerning the Glutamic Acid Content.

Except for the sardines, the free glutamic acid content in seafood is relatively low. It is also relatively low in those fishes such as eel, sea bream and blowfish that are held to have a good taste. Among the fish that were analyzed on this occasion, only the sardines had an extremely high free glutamic acid content. Since it cannot be said that this is a general tendency in sardines on the basis of this analytical sample alone, it is hoped that the analysis can be repeated again on some other occasion.

The free glutamic acid content of the shellfish is relatively high and a particular large quantity was detected in the ark shell, the short neck clam and the oyster. It was originally held that the cause of the

taste in the shellfish is succinic acid.[14] On the basis of the present analysis, however, it is theorized that the free glutamic acid also has a great effect on taste.

Further, a high quantity of free glutamic acid was also detected in the sagittated calamary, which was also classified as a soft-bodied type.

Among the meats, a quite high free glutamic acid was detected in the chicken meat, however, the content was low in the beef round specimens used.

Among the vegetables and potatoes, the glutamic acid content of the tomato was high. There was about twice the free glutamic acid content in ripe tomato as there was in the unripened tomato. There was a higher free glutamic acid content in squash and sweet potato than there was in the other vegetables.

An analysis was performed on eight varieties of mushrooms and all of these were found to have a relatively high free glutamic acid content. There was over 100 mg% (0.1%) in the champignon, and the Enoki mushroom, etc., a higher content than the other foodstuffs.

Since the Lactobacilli, which are used for microbiological determination, can utilize certain of the peptides,[15-17] it is generally thought that lactic acid bacteria available peptides, which have a relatively high activity for glutamic acid, are present in the mushrooms. It is believed necessary that additional tests be made of this point.

Among the soup stock types, there was a particularly high free glutamic acid content in soup stock kelp.

The free glutamic acid content of beef round, chicken bones (sic),

Table 3. The Free Glutamic Acid Content of Foods

Sample	Water content	Free glutamic acid content: Within the sample		Free glutamic acid content: Converted to a dry state		pH	Remarks
		Glutamic acid	Amino-N	Glutamic acid	Amino-N		
Mushrooms	%	mg%	mg%	mg%	mg%		
Fresh Cortinellus shiitake	91.9	67	6.4	830	79	4.95	Stem removed
Armillaria edodes (shimshu) product	89.1	66	6.3	550	52	5.65	Base of the stalk removed
(Kansai product)	89.1	85	8.1	780	74	5.67	"
Nameko (Nagano product)	94.9	49	4.7	960	92	5.47	Cap removed
(Yamagata product)	95.6	77	7.3	1750	166	5.32	"
Champignon	86.3	114	10.9	830	80	6.08	Base of the stalk removed
Enoki mushroom	90.6	133	12.7	1410	135	6.55	"
Mushroom	90.7	180	17.1	1940	184	5.35	
Soup stock							
Bonito cake	8.26	26	2.5	38	3.6		
Dried small sardines	15.6	50	4.8	65	6.2		
Soup stock kelp	9.72	420	4.0	460	44		
Meat extract	38.0	60	5.7	97	9.2		
Dried cuttlefish	24.0	280	26	360	34		
Mushroom (dried)	13.3	240	[illegible]	[illegible]	[illegible]		

dried sardines and bonito cake, and such similar foodstuffs as may be used in soups or soup stock was relatively low. It is believed that this may be attributable to the fact that there are a large number of other components which cause a taste in addition to the free glutamic acid.

To draw a conclusion from the above analytical values, it may be observed that as a general rule there tends to be a high free glutamic acid content in the foodstuffs that have relatively strong taste.

(Summary)

1) Measurements were made by microbiological quantitative analysis of the free L-glutamic acid content of 53 types of foodstuffs comprising marine products, meats, fresh vegetables and fruits, mushrooms and soup stock foods.

2) A particularly high quantity of free L-glutamic acid was detected in tomato, ark shell, short neck clam, oysters, squid, mushrooms, sagittated calamary and soup stock kelp.

In conclusion, thanks are expressed to Aji-no-moto Company, Ltd. for granting permission to publish this report and to Managing Director Shigeo Fuzaka, the Chief of the Foodstuffs Research Laboratory, as well as to Dr. Fumikazu Toi.

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食品中の遊離Lグルタミン酸含有量について

The Content of Free L-Glutamic Acid in Various Foods.

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緒　言

遊離グルタミン酸が食品の旨味の一因子として重要な役割を演じている事は1908年池田菊苗博士のコンブの旨味成分の[1]遊離以来[illegible]の事実である。天然食品の旨味性と遊離グルタミン酸含有量との関係は極めて興味ある問題である。

[illegible]の[illegible]並びに食品中の遊離グルタミン酸に関する文献は多くあるが、その主なものをあげると文献[2]の如きものがある。

[illegible]または天然食品中の遊離グルタミン酸の含有量はその生物体の生育的条件、試料採取時の条件、試料調製法及びグルタミン酸の定量方法等によつて差異を生ずるものと考えられる。

著者等は[illegible]食用に供される状態に於て、その食品の旨味性と遊離グルタミン酸含有量とが如何なる関係にあるかを知る為に次の実験を行つた。即ち試料として魚介類、肉類、生鮮蔬菜類、イモ類及び[illegible]全く加工を施されていない天然食品並びにダシコンブ、カツオブシ等一般に"だし"として用いられる食品を選び、その遊離グルタミン酸含有量を微生物定量法に依り測定した。

この測定結果より食品の旨味性と遊離Lグルタミン酸含有量との関係性についてある程度の知見を得たのでその結果を報告する。

〔実験〕

試　料

天然食品中の遊離グルタミン酸は食品の種類、成熟度大きさ、[illegible]の差異等により当然その含有量が異る事が考えられる。特に魚類に於ては漁獲後の処理、経過時間等により自己消化（Autolysis）の程度も異り、従つて遊離グルタミン酸含有量も変化すると思われる。

今回の実験に於ては、食品の一般的傾向を知るために市販の食品について実際の調理条件に可及的近い状態で試料採取を行つた。

第1、2、3表に示した如く魚類20種、肉類5種、蔬菜類12種、茸類8種、だし類6種、その他2種、合計53種類の食品を試料とした。

各試料は水分並びに遊離グルタミン酸含有量を測定し、更に生鮮試料についてはpHをも併せ測定した。

試料調製法

魚介類、肉類、だし類の場合pH、水分の測定は乳鉢ミキサーまたはホモゲナイザーにて均一とした試料につき行つた。

遊離グルタミン酸の定量はこの試料の一定量を秤量、採取試料の約2倍量の熱水を加え加温、遠沈後その上澄液を取り、その残渣につき同様抽出を行い、これを8～10回繰返して遊離グルタミン酸の抽出を行つた。この各回の抽出液を合し、pHを6.0に調整して一定容とし試料とした。更にこれをアンプル中に封じ、110°C 10分間加圧殺菌し、常温に保存して必要に応じグルタミン酸の定量に供した。

高等植物の組織中には糖類の如く活性の高いグルタミン酸脱炭酸酵素が含まれているので、生鮮蔬菜類及びイモ類の場合には試料を截切、秤量し100°C附近の熱水中に約一時間加温してグルタミン酸の破壊を避けた。その後は上述の如くサンプリングを行つた。

pH、水分の測定はミキサーで磨砕、均一にした試料につき行つた。

また茸類についても蔬菜類と同様な方法で試料の採取を行つた。

尚何れの試料の場合も実際の調理条件に近付けるため試料採取はなるべく所謂"可食部分"（Edible Part）について行つた。その詳細は第1,2,3表の備考に示した。

微生物定量法

グルタミン酸の定量は赤堀、水島編"蛋白質化学"[10]の微生物定量法にならつて行つた。

使用菌株は Lactobacillus arabinosus 17-5で、1週間乃至10日目毎に保存培地（パン酵母10%、ブドー糖1%、ポリペプトン0.5%、無水酢酸ソーダ0.5%を含む寒天培地）に植継ぎを行い後冷蔵庫中に保存した。

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浸出液中の各成分（アミノ酸類、ビタミン類、有機酸類、糖類等の他）は各々一定濃度溶液として冷凍庫中に保存した。

定量に際しては、上記各成分を一定容採り、グルタミン酸を欠いた定量用緩衝液（pH 6.0）を調製した。

この溶液 1cc を試験管に取りこれに標準グルタミン酸液 1cc または試料溶液 1cc を加えて分析を行った。尚標準溶液としては 1～20 γ/cc の濃度範囲で 14 段階、試料溶液としては 3～5 段階で行った。

分析後 110°C 10 分間加圧滅菌を行った。

接種菌は前培養培地（保存培地より寒天を除き蒸留水を加えたもの）2cc に保存菌を接種し、37°C で 16～20 時間前培養を行った。

この前培養を滅菌遠心管に移し 1,500 g 15 分間遠心分離、その菌体を 0.9% 食塩水で洗浄して後菌体を懸濁して後菌体濃度を測定した。この操作を 2 回繰り返して後 5 倍法（10cc）の 0.9% 食塩水に菌体を懸濁せしめた。この接種菌一白金耳を試験管ピペットで各試験管に無菌的に接種して後 37°C で 72 時間培養を行った。

培養後この試験管を沸騰水中で 5 分間加熱して菌の生育をとめ、$\frac{1}{20}$ N 苛性ソーダで滴定して生育曲線を得た。これより検量線を作製して試料の分析を行った。

【結果及び考察】

第 1,2,3 表は水分及び pH の測定結果並びに遊離グルタミン酸含有量を示した。

第 1 表 食品中の遊離グルタミン酸含有量

試料	水分	遊離グルタミン酸含有量 試料中 グルタミン酸	試料中 アミノ-N	溶液換算 グルタミン酸	溶液換算 アミノ-N	pH	備考
	%	mg%	mg%	mg%	mg%		
魚介類							
魚類							
イワシ	68.3	280	27	860	85	6.15	頭、尾、骨除去
コイ	75.0	12	1.1	48	4.4	6.33	さし身
ソナ	76.5	13	1.2	55	5.1	6.61	〃（皮を含む）
ウナギ	62.2	10	0.95	27	2.6	6.53	頭、尾、内臓、骨除去
サンマ	53.8	36	3.4	78	7.4	6.12	頭、尾、骨除去
アジ	71.6	19	1.8	75	7.1	6.37	頭、内臓、骨、尾、皮除去
マグロ（キハダ）	77.3	8.9	0.85	39	3.7	5.95	一部分を前・中後三部より採取
マグロ（ビンチョウ）	73.6	5.3	0.50	20	1.9	6.30	〃
タイ	78.1	9.3	0.89	42	4.0	6.47	さし身（皮を含む）
フグ	80.3	6.8	0.65	35	3.3	5.87	肉部
カレイ	78.1	38	3.6	174	16.4	6.45	頭、尾、骨、内臓、皮除去
甲殻類							
クルマエビ	75.1	43	4.1	173	16.5	7.05	殻、せわた除去
〃 *	76.1	51	4.9	210	21	7.17	
貝類							
ハマグリ	88.7	41	3.9	360	35	6.51	むき身
アカガイ	85.6	151	14.4	1050	100	6.63	〃
シジミ	89.3	23	2.2	214	20.4	6.83	〃
アサリ	86.1	136	12.9	980	93	6.51	〃
アワビ	80.3	72	6.9	370	35	6.00	〃
カキ	81.0	137	13.0	720	69	6.17	〃
頭足類							
スルメイカ	77.5	146	13.9	650	62	6.03	内臓を含む

* 死後一日冷蔵庫中に放置したもの。

第2表 食品中の遊離グルタミン酸含有量

試料	水分	遊離グルタミン酸含有量 試料中 グルタミン酸	試料中 アミノ-N	常量摂取量 グルタミン酸	常量摂取量 アミノ-N	pH	備考
	%	mg%	mg%	mg%	mg%		
肉類							
ウシヒレ肉	75.9	33	3.1	140	13.1	5.55	一頭
ウシスネ肉	75.5	11	1.0	45	4.3	5.47	
ブタヒレ肉	72.0	23	2.2	82	7.8	5.85	一頭
ニワトリササ身	75.6	41	4.2	160	17.1	5.97	一羽
ニワトリガラ	48.6	40	3.8	78	7.4	7.82	〃
蔬菜類							
果菜類							
ナス	93.7	16	1.5	250	24	5.60	へた除去
トマト (未熟)	95.0	58	5.5	4160	110	3.89	〃
〃 (成熟)	93.9	140	13.3	2280	217	4.10	〃
キウリ	95.4	23	2.2	500	48	5.85	両端除去
シロウリ	96.1	16	1.5	410	39	5.78	両端、種除去
カボチャ	92.9	47	4.5	660	63	5.83	へた、種除去
エダマメ	71.1	19	1.8	66	6.3	6.57	莢、薄皮除去
葉菜類							
キャベツ	93.3	37	3.5	550	52	6.14	へた除去
ネギ	92.1	18	1.7	228	22	5.13	両端、薄皮除去
ホウレンソウ	91.5	39	3.7	460	44	6.11	根除去
根菜類							
タマネギ	91.7	21	2.0	250	24	5.17	皮、根、除去
ニンジン	89.1	33	3.1	300	29	5.88	
イモ類							
サツマイモ (金時)	66.4	60	5.7	179	17.0	6.22	
〃 (大白)	66.2	51	4.9	151	14.5	5.80	

各食品のpHを見ると、トマトがやや低く、エビ及びニワトリガラがやや高い。その他のものは略々一定範囲内のpH値を示した。

遊離グルタミン酸含有量について

魚類ではイワシを除いては遊離グルタミン酸含有量が比較的少く、ウナギ、タイ、フグ等の如く美味なりといわれているものにも比較的少い。ただ今回分析を行つた極の魚類の中イワシのみが非常に高い遊離グルタミン酸含有量を示している。しかし唯一回の分析値のみでイワシの一般傾向を云々する事は出来ないので更に機会を得てこの分析を繰り返したい。

貝類に於ては遊離グルタミン酸の含有量は比較的多く特にアカガイ、アサリ、カキに多く検出された。元来貝類の香味はコハク酸に由来するものといわれているが、この分析値より判断すると遊離グルタミン酸もその香味に多大に関与しているものと考えられる。

また貝類と同じ嗜好的価に属するスルメイカにも遊離グルタミン酸が多く検出された。

肉類中鶏肉には遊離グルタミン酸がやや多く検出されたが、だしに用いられる牛のスネ肉にはその含有量は少かつた。

蔬菜類、イモ類ではトマトに多く含有され、特に成熟したトマトは未熟なものに比し2倍以上の遊離グルタミン酸含有量を示した。またカボチャ、サツマイモには他の蔬菜類に比して多くの遊離グルタミン酸が含有されていた。

茸類は8種類について分析を行つたが、何れも遊離グルタミン酸が比較的多い。シメジ、エノキダケ Mushroom 等には100mg% (0.1%) 以上存在し、他の食品に比してその含有量は多い。しかし微生物定量に用いられ

第3表 食品中の遊離グルタミン酸含有量

試料	水分	遊離グルタミン酸含有量 試料中 グルタミン酸	試料中 アミノ-N	固形物換算 グルタミン酸	固形物換算 アミノ-N	pH	備考
	%	mg%	mg%	mg%	mg%		
野菜							
生シイタケ	91.9	67	6.1	830	79	4.95	柄除去
マツタケ (信濃産)	89.1	66	6.3	550	52	5.65	イシヅキ除去
〃 (関西産)	89.1	85	8.1	780	74	5.67	〃
ナメコ (長野産)	91.9	49	4.7	960	92	5.47	傘部
〃 (山形産)	95.6	77	7.3	1750	166	5.32	〃
シメジ	86.3	114	10.9	830	80	6.08	イシヅキ除去
エノキダケ	90.6	133	12.7	1410	135	6.55	〃
Mushroom	90.7	180	17.1	1940	184	5.35	
だし類							
カツオブシ	8.26	26	2.5	38	3.6		
ニボシ	15.0	50	4.8	65	6.2		
ダシコンブ	9.72	420	4.0	460	44		
肉エキス	38.0	60	5.7	97	9.2		
スルメ	24.0	280	26	360	34		
シイタケ (干)	13.3	240	23	270	26		

る栄養理論による有効のペプタイドを利用出来るので特別に生理的強いグルタミン酸性を有する "Lactic Acid Bacteria Available Peptide" が存在する事も一応考えられる。これらの点について更に検討を加える必要があると考えている。

"だし" 類中ダシコンブには遊離グルタミン酸含有量が多い。

スープまたは "だし" として用いられる牛のモモ肉、ニソトリガラ、ニボシ、カツオブシ等の場合は遊離グルタミン酸が相当的少い。これらは遊離グルタミン酸以外の風味成分を多く含有するものと考えられる。

以上の分析値から推察すると一般に比較的味の強い食品は遊離グルタミン酸含有量が多い傾向にある事が認められる。

(要約)

1) 魚介類、肉類、生鮮蔬菜類、野菜、"だし" 類等53種の食品について遊離Lグルタミン酸含有量を微生物定量法に依り測定した。

2) これらの食品の中で、トマト、アカガイ、アサリカキ、イカ、野菜、スルメ、ダシコンブ等に多くの遊離Lグルタミン酸が検出された。

終りに臨み本研究の発表を許可された、味の素株式会社並びに試料提供の御助言を賜った食品研究室及び技術研究部、丁用文一博士に深謝する。

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葉菜カロチンの料理による変化（第I報）

Studies on the Variation of Carotene Content in Vegetable Leavies during Cooking (Part I)

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Reiko Ayukawa, Satomi Nishikawa

I 緒言

緑黄色野菜その他多くの統計によると我々日本人のビタミンA給源の85～95%が葉菜類中のカロチンによっている。ある調査報告は指摘されたように、でもなお必要な5000 IUをはるかに下廻っている状態である。それは後者に比べて日本の場合、肉よりはむしろ魚を多く食べる日本人の嗜好など外的条件によるが、一方葉菜類中のカロチンの吸収率が50%以下であることにもよる。

この対策として、ジャム、マーガリン等へのビタミンA、或はカロチンの添加、植油の利用、日本人の嗜好からみた鮫などを主とした魚介類、葉菜類調理中の損失を少くする事等が考えられる。

スカロチンがプロビタミンAと称される様になって以来、実際にカロチンを摂取する面での今までの研究は主として外国に於て行われ、高度な設備で葉菜類を豊富に食べる様な国に関係のある日常の料理法を中心とした系統的な研究報告は極めて少い。

筆者はこの際に日本人にとって重要なビタミンA給源である葉菜類中のカロチンが料理によっていかに変化するかを調べたいと思い実験を始めてみたが、今回は活性アルミナによるカラムクロマトグラフで α,β カロチンのみを抽出し、このカロチンの料理過程における変化の要因について実験を行った。

II 実験方法

(1) 試料――本学調理実習室に栽培され当日の朝8時頃に採取したほうれん草と、しゅくしゃ菜及び白菜と市販のかぶらの葉と、学園のクローバーの葉部を採取し前処理[1]した試料を得る為直径 0.6 cm の口径で葉の有部分を抜き取って試料とした。葉菜は全て生葉の一定量 (0.5 g) を所定に有害な操作を行った。

(2) 定量法――アメリカの公定法[2]のクロマトグラフ法[3]と我国の標準定量法である鹿田法[4]法で定量を行った。

(3) 計算法――次の式により算出した。

a： 試料(g)
m： 浸出液の容積
V： 浸出液の稀釈倍数
E： 分光光度計の E 値

$$総カロチン = 860 \times \frac{EmV}{a}$$ [5]

II 実験結果

1. 加熱によるカロチンの変化

葉菜類の料理法として代表的なゆで物について実験を行った。

a. ゆで物（蒸留水使用）の加熱時間とカロチン。ゆで方を一定にした時間によってカロチンがどのように変化するかを見た。ゆで方については、ゆで水の温度を一定に保ち試料に熱が完全に伝り、同時に変化を受けること無くしかも多数の試料片が一時に取り出せる点を特に考慮し次の様な器具を用いた。

第1図の如く蒸留水を沸騰せしめ第2図の如く熱の器を取り付けてガーゼの袋に入れた試料をガラス棒の先に結びて出し入れを行った。その時の温度変化を示す。

*奈良女子大

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ALEKSANDRA MAZUROWA, ALEKSANDER MROZIKIEWICZ
AND TADEUSZ WROCIŃSKI

THE CIRCULATORY EFFECTS OF THE GLUTAMIC ACID ADMINISTRATION IN EXPERIMENTAL ANIMALS

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MAZUROWA A., MROZIKIEWICZ A., WROCIŃSKI T.: *The Circulatory Effects of the Glutamic Acid Administration in Experimental Animals.* Acta Physiol. Polon. 13 (6): 797—806, 1962. — Among the basic components of vegetable and animal protein, glutamic acid produces important metabolic effects. In 1943 it was introduced into therapy and gained a vast application in many pathological conditions. The mechanism and scope of the biochemical and pharmacodynamic effects of glutamic acid are not yet fully known. The authors tried in the present work to establish the influence of glutamic acid on the circulatory system. Rabbits, frogs, and cats were used for the experiments. Glutamic acid was applied as 15 percent solution of sodium glutamate, 5 percent calcium glutamate and 0·85 percent glutamic acid. The effect of orally or parenterally given glutamic acid was studied: 1) on the rabbit heart muscle condition using *Triplex* electrocardiograph; 2) on the blood pressure and respiratory fluctuations in normal and decapitated cats; 3) on the blood vessels using an isolated preparation of the cow's ear; 4) on the frog's heart isolated according to *Straub*, 5) and on the cat's isolated heart according to *Langendorff's* method. In the result of experiments it was established that after continued intravenous administration, large doses of glutamic acid caused in rabbits electrocardiographic changes, interpreted as symptoms of myocardial lesions. Continued oral administration of glutamic acid was not followed by substantial changes in the electrocardiographic pattern. Arterial hypotension, induced by glutamic acid preparations was demonstrated to be of central origin. In experiments with the isolated heart, large doses of glutamic acid were found to slow heart action, increase systolic amplitude, and constrict coronary vessels. Very large doses arrested cardiac action totally. The investigations performed have demonstrated varying effects of glutamic acid on the circulatory system and may serve as a warning against indiscriminate use of glutamic acid, especially in intravenous injections.

Among the basic components of vegetable and animal protein, glutamic acid produces important metabolic effects. In 1943 it was introduced into the therapy and gained a vast application in many pathological conditions. Special attention is paid to the effect of this drug on the central nervous

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(1962)

system and on the metabolic transformations in the liver. Glutamic acid finds the application in mental deficiencies, mainly in children, in psychoses, epilepsy, mental depression, memory defects, exhaustion. Liver dysfunction, hypochloric gastritis and pernicious anemia present also indications for the application of the drug. Cardiac patients often ask the doctor to apply this wonderful — in their opinion — drug. The mechanism and scope of the biochemical and pharmacodynamical effects of glutamic acid are not yet fully known. It influences the acid-base balance, stabilizes the mineral metabolism, plays an important role in the acetylcholine synthesis, takes part in the formation of organic proteins and carbohydrates.

So vast being its range of effects and applications, we tried to establish the effect of glutamic acid on the circulatory system. The easiest way consists in performing appropriate experiments.

METHODS

Rabbits, frogs and cats were used for the experiments. Glutamic acid was applied as 15 percent solution of sodium glutamate, 5 percent calcium glutamate and 0·85 percent glutamic acid in 10cc ampoules, produced by Spoldzielnia Pracy Chem. Farm. „Polon" in Lodz. The pulverized glutamic acid, produced by Spoldzielnia Pracy Chemikow „Xenon" in Lodz, was used to prepare 5 percent aqueous solution for oral application.

Initially the effect of orally or parenterally given glutamic acid on the heart muscle was investigated. Rabbits of various sex and race, weighing 2000 g to 3000 g, were used in this part of the experiments. The heart muscle condition was followed using Triplex electrocardiograph in three classical and three precordial leads V_1, V_2 and V_6. Before applying electrodes the coat was removed, the animals placed supine and fastened. No anesthesia was used for these experiments. Electrocardiograms were estimated according to the normal pattern given by *Slepak* and *Hermanek*, confirmed in our own practice. First group of ten rabbits received 5 percent aqueous solution of glutamic acid in the dosis of 0·25 g per kg daily for 28 days, by means of a gastric tube. Before, during and after the application of the drug electrocardiograms were taken. In the second group the drug was applied intravenously. In seven rabbits the immediate effect of intravenous 15 percent sodium glutamate on the electrocardiographic pattern was investigated. Before and after injection ECG traces were taken every 15 minutes for three hours. In another group of 10 rabbits 15 percent solution of sodium glutamate was applied intravenously in the dosis of 0·75 g per kg for 14 days and the electrocardiographic pattern was checked every seven days.

Along with the observation of the electrocardiographic changes, the blood pressure and respiratory fluctuations under the influence of various glutamic acid preparations were studied in ten cats. Acute experiments were performed in these cats of both sexes, weighing 2000 g to 3200 g, in urethane anesthesia. Twenty percent ethyl urethane was administered in the dosis of 9 ml per kg of body weight. A tube connected with a Ludwig's manometer was placed into the common carotid artery. After tracheostomy, the trachea was connected to a Marrey's drum, recording the

respiratory excursions. Various glutamic acid preparations were injected into the femoral vein in the doses 0·25, 0·5, 0·75, 1·0, 2·0, 3·0, 4·0 and 5·0 ml per kg of body weight. The similar experiments were also performed in decapitated cats.

The effect of glutamic acid on the blood vessels was investigated using an isolated preparation of the cow's ear. Into the dissected artery of the ear a tube was introduced, connected with a burette containing a nutritive fluid, which perfused the preparation at the flow of 1 ml per minute. Through a needle introduced into the same artery the investigated drugs were infused at the same speed. The number of drops flowing out of the preparation was recorded on a smoked drum. An increased number of drops results from the dilatation of vessels, a decrease follows their constriction.

As the next step the effect of glutamic acid was investigated on the frog's heart isolated according to *Straub*. Thirty meadow frogs, weighing 15 to 30 g, were used in this series. From 0·2 to 1·0 ml of various glutamic acid preparations were added to the nutritive fluid, and the heart action was recorded.

In the last series of experiments the effect of glutamic acid on the cat's isolated heart was investigated. The action of an isolated mammalian heart can be preserved, if the defibrinated blood is pumped into its coronaries. According to *Langendorff's* method, applied in our study, the blood is pumped in the direction of the heart through a cannula inserted into the aortic stump. The aortic valves are closed under the pressure of blood and it flows into the coronary vessels. Then the blood flows out of the sinus in the right atrium. The blood drops are recorded on a smoked drum, using a simple scriber. The heart contractions are recorded on the same drum. The heart contractions are recorded on the same drum. The investigated drugs were injected by means of a syringe into a rubber tube connected with the cannula, at the speed of 1 ml per minute. When the drug in question constricts coronary vessels, the coronary flow diminishes and so does the number of blood drops flowing out of the heart. On the contrary — dilatation of the coronaries results in an augmented flow and in an increased number of blood drops.

RESULTS

In ten rabbits 5 percent aqueous solution of glutamic acid was introduced by a gastric tube into the stomach in the dosis of 0·5 g per kg daily for 28 days. The dosis and the oral application corresponded to the dosage administered by some clinicians in the human patients. The initial electrocardiogram did not reveal irregularities in any of these rabbits. The frequency of impulses and the duration of individual portions of the electrocardiographic tracing were within the normal limits, as determined by *Slepak* and *Hermanek*, as well as by other authors. The oral application of glutamic acid for 28 days in the forementioned dosage did not substantially change the electrocardiogram in nine rabbits, nor after fourteen neither twenty eight days of the administration. Although in three animals there was some lowering of ST segment in the lead CR4 after fourteen days, in the conclusive record no distinct irregularities were found, when compared with the initial tracing. In one rabbit after 28-day

application of glutamic acid the heart action accelerated from 315 up to 400 per minute. The T wave was flattened in the II and III lead with the lowering of ST segment. In general, however, the gastric administration of glutamic acid in rabbits was not followed by substantial changes in the electrocardiographic pattern (Fig. 1).

In the next groups of seven rabbits 15 percent sodium glutamate was applied intravenously and the immediate effect of the injection on the electrocardiogram was observed. In five animals sinusal bradycardia, an increased voltage of T waves and, sometimes, of the QRS complexes immediately followed the injection. Such a picture of vagal stimulation lasted for 15 minutes, then disappeared. Sixty minutes later the heart action was accelerated, the increased T waves flattened and lowered. These changes persisted until the next day. In the last two rabbits no distinct electrocardiographic irregularities were observed.

Ten rabbits received intravenously 15 percent sodium glutamate daily for fourteen days and their electrocardiograms were taken every seven days. In six animals the flattened T wave and lowered ST segment appeared and persisted, mainly in the CR4 lead. These changes could be interpreted as the myocardial lesion. In the other four animals no substantial changes were noted in the electrocardiogram (Fig. 2).

As an addition to the electrocardiographic investigations, the alterations in the blood pressure and respiration under the influence of glutamic acid were studied. These investigations were carried on cats, in which 15 percent solution of sodium glutamate was applied intravenously, the dosage being: 0·25, 0·5, 0·75, 1·0, 2·0, 3·0, 4·0 and 5·0 ml per kg. The blood pressure drop was always noted during the infusion, then the normal blood pressure was slowly regained. The most important blood pressure decrease, of 20 mm Hg, followed the application of 3 ml of glutamic acid, equal to 0·45 g per kg of body weight. The normal blood pressure was restituted after 15 minutes. The dosis of 4·0 and 5·0 ml per kg did not produce more important pressure drop, nor did it last longer than 15 minutes (Fig. 3 and 4). When 5 percent calcium glutamate and 0·85 percent glutamic acid were applied, the effects were similar to those after 15 percent sodium glutamate, however not so intense. In order to elucidate the mechanism of this pressure drop the same were administered in the decapitated cats. In all these experiments glutamic acid led to an insignificant rise in the blood pressure, by 5 to 10 mm Hg (Fig. 5). It proves that the point of hypotensive action is placed in the central nervous system.

In the experiments with the cow's isolated ear the application of 3 ml of 15 percent sodium glutamate resulted always in an inconsiderable diminution in the number of drops flowing out of the preparation, which indicated to some constriction of the blood vessels. The similar results were

in the behavior of body temperature in young and old animals. They obtained, when the other forms of glutamic acid were given.

The influence of intravenously applied glutamic acid on the respiration consisted in an increased rate of respiratory movements. These changes were more readily seen after small dosage of the drug.

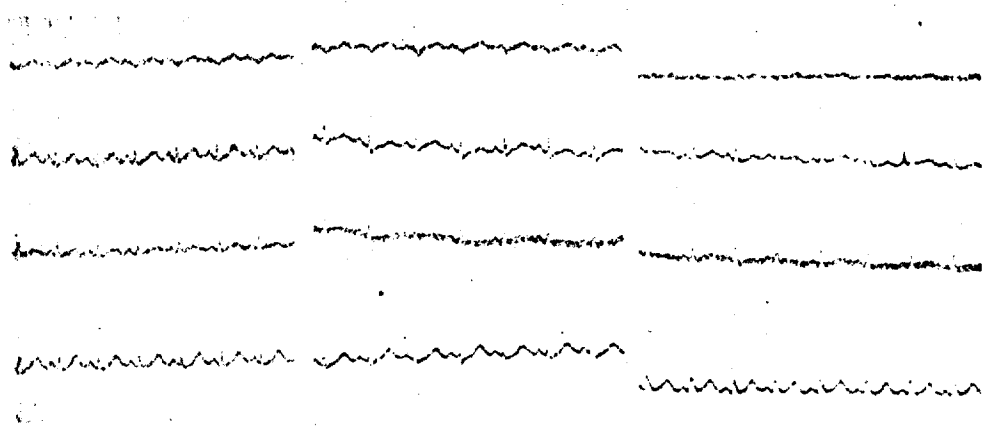

Fig. 1. The electrocardiographic pattern of a 3·5 kg rabbit after prolonged oral administration of 5 percent aqueous solution of glutamic acid in the dosis of 0·5 g per kg of body weight.

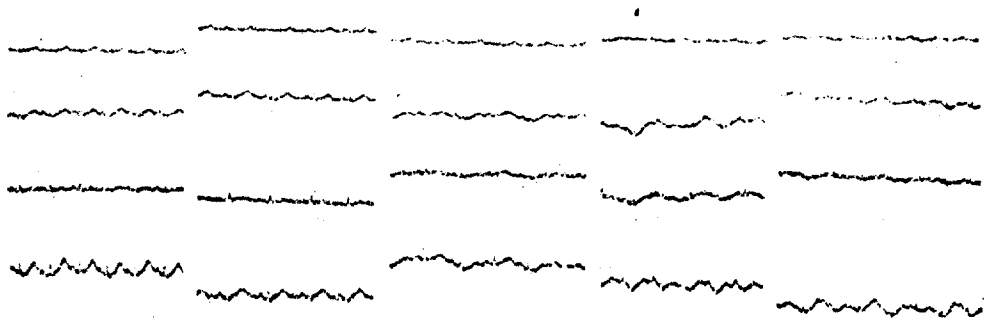

Fig. 2. The electrocardiographic pattern of a 3·0 kg rabbit after direct and prolonged intravenous application of 15 percent sodium glutamate in the dosis of 0·75 g per kg of body weight.

Later on, the effect of the glutamic acid preparations on the frog's heart, isolated according to *Straub's* method, was investigated. Thirty frogs 15 to 30 g of weight were used for the experiments. Fifteen percent solution of sodium glutamate was applied in the dosage: 0·2, 0·3, 0·4, 0·5 etc. up to 1·0 ml. The doses of 0·2 and 0·3 ml led to a diminution in the heart rate, lasting from several to over ten minutes. The doses of 0·4 and

0·5 ml were followed by a constant bradycardia and arrhythmia. The doses of 0·7 and bigger resulted always in a considerable slowing of the heart rate, extrasystole and complete arrhytmia up to cardiac arrest. The amplitude of the cardiac contractions did not sustain significant modifications (Fig. 6).

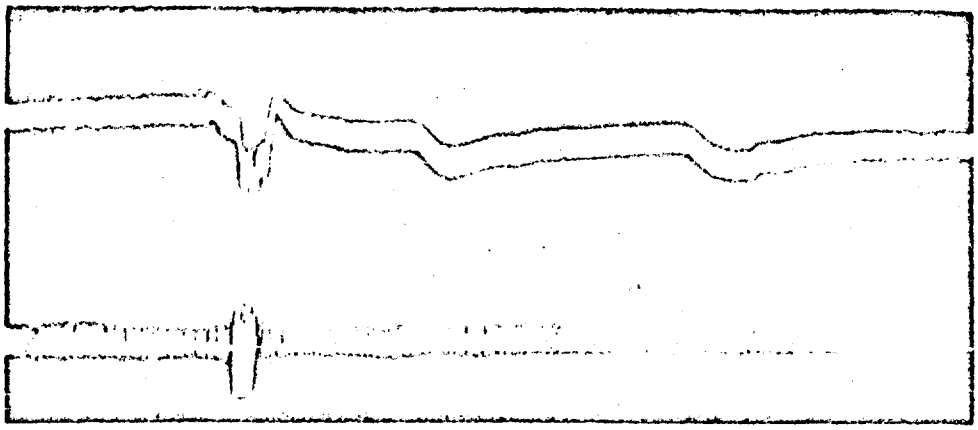

Fig. 3. The arterial blood pressure of a 3·3 kg cat under urethane anesthesia, after intravenous administration of 15 percent sodium glutamate in the doses of 0·25, 0·5, 0·75, and 1·0 ml per kg of body weight.

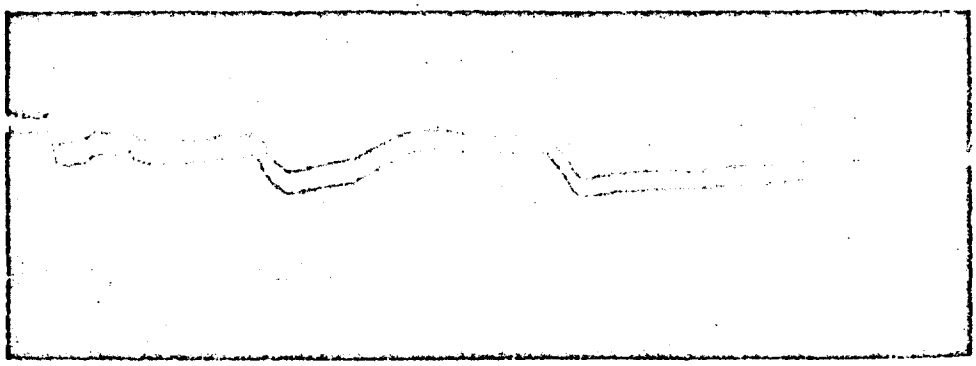

Fig. 4. The arterial blood pressure of a 3·3 kg cat under urethane anesthesia, after intravenous administration of 15 percent sodium glutamate in the doses of 2·0 and 3·0 ml per kg of body weight.

Three of the above mentioned glutamic acid preparations were checked on the isolated heart of a cat. When 15 percent sodium glutamate was applied in the dosage 1·0, 1·5, 2·0, 2·5 ml, bradycardia occurred as a rule, the amplitude raised and the number of drops flowing out was considerably diminished. If the dosis of 2·5 ml was exceeded, arrhythmia resulted, eventually leading to cardiac arrest. The application of 5 percent

calcium glutamate and 0·85 percent glutamic acid was followed by similar effects, however not so pronounced. A diminution in the number of drops, slowing of the heart rate and an increase in the amplitude of contractions

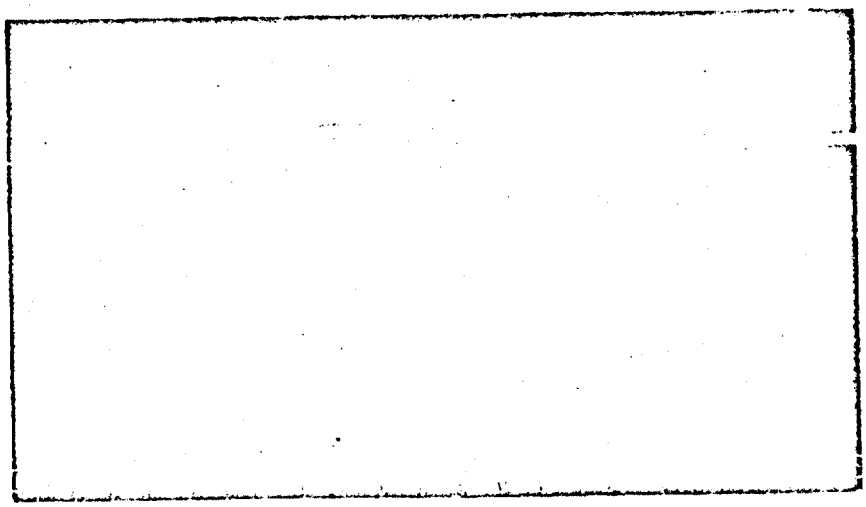

Fig. 5. The arterial blood pressure of a decapitated 3·8 kg cat under urethane anesthesia, after intravenous administration of 15 percent sodium glutamate in the dosis of 3·0 ml per kg of body weight.

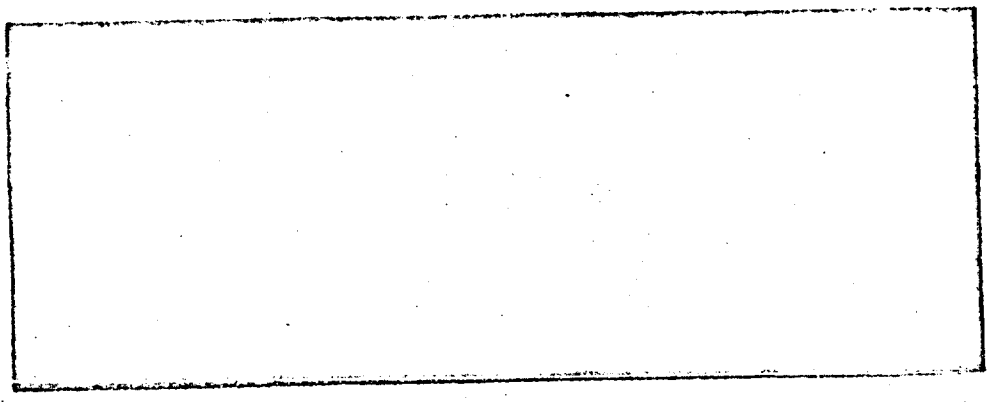

Fig. 6. The action of a frog's isolated heart, after administration of 0·4 ml of 15 percent sodium glutamate.

were observed under these conditions after 1·5, 2·0, 2·5 and 3·0 ml of the solution. Arrhythmia and cardiac arrest occurred after 3·0 and 3·5 ml of this solution. The described changes, the diminution in the number of drops in particular, are the result of the diminished coronary flow secondary to the constriction of these vessels by the drug applied.

DISCUSSION

As follows from our experiments, glutamic acid exerts various effects on the circulation of experimental animals. The oral application of this drug did not produce any substantial changes in the electrocardiographic pattern of rabbit. The intravenous application in rabbits led to instant electrocardiographic changes, typical for the parasympathetic stimulation. These changes regressed after 15 minutes, but until the next day the following irregularities were seen: an acceleration of the heart rate, flattening of T waves, which were previously increased. The daily intravenous administration of glutamic acid in rabbits was followed by the electrocardiographic changes, which can be interpreted as the myocardial lesion.

In the experiments on the isolated heart of a cat the small dosage of glutamic acid resulted in the slowing of the heart rate, an increase in the amplitude of its contractions, and in the coronary constriction. The latter effect may be one of the factors leading to the lesion of the myocardium, which followed the prolonged use of the drug and was established by means of the electrocardiogram. Higher dosage of the drug produced arrhythmia and eventually — cardiac arrest.

The results of the experiments on animals cannot be applied without reserve to the human beings. Nevertheless the circulatory changes observed in the experimental animals under the influence of glutamic acid, such as the coronary constriction after the smaller dosis of the drug and cardiac arrest after higher dosis, may serve as a warning against too liberal application of the drug in the clinical practice. The intravenous administration of glutamic acid has to be performed slowly and in diluted solutions (drip infusions), using limited doses. These precautions are of special importance at the present, when the intravenous administration of glutamic acid is recommended in hepatic, uremic and hypoglycemic coma.

On the other hand, the blood pressure drop observed after the administration of this drug, especially if the oral way is chosen, is unlikely to be followed by the side effects. Worth mentioning is the fact that the hypotensive effect has the central point of action.

Among the three injectable glutamic acid forms, offered by the Polish Pharmaceutical Industry, the most active one is 15 percent solution of its sodium salt, the other forms exert similar effects, but the intensity of their action is lower.

CONCLUSIONS

1. Large doses of glutamic acid after continued intravenous administration caused in rabbits electrocardiographic changes, interpreted as symptoms of myocardial lesions. Continued oral administration of glutamic acid was not followed by substantial changes in the electrocardiographic pattern.

2. Arterial hypotension, induced by glutamic acid preparations was demonstrated to be of central origin.

3. In experiments with the isolated heart, large doses of glutamic acid were found to slow heart action, increase systolic amplitude, and constrict coronary vessels. Very large doses arrested cardiac action totally.

4. The investigations performed have demonstrated varying effects of glutamic acid on the circulatory system and may serve as a warning against indiscriminate use of glutamic acid, especially as intravenous injections.

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To the Editor Until I read the correspondence of Dr. Ho Man Kwok in the April 4 *journal*, I was convinced that my own Chinese-restaurant syndrome could easily have been remedied with a placebo.

The first experience came at lunchtime in a New York City establishment in 1956. Subsequent four or five occurrences took place during evening meals at a Chinese restaurant in Chicago, during 1961 and 1962. From 1962 to 1964 I lived on a fairly consistent Chinese diet in the Hawaiian Islands — uneventfully. Upon return to the mainland at the end of 1964 and during the period of 1965 and 1966, I dined fairly often at Chinese restaurants in southern California and in New Hampshire, with a renewal of the experience each time. It was only at the New York restaurant and once at the New Hampshire restaurant that the attack was most severe. My most recent visits during the past two years to a Chinese restaurant in Harrisburg, Pennsylvania, were uneventful.

Like Dr. Ho Man Kwok's, my trouble began about 20 minutes after I started the meal. However, the symptoms differed: they began with severe aching in the muscles down both sides of the back of the neck and spread into the shoulders, shoulder blades and upper spine. This was accompanied on each occasion with a profuse, cold sweat about the face and under the armpits. The climax to each experience was a most uncomfortable tightness on both sides of the head just forward of the ears in the temple area. It felt as though, at any second, the sides of my head would burst; this symptom lasted no more than a minute, and the entire syndrome disappeared quite rapidly.

It should be pointed out that there were occasions, but few, when I dined in the New York, Chicago, southern California and New Hampshire restaurants when the syndrome did not appear at all.

THOMAS J. MCCAGHREN
Managing Editor
Pennsylvania Medicine

Lemoyne, Pa.

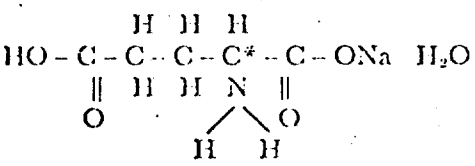
SCIENCE ON THE MARCH

MONOSODIUM GLUTAMATE—IMPROVER OF NATURAL FOOD FLAVORS

RADIO'S singing commercial relies on catchy phrases tied in with a melody to make the consumer conscious of the virtues of a manufacturer's product. "Accentuate the positive, eliminate the negative" might well become the theme song for monosodium glutamate, particularly now that it is being sold directly to the housewife as the third shaker for the dining table.

Because earlier preparations of monosodium glutamate of relatively low degree of purity exhibited a meatlike flavor, the general belief existed (and still exists in many quarters) that glutamate should be used as a seasoning for imparting meat flavors. Continued refinement in production methods has been responsible for progressive increases in purity, until the present commercial product (the monohydrate) is seldom less than 99 per cent pure. Only when the pure product became available was its unique property of accentuating natural food flavors and eliminating undesirable qualities fully appreciated.

The molecular structure of commercial monosodium glutamate monohydrate may be presented as follows:



The starred atom is asymmetric. The naturally occurring form of glutamic acid, that derived from protein hydrolysates or other natural sources, is the dextrorotatory form, l (+) glutamic; the "l" refers to its structural relationship to the other amino acids, most of which are levorotatory, the (−) to the direction of rotation. Frequently the naturally occurring form is indicated as d-glutamic acid, but l (+) glutamic acid is a more proper designation. The 1 per cent impurity in commercial monosodium glutamate monohydrate consists mainly of free moisture, salt, and traces of other amino acids.

The introduction of monosodium glutamate into foods for human consumption, its method of production, the mechanism by which it enhances food acceptability, and its physiological functions in the mammalian organism are all chapters in an interesting story. To hear this story firsthand from those who were largely responsible for introducing monosodium glutamate into the American diet and from those currently engaged in research investigations of this chemical compound, more than 120 food technologists from all parts of the country gathered in Chicago one day in March 1948 to participate in a symposium on the "Flavor and Acceptability of Monosodium Glutamate." This meeting was sponsored by The Quartermaster Food and Container Institute for the Armed Forces and the Industrial Associates of that Institute. The interest of the U. S. Armed Forces in increasing the acceptability of ration items is matched only by that of progressive food processors striving to gain competitive advantages for their own products. This meeting was effective in bringing to the attention of the American food industry as a whole the advantages of adding glutamate to food products, not for imparting a flavor of its own but for physiologically promoting and prolonging sensory acuity in the consumer for the desirable natural flavors in the products so supplemented.

History of Monosodium Glutamate

The German chemist Ritthausen was the first to isolate glutamic acid from an acid hydrolysate of gliadin, a component of wheat gluten; gliadin containing approximately 46 per cent of glutamic acid is the richest natural source of this material. Although synthetic glutamic acid is available (synthesis first accomplished by Wolff in 1890), the synthetic approach is far more costly than that based upon isolation of the amino acid from natural sources. Furthermore, the synthetic product is usually the racemic mixture, from which it is not commercially feasible to isolate the active, or dextrorotatory, form, and it is only the latter that exhibits flavor-accentuating value.

The contribution of glutamate to the building up of food flavors undoubtedly originated in the discovery in the Orient of a process for converting soybeans into soy sauce. Enzymic digestion occurs in this conversion process, with the result that free amino acids, including glutamic, are found. Soy protein contains approximately 20 per cent of glutamic acid. The protein in addition is rich in amide nitrogen, also susceptible to enzymic hydrolysis, with the result that Oriental soy sauces contain, as

shown in recent years, ammonium salts of the amino acids, including ammonium glutamate.

In 1912 a Japanese chemist, Ikeda, reported before the Eighth International Congress of Applied Chemistry meeting in New York City that glutamic acid recovered from the hydrolysate of a dried seaweed, *Laminaria japonica*, and partially neutralized with sodium hydroxide, exhibited a desirable meatlike taste. Patentable claims had been granted a number of years prior to the report (British Patent No. 9440). Suzuki and Company, of Tokyo, became interested in Ikeda's process and soon began producing monosodium glutamate on a commercial scale, the product being sold under the trade-mark, *Aji-no-Moto*, meaning "the essence of taste." The basic Suzuki-Ikeda patents expired in 1929, and by 1936 there were some seventy small firms manufacturing glutamate in Japan, basing the process for the most part on hydrolysis of wheat gluten. Suzuki and Company, however, remained the major producers, accounting for more than half the total Japanese output. The yearly production was sizable, the annual output of all Japanese manufacturers in 1933 being 9,000,000 pounds.

The growth of the glutamate industry in Japan during the first twenty years of its history has exceeded that in the United States—an observation calling for some comment. Oriental diets are greatly restricted as compared to those generally available to the over-all population in this country. The monotony of taste of Oriental dishes undoubtedly prompted the development of soy sauce and ultimately led to glutamate supplementation of soups, of rice and fish meals, and of soy sauce itself.

Suzuki and Company tried with but little success to introduce monosodium glutamate into the United States. The first successful American manufacturer of glutamate in this country was the Huron Milling Company, of Harbor Beach, Michigan. As manufacturers of various starches, including wheat, this company had a wheat gluten by-product, a logical base for glutamate production. In August 1934 commercial manufacture of the product was initiated by the Huron Milling Company under the direction of Patten, who had spent a number of years in Germany working with Kossel on problems of amino-acid technology.

All methods of glutamate production up to this time depended upon acid hydrolysis of a protein-bearing material, particularly wheat gluten. Concurrent with the attempts of the Huron Milling Company to devise a commercial method for the preparation of glutamate in this country, a beet-sugar entrepreneur by the name of Larrowe conducted large-scale plant experiments at Rossford, Ohio, with the object of isolating glutamic acid from Steffen's waste, the mother liquor remaining after the crystallization and removal of sugar (sucrose) from the beet juices. The acid-hydrolytic methods employed by the Japanese Suzuki and Company proved to be commercially not feasible for the isolation of glutamic acid from Steffen's waste. Marshall, now president of the Rumford Chemical Works, was retained by Larrowe as a consultant for setting up an efficient manufacturing method. Marshall found that, contrary to general belief, racemization of glutamic acid to the optically inactive mixture need not occur during alkaline digestion of the Steffen's waste. By a judicious choice of alkali concentration, temperature, and reaction time, it was possible to obtain l (+) glutamic acid in satisfactory yield. The successful commercial production of monosodium glutamate from Steffen's waste led to the founding of the Amino Products Company, which subsequently became a subsidiary of International Minerals and Chemical Corporation. Today the Huron Milling Company, operating out of Harbor Beach, and the Amino Products Company, with plants in Rossford, Ohio, and San Jose, California, are responsible for more than 80 per cent of the total glutamate production in this country. The latter figure approximates 7,000,000 pounds yearly, with no indication that a plateau in consumption is in sight. During recent years, the Staley Manufacturing Company at Decatur, Illinois, and General Mills, Inc., at Keokuk, Iowa, have entered the field of glutamate manufacture.

Methods of Manufacture

Glutamic acid is one of the most common of the amino acids, being a constituent of practically all proteins. Wheat gluten is one of the richest and probably the most economical source of this amino acid, the proteins in wheat gluten containing approximately 40 per cent glutamic acid. In Steffen's waste the substance is present as pyrrolidone carboxylic acid (the internal anhydride of glutamic acid) in variable concentrations up to 15 per cent of the total solids.

Any one of three hydrolytic methods can conceivably be used in the first phase of monosodium glutamate manufacture; these involve the use of proteolytic enzymes, acids or alkalis. Enzymic digestion is not favored in this country because of the long holding periods required to effect a substantial (never complete) hydrolysis of the protein, the possibility of microbiological contamination of the digestion mixture, and the inability to obtain at the end item monosodium glutamate of the high

purity required (in the neighborhood of 99 per cent). Hydrolysis with acid is free from the criticisms mentioned. The only objection to the use of acid is its corrosive action on equipment. Hydrolysis with alkali is better tolerated by processing equipment; however, the products found in alkaline digests of protein are optically inactive and hence contain only about 50 per cent of the desired l (+) isomer. Nevertheless, alkaline hydrolysis according to a specific procedure is currently used in the commercial manufacture of the monosodium glutamate from beet-sugar solution, practically without any racemization occurring. Acid hydrolysis is the preferred method when the substrate is protein, such as that found in wheat or corn gluten.

In preparing monosodium glutamate from wheat gluten, the protein is completely hydrolyzed by boiling or autoclaving with strong hydrochloric acid. After the humin is eliminated by filtration, much of the excess hydrochloric acid is removed by evaporation under reduced pressure. From the strongly acid hydrolysate the glutamic acid hydrochloride precipitates. The crystalline hydrochloride is dissolved in a minimal volume of water, treated with charcoal to remove color, filtered, and the solution adjusted to pH 3.3, the isoelectric point of glutamic acid. From this solution glutamic acid crystallizes. The glutamic acid is suspended in a minimal quantity of water and brought into solution with sodium hydroxide additions until pH 7 is attained. Upon concentration and chilling of the solution, the monosodium salt crystallizes. The dried product containing one molecule of water of hydration is the article of commerce. An approximately 67 per cent yield of the glutamic acid, known to be present in the wheat gluten, is recovered by this process. Considering the complexity of the process, particularly on a plant scale resulting in a daily production of about 10,000 pounds of monosodium glutamate, the yield of product is remarkably good.

The manufacture of monosodium glutamate from Steffen's waste follows a different procedure. Some sugar-beet juices contain sufficient quantities of glutamine to make practical the isolation of glutamic acid from this source. Other lots may contain little or no glutamine. The reasons for the differences are obscure; however, beets from certain geographical localities are uniformly high, and from others low, in glutamine content.

During the Steffen's process of liming (treatment with calcium oxide) beet molasses for the recovery of beet sugar, the glutamine present is largely converted to pyrrolidone carboxylic acid. The filtrate is treated with carbon dioxide to remove calcium ions and the clarified filtrate concentrated twenty-five fold to 60 or 70 per cent total solids content. In this form the material keeps indefinitely and may be shipped to distant plants for subsequent processing. The concentrated Steffen's waste is hydrolyzed with a strong sodium hydroxide solution. The hydrolyzed material is acidified with hydrochloric acid and concentrated *in vacuo*. After the centrifugal separation of precipitated inorganic salts, glutamic acid is recovered from the solution by adjusting the pH to 3.3 and holding at low temperatures. Sufficient glutamic acid is dissolved in a sodium hydroxide solution to bring the pH to 7, and the resulting solution is decolorized with charcoal. Following concentration and chilling of the solution, the monosodium glutamate crystallizes. The final product is dried in a rotary air drier and screened. Plant production by this process exceeds 9,000 pounds of monosodium glutamate per day, and the yield is variable, being a function of the initial glutamine concentration.

The price of monosodium glutamate to food processors is about $1.50 per pound. Its use as a food adjunct in such low concentrations as 0.05–0.5 per cent, however, negates its apparent high cost. Only because a constant market exists for by-products obtained in the manufacture of monosodium glutamate is it possible for the price to remain at its present level. The major by-product is the concentrated and subsequently neutralized filtrate (as a liquid, paste, or powder) obtained following the removal of the bulk of the glutamic acid hydrochloride from the wheat gluten hydrolysate. These concentrates of amino acid, containing in addition sugar-amino acid reaction products, have pronounced meaty flavors akin to those of the broth from well-browned beef, and when added to foods in sufficient amounts impart a dominant flavor to the final product. Frequently both a vegetable protein hydrolysate and monosodium glutamate are added to foods.

In all methods for the manufacture of monosodium glutamate, corrosion problems are of great magnitude. In solving the problems encountered, the glutamate industry in this country has contributed much to the nation's fabricating techniques for building equipment out of materials resistant to hydrochloric acid.

"Accentuating the Positive and Eliminating the Negative"

In a report on the *The Flavor of Meat and Meat Products* by the U. S. Department of Agriculture, Howe and Barbella stated as early as 1937 that purified monosodium glutamate is devoid of a

meatlike flavor. This report was largely neglected until Crocker, of the Arthur D. Little Laboratories, demonstrated how monosodium glutamate enhances food flavors.

Crocker investigated by direct study the generally held belief that monosodium glutamate imparts a meaty or chickenlike flavor to the foods supplemented. Meat products, including beef, pork, lamb, and chicken, were divided into juice and fiber fractions, and each examined separately by an objective taste panel for flavor in the cold raw state and after heating. It was found that the characteristic meaty flavor developed on heating is derived from the meat fiber rather than from the juice. The flavor of the meat fibers is not detectable when the nose is pinched, indicating that the favorable organoleptic properties of the cooked meat are due almost entirely to aroma. The volatile constituents are a mixture of hydrogen sulfide, various acids, and amines, presumably degradation products of the amino acids in the protein.

Solutions of monosodium glutamate, on the other hand, are entirely devoid of odor when pure. All four taste components (sweetness, sourness, saltiness, and bitterness) are detectable in flavoring such solutions. It is possible to match the taste of two thresholds of concentration of glutamate with a solution having 0.6 threshold of sweetness (sucrose), 0.7 of saltiness (sodium chloride), 0.3 of sourness (tartaric acid), and 0.9 of bitterness (caffeine). However, the synthetic flavor fails to match glutamate in enhancing natural food flavors. Monosodium glutamate not only increases salivary secretion for some time but also stimulates a tingling feeling in the mouth, and the taste sensation persists. This persistence effect occurs in the whole of the mouth region, including the roof of the mouth and the throat. This glutamate sensation, independent of true taste, has been described as a "feeling of satisfaction" and adds psychologically to the flavor of food eaten. Crocker postulates that this is due to the glutamate acting as a functional amine and in this capacity reaching and stimulating nerve endings in the buccal cavity. The fact that isomers and homologues are inactive points to the need for a specific molecular structure to obtain the glutamate effect, and supports Crocker's hypothesis.

The other amino acids, as such, or as their neutral salts, are also without odor. At pH 7 they are all sweet but usually somewhat bitter. Saltiness and sourness, associated with glutamic acid and its sodium salt, are not detectable in flavoring solutions of the other amino acids, nor do these other solutions leave a persistent tingling feeling in the mouth.

Crocker concludes that since the flavor of cooked meat is predominantly odor and pure monosodium glutamate is odorless, meat flavor cannot be due to, or be simulated by, glutamate. The pure glutamate commercially available in this country, nevertheless, has greater value in foods than the old odoriferous glutamates, because of the ability of the former to reinforce or accentuate natural food flavors without introducing a flavor of its own.

Studies conducted concurrently by Galvin, of the Huron Milling Company, on pure solutions of amino acids yielded results similar to these reported by Crocker. Galvin, however, emphasizes the importance of salt in augmenting the glutamate effect. The glutamate taste is also affected by pH, optimal results being achieved between pH 6 and 8. Taste diminishes slightly at pH 5 to approximately 80 per cent of original intensity, and progressively greater reductions are apparent with decreasing pH of the solution. Bearing in mind that monosodium glutamate accentuates sweetness also, Galvin points out that, for the most economical use of this material, adjustments should be made in saltiness, in pH, and in sweetness by the addition of conventional and low-cost flavoring agents. In this way full advantage is taken of the glutamate effect in promoting and prolonging sensory acuity in the consumer for desirable natural flavors.

In attempting to make objective measurements on flavors of foods, the investigator is handicapped by the usual limitations of subjective impressions, prejudices, and preferences. Nevertheless, it is possible by open-panel discussion to determine the principal notes or points of odor and flavor on unseasoned foods. A composite of these points has been called the flavor profile. Subsequent observations may then be made on the effect of various supplements upon the specific aspects of the flavor profile, without reliance upon preference. Such studies by Cairncross and Sjostrom, also of Arthur D. Little, Inc., have shown that monosodium glutamate, even in subthreshold concentrations, accentuates sweet and salty tastes in food when these are present in less than optimal amounts. Sourness and bitterness appear to be suppressed. These effects are noted with concentrations of glutamate that in themselves are not detectable. The improvement in flavor appeal is most readily detected in meats, sea foods, stews, soups, chowders, vegetables, and in dehydrated foods on reconstitution. Foods supplemented with glutamate and held on steam tables for extended periods do not suffer in flavor acceptability to the same degree as the foods without the supplement. The flavor-accentuating property of glutamate is retained indefinitely regardless

of the holding conditions to which the food may be exposed. Undesirable flavors in certain foods are suppressed by glutamate: these include the sharpness in onion flavor, rawness in many vegetables and some meats, the flavor of peel and earthiness in vegetables, such as in potatoes, and a fishy note sometimes present in Lima beans. Monosodium glutamate should be added to foods prior to complete seasoning; otherwise it might upset the balance attained with these other agents alone.

Recent studies by Hac, Long, and Blish, of International Minerals and Chemical Corporation, have shown that free glutamic acid occurs naturally in a large variety of vegetables, and in fish and meat products, in the range of 0.005–0.23 per cent. Because young or freshly harvested vegetables have a higher free glutamic acid content, and because the free glutamic acid decreases as the vegetables approach maturity or as a result of short storage in the refrigerator, a plausible explanation for the greater flavor acceptance of such products is at hand. A high free glutamic acid content (0.13–0.23 per cent) may be one of the reasons, too, why mushrooms augment the flavors of the many dishes to which they are added.

For a time the use of monosodium glutamate by food processors was complicated by the ruling of the Food and Drug Administration that a product supplemented with glutamate must bear the label "Artificially flavored with monosodium glutamate." The recent decision of the Administration that the "artificial-flavoring" designation need no longer be included in the label declaration was in part prompted by the demonstration that free glutamic acid occurs naturally in prepared foods and largely by the demonstration that it was used widely in processed foods to enhance natural flavors and not to impart a flavor of its own. Nevertheless, the presence of *added* glutamate must still be declared.

Not all foods are improved in flavor by glutamate supplementation, however; in this category are sweet products and fatty foods. In fact, supplemented carbonated sweet drinks suffer in flavor appeal, since the desirable evanescent taste (one that disappears quickly) of such products is converted to a lingering, persistent flavor: the flavor is reminiscent of the unsupplemented product after the carbonation has been dissipated.

Anson, at Continental Foods, Inc.—a company that makes a noodle soup mix dependent for its flavor in part on a balance of flavoring agents, including monosodium glutamate and a wheat gluten hydrolysate—has directed attention to an important problem associated with glutamate fortification of dry mixes. This is concerned with the changes in flavor that may develop during extended storage of the supplemented food. Various carbohydrate substances, particularly the reducing sugars, yield brown substances when heated at a neutral pH. This decomposition and polymerization of sugars is catalyzed by compounds having free amino groups and is frequently referred to as the Maillard, or browning, reaction. Since monosodium glutamate contains a free amino group and is used in products of neutral pH, this material has been involved in the browning observed during storage of certain supplemented foods. This undesirable property is not peculiar to glutamate, since the other amino acids behave similarly. Thus, protein hydrolysates, also used for flavoring purposes, participate in this reaction. The end products of the reaction not only are objectionable in the brown color but also in the bitter and caramel-like flavor imparted to the food. In all fairness it should be pointed out that all concentrated food products containing reducing carbohydrates (even in very small concentrations) and protein as the sole source of free amino groups are susceptible to this form of deterioration. In an amino-acid supplemented food, however, the number of free amino groups per unit of weight is obviously high, and hence the browning reaction is more likely to proceed in such foods to a greater extent. Normally the rate of browning is at a maximum in foods containing 10–15 per cent moisture. Thus the browning reaction is a serious problem in dry and dehydrated foods containing the essential reactants, and not in dilute products, such as soups. Reducing the moisture content of the foods to 0.5–5 per cent, storage of the products at low temperatures, sulfite treatment, and adjustment of the pH to an acid reaction have proved effective in inhibiting browning of dehydrated foods. In the latter method a solid alkalizing agent in a potentially active form, but physically isolated in the dry product from the other materials present, is added so that the pH of the reconstituted product is in the proper range of acceptability.

Physiological Functions of Glumatic Acid

Glutamic acid is a so-called nonessential amino acid; the organism is not dependent upon a dietary source of this amino acid for protein synthesis. And yet glutamic acid exhibits a number of interesting functions. It has a rather marked specific dynamic action, being exceeded in this regard only by phenylalanine and tyrosine. It is one of the amino acids which can be converted in vivo to glucose or glycogen, and it is one of the few amino acids that has lipotropic activity. It is also involved in reversi-

ble transaminase reactions in the body, converting keto acids to amino acids, it in turn being converted to alpha ketoglutaric acid. It is an important constituent of many biologically active compounds such as glutathione, folic acid (peteroyl glutamic acid--a vitamin), and insulin. It is unique among the amino acids in that it alone is utilized by cerebral tissue; it is converted to the acid amide, glutamine, in which form it may be used by brain tissue as a source of energy. In clinical trials with moderate doses given to mentally retarded children, it has been reported to increase the IQ and produce other beneficial effects, provided there is no cessation of dosage.

With recent discoveries of the important physiological roles played by the so-called nonessential amino acids, it has been facetiously remarked that the nonessential amino acids are so essential that the body cannot rely on outside sources but must synthesize them itself. This interpretation would seem to apply to glutamic acid in view of the recognized functions of this amino acid.

With monosodium glutamate in hand, the chemist need not hesitate to descend from his ivory tower and enter the sanctum sanctorum of the home (sometimes referred to as the kitchen) and demonstrate to his hitherto skeptical wife tangible evidence of real scientific accomplishment.

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To the Editor: The brief description of a "Chinese-Restaurant Syndrome" by Kwok appears to be somewhat different from the side effects that my family experienced after eating Chinese food. We noted a pounding, throbbing sensation in the head, vise-like in quality, but without real pain. I thought that the effects might conceivably be due to a vasoconstrictor substance.

Recently, I encountered a 57-year-old man without a previous history of vascular disease who suffered a cerebrovascular thrombosis three hours after eating a Chinese dinner. Although this association may, of course, be coincidental, the onset of the occlusion during waking hours at least raises the question whether an association might not exist.

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(4930)

To the Editor Regarding Dr. Kwok's article on the "Chinese-Restaurant Syndrome" I was happy to note that some thought has been given to this problem since my wife suffers from it. The symptoms that he mentions are identical to hers.

For the information of the *Journal's* readers, we are unable to recall any occasion on which she had an attack while eating Chinese food in London. Perhaps this is a lead for investigation by Dr. Kwok. We also failed to notice these symptoms when other foods high in sodium or prepared with cooking wines were used.

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PHOTOMETRIC NINHYDRIN METHOD FOR USE IN THE CHROMATOGRAPHY OF AMINO ACIDS

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For the investigations on the chromatographic separation of amino acids outlined in the preceding communication (1), it was necessary to have available a suitable quantitative method for the determination of the concentration of amino acids in the effluent from the column. For this purpose, the method should be sufficiently general to include the determination of most of the amino acids and peptides likely to be encountered in protein hydrolysates or other material of biological origin. The method should have as high a sensitivity as possible to permit the determination of low concentrations of amino acids in the effluent from the chromatogram. Also the laboratory procedure should be fairly simple to permit the method to be applied conveniently to large numbers of effluent samples.

It appeared probable that a photometric method would best fulfil these requirements. The two colorimetric methods of this type which had received the most study were the procedures based on the use of β-naphthoquinonesulfonic acid and ninhydrin (triketohydrindene hydrate) as reagents. For reasons which will be described, the ninhydrin reaction was selected for further investigation.

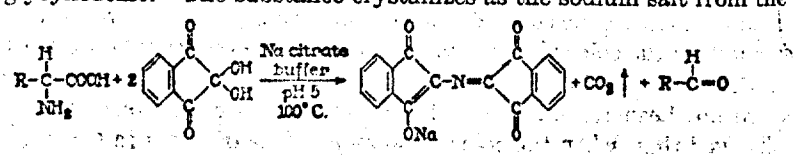
The color reaction between α-NH_2 acids and ninhydrin has been studied extensively in the past. It has been established that colored compounds are formed not only with amino acids, but also with peptides, proteins, and other classes of substances possessing free amino groups. The reaction is known to be extremely sensitive for qualitative work. In earlier attempts to render the color reaction quantitative (2–8), however, it has been found that the color yield per microgram of amino acid decreased markedly as the concentration of amino acid was reduced. In addition, the results have not been reproducible. In the present investigations, it has been observed that, when the color development is carried out in tubes exposed to the air, these difficulties appear to result primarily from the influence of dissolved oxygen. Improved results can be obtained when the reaction is performed in tubes evacuated to 20 mm. Under these conditions, the relationship between color yield and amino acid concentration is more nearly linear, although the deviations are still marked. By the addition of a strong reducing agent directly to the reaction medium, however, the oxidative side reaction has been eliminated. In preliminary experiments

the reduced form of ninhydrin, as hydrindantin (9), was added. Subsequently, it proved simpler to add stannous chloride to the reaction mixture. The stannous chloride serves to reduce part of the ninhydrin, and the preparation of hydrindantin in crystalline form is not required. Although ascorbic acid used as an antioxidant increases the sensitivity of the reaction for qualitative use (10), it is unsuitable for quantitative work because it can also give colored condensation products with amino acids (*cf.* (11)).

When conditions had been established which would give thoroughly reproducible photometric readings with a given amino acid, a study was made with several amino acids to determine the effects upon the reaction of variations in pH, temperature, time of heating, and amounts of reagents. At the same time, the mechanics of the procedure were developed to permit the analysis of large numbers of samples in a routine manner.

The method still possesses one important disadvantage. Although reproducible results are obtained for a given amino acid, the different amino acids do not all yield the same amount of color per mole. For chromatographic analysis, this is not a serious disadvantage. In those instances in which the starch column can separate an amino acid completely from the other components of the mixture, the ninhydrin reaction can be made to give quantitative values by the use of a factor appropriate for the amino acid in question.

Some of the possible causes for variations in the color yield per mole have been studied. The absorption spectra indicate that all the α-NH_2 acids (except cysteine) give the same major colored end-product. This blue coloring matter was prepared by Ruhemann (9) by the reaction of ninhydrin with alanine and by the condensation of hydrindantin with ammonia. To this compound Ruhemann assigned the structure of diketohydrindylidene-diketohydrindamine. In the present work, the product has also been isolated from the mixtures obtained in the reactions of ninhydrin with glycine and glycylleucine. The substance crystallizes as the sodium salt from the



citrate buffer solution used in the present experiments. The absorption spectrum of a standard solution of diketohydrindylidene-diketohydrindamine can be compared with the spectrum of the unfractionated reaction mixtures from ninhydrin and α-NH_2 acids. The comparison shows that in the leucine reaction, for example, the colored product is formed in only 93

per cent of the theoretical yield. A modification of the method which would render the yields quantitative in all cases would be an improvement. For a given amino acid, the percentage yield of the colored product is independent of the initial amino acid concentration. This fact indicates that the low yield is characteristic of the mechanism of the reaction under the experimental conditions employed and is not due to destruction of part of the color by a trace of oxygen.

With proline and hydroxyproline, as shown by Grassmann and von Arnim (12), the reaction follows a different course than with the amino acids containing an α-NH_2 group. These two amino acids give products with a maximum absorption at 440 mμ. The present procedure can also be used to determine proline and hydroxyproline, although the sensitivity is less than in the case of the amino acids which form diketohydrindylidene-diketohydrindamine.

It has long been known that colorimetric ninhydrin methods are not specific for the NH_2 groups of amino acids. The NH_2 groups in peptides give good color development, many amines such as histamine and tyramine will react and the presence of hydrindantin, used in this procedure, causes NH_3 to give a nearly quantitative yield of the blue reaction product. For chromatographic experiments with amino acids and peptides, the generality of the reaction extends its usefulness. For work with unfractionated biological material, the lack of specificity would be a disadvantage, as was recognized by Harding and MacLean (2). The specificity of the photometric ninhydrin method is similar in a number of respects to that of the nitrous acid reaction for amino nitrogen. The method may be of value in instances when the nitrous acid reaction is useful. For the estimation of free amino acids in the presence of peptides, the photometric method, of course, lacks the specificity of the gasometric amino acid carboxyl determination of Van Slyke, Dillon, MacFadyen, and Hamilton (13).

The photometric ninhydrin method, with the present modifications which have rendered the results fully reproducible, appears to possess some advantages over β-naphthoquinonesulfonic acid procedures for those applications for which these methods are suitable. The reaction of amino acids with β-naphthoquinonesulfonic acid, as employed by Folin, recently modified by Frame, Russell, and Wilhelmi (14), and compared with the CO_2 method by Chinard and Van Slyke (15), involves the additional operation of bleaching of the excess reagent. The ninhydrin reagent solution possesses the advantage of being stable, and for routine use can be stored under nitrogen for a month or more. Fading of the color in the ninhydrin method proceeds at a much slower rate than that reported for the naphthoquinone procedure (15). The ninhydrin reaction yields the same end-product from all the α-NH_2 acids (cysteine excepted), whereas the chemistry of the β-naphtho-

quinonesulfonic acid reaction is less well defined and the absorption maxima of the colored products obtained from different amino acids, though similar, are not identical (14).

Apparatus

Pipettes—For the pipetting of large numbers of small samples of amino acid solutions for analysis, modified self-adjusting transfer pipettes are used in 0.05, 0.1, 0.2, and 0.5 cc. sizes.[1] The accuracy of these pipettes is increased by operating them on a manifold connected to both compressed air and vacuum lines. For long series of analyses, this arrangement is also much more convenient for the operator. The arrangement of the pipetting stand is illustrated in Fig. 1. The manifold is made from three T-tubes. The third tube is mounted behind the rubber stopper (size 13). The connections are made with soft rubber hemocytometer pipette tubing. A slight vacuum (or pressure) is applied to the micro pipette by touching the top of the appropriate T-tube and greater vacuum (or pressure) by simultaneously pressing on the adjacent rubber tubing. The stop-cocks are closed only during the adjustment of the vacuum to about 60 mm. and the pressure to about 15 mm. Before use, the tip of the micro pipette should be bent, as in Fig. 1, and fire-polished to give a delivery time of 8 to 12 seconds at 15 mm. pressure. At this rate, and with wiping of the pipette tip before delivery, the reproducibility of delivery is 0.1 to 0.2 per cent. The hold up is about 2 per cent. A series of tubes from a chromatogram is run through without washing the pipette between samples. For each solvent employed the pipette must be calibrated gravimetrically. A table of calibration factors expressed in terms of the fraction of the rated delivery is prepared for each pipette. The metal holders for the 150 × 18 mm. sample tubes and photometer tubes are cut from brass tubing of 20 mm. inner diameter.

Photometer Tubes—For the chromatographic procedure, it has been necessary to accumulate a matched set of over 1000 tubes for use with the Coleman junior spectrophotometer, model 6-A. The tubes have been selected from strain-tested soft glass test-tubes, 150 × 18 mm., without lips.[2] A solution of methyl red in 0.03 N HCl is prepared of such a strength as to give a reading of 0.60 to 0.70 on the optical density scale when read at 525 mμ against a water zero. About 100 tubes are filled with 5 to 10 cc. of the methyl red solution. It is important that all tubes receive the methyl red solution from the same reservoir bottle. Pouring the solution

[1] The pipettes are made to the design of Dr. P. L. Kirk by the Microchemical Specialties Company, 1834 University Avenue, Berkeley 3, California (catalogue No. 283-B).

[2] Catalogue No. 9446, Arthur H. Thomas Company, Philadelphia, Pennsylvania.

from tube to tube can introduce significant errors. The largest group of tubes giving readings within 0.005 unit of the same value is selected. The side of the tube facing the light source is marked temporarily at the time of the test, and subsequently with a glass-marking tool,[3] to indicate the correct position for the tube in the spectrophotometer. About a dozen of

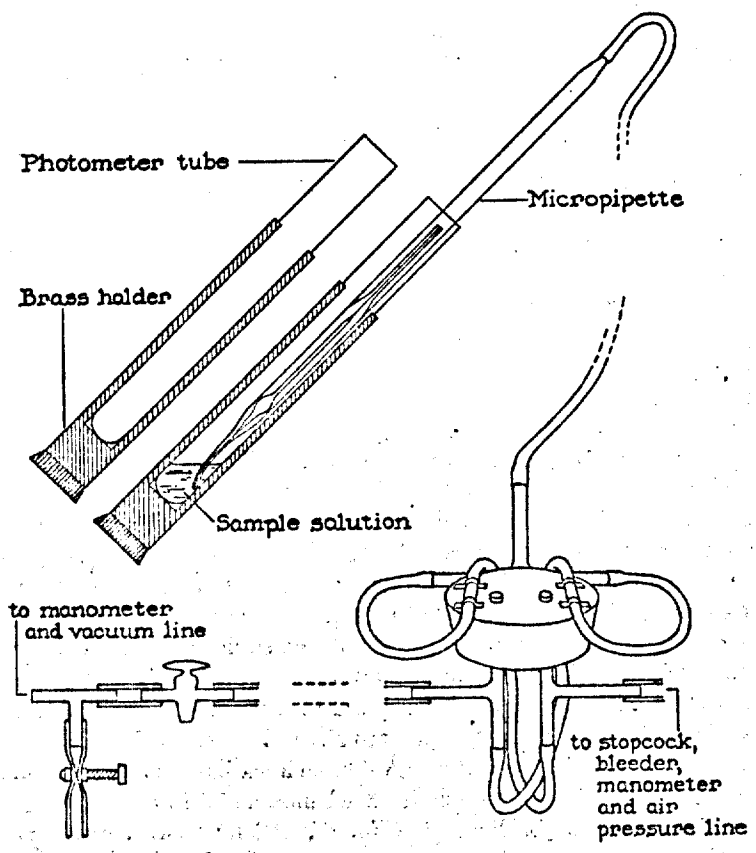


FIG. 1. Arrangement for the pipetting of samples for analysis

these tubes are marked as standards and reserved for use only in the checking of new sets.

In calibrating subsequent groups of 100 tubes against standard tubes filled with methyl red solution, each new tube is rotated in the instrument to determine whether it can be set to read within 0.005 unit of the standard

[3] Catalogue No. 3008, Chicago Wheel and Manufacturing Company, 1101 West Monroe Street, Chicago, Illinois, with additional grinding wheels No. 5-B-44.

reading. The satisfactory tubes are appropriately marked for position. The selected tubes are permanently numbered in sets of 200 tubes each. Each set can be subjected to an additional test by observing the zero reading when the tubes are filled with distilled water. In general, tubes selected by the methyl red procedure have given uniform zero readings.

When measured by calipers below the rim, the tubes selected for this study had an inner diameter of 16.25 ± 0.15 mm. The outer diameter averaged 18.3 mm. Once a calibrated set has been selected with tubes of a given average diameter, such as 18.3 mm., it is necessary that subsequent lots of tubes obtained from the manufacturer have an average diameter in the same range. Under these conditions, twenty-five to 50 tubes per hundred may prove acceptable. If such specification is not made, some lots may run all high or all low.

When the sets have been handled in wooden or aluminum racks,[4] in order to protect the tubes from being scratched, and washed with boiling soap solution, the tubes have remained accurate during constant use for more than a year.

To cover the photometer tubes during the analysis, Aloe-Willett aluminum caps[5] are used.

Water Bath—A vigorously boiling water bath is required. An open bath can be used, but the amount of evaporation of the solvent (*cf.* Table III) from the tubes is less reproducible. The present experiments have been carried out in a specially designed, electrically heated, covered bath[6] with a constant level regulator. For these analyses, the bath is operated with the thermostat at the maximum setting, so that heat is constantly, not intermittently, applied. The tubes are immersed to a depth of about 2 inches. The rate of heat supply should be sufficient to bring the bath back up to 99–100° within 2 minutes after the insertion of a full rack of 50 tubes. Only one rack should be inserted at a time. The rear corners of the cover are bent downward slightly to allow the escape of steam without the collection of condensed water. A double hook handle for insertion and removal of the racks can be made from a ¼ inch metal rod.

Pipetting Machines for Reagents—For a small number of analyses, the reagents can be added by a burette or pipette. When large numbers of samples are being run, the ninhydrin solution can be stored under nitrogen in a 1 liter brown or red glass reservoir attached to a pipetting machine.[7]

[4] Suitable aluminum racks are described in the preceding communication (1).

[5] Catalogue No. JL-78300 (for 18 mm. tubes). A. S. Aloe Company, St. Louis, Missouri.

[6] Catalogue No. 405/3/R, Electric Heat Control Apparatus Company, 507 Fifth Avenue, New York 17.

[7] Model No. 40-SS-10, Brewer Automatic Pipetting Machine, Baltimore Biological Laboratory, Inc., 500 North Calvert Street, Baltimore 2, Maryland.

The flexible connections are made with ⅛ inch inner diameter Neoprene tubing. A 250 cc. dropping funnel, for use in filling the reservoir, is mounted on the bottle through a 3-hole rubber stopper. The reservoir is connected to the top of a second bottle of the same size by a U-tube, and the second bottle is connected through a 2-hole stopper to a third 1 liter bottle by a U-tube which reaches to the bottom of each. The second bottle is initially filled with water, and the air in the system is replaced by nitrogen introduced through the dropping funnel at the time the apparatus is set up. The reservoir can be refilled without replenishing the nitrogen, except when the apparatus is disassembled for cleaning. The pipetting machine is equipped with a 3 cc. syringe and set for 1 cc. delivery. The valves (ungrooved) may require regrinding by hand with a fine emery or rouge to insure smooth performance with organic solvents. Fire polishing of the glass delivery tip, when small volumes such as 1 cc. are being delivered, helps to eliminate a hanging drop or back flow.

A second pipetting machine, equipped with a 10 cc. syringe, is used to deliver 5 cc. of the diluent solution from a 2 liter storage bottle. The flexible connections can be made with 3/32 inch inner diameter Tygon tubing or ⅛ inch Neoprene tubing. For convenience in the analysis of large numbers of samples, the reaction mixtures are diluted with a given volume of solvent from the pipetting machine to avoid the procedure of bringing the solutions to a prescribed volume in calibrated glassware.

Reagents

Ninhydrin—To insure a low blank reading in the photometric procedure, the ninhydrin, prepared commercially according to the method of Teeters and Shriner (16), has usually been recrystallized within several months of the time of use. To 250 cc. of water, 100 gm. of ninhydrin are added. The hot solution is treated with about 5 gm. of decolorizing carbon. The filtrate is stored at 4° overnight. The ninhydrin is washed on the filter four or five times with 20 cc. portions of cold water. The air-dried crystals are stored in dark glass. The recovery is 85 to 90 per cent.

Citrate Buffer—The buffer, pH 5 (0.2 M), is prepared from 21.008 gm. of citric acid, $C_6H_8O_7 \cdot H_2O$ (reagent grade) and 200 cc. of N NaOH diluted to 500 cc. Several times this quantity can be prepared and stored in the cold with thymol. The pH of the buffer when diluted with an equal volume of water should be 5.0 ± 0.1.

Methyl Cellosolve—The samples of methyl cellosolve should give a clear solution when mixed with an equal volume of water[3] and should give a negative or very faint peroxide test with 10 per cent aqueous KI.

[3] Turbidity from several samples of methyl cellosolve was found to result from the fact that the solvent had been repackaged in lacquered cans. Purchase of the solvent packaged in glass or in the manufacturer's original containers is to be preferred.

Ninhydrin Solution—Dissolve 0.80 gm. of reagent $SnCl_2 \cdot 2H_2O$ in 500 cc. of the citrate buffer, pH 5. Add this solution to 20 gm. of recrystallized ninhydrin dissolved in 500 cc. of methyl cellosolve. Transfer the reagent solution to the reservoir bottle. If the system is not already filled with nitrogen, run a stream of nitrogen through the dropping funnel for about 30 minutes. When stored in this manner the solution can be kept for at least a month without deterioration. The adequacy of the preservation of hydrindantin in the reagent solution can be checked by determining the color yield on a standard amino acid solution of relatively low concentration (1 mM). If unrecrystallized ninhydrin is used, and the reagent solution is clarified with carbon, high blanks are likely to result from nitrogenous materials on the carbon.

If only a few analyses are being run at one time, and a pipetting machine is not required, the necessary small quantity of the reagent solution can be prepared for immediate use and the 1 or 2 cc. aliquots run into the photometer tubes from a burette.

Diluent Solution—Mix equal volumes of water and *n*-propanol (C.P.).

Standard Amino Acid Solutions—For the analysis of 0.1 cc. samples, 1.6 to 2.0 mM solutions of amino acids are convenient. The solutions are diluted 1:1 or 1:4 for analyses in which 0.2 or 0.5 cc. samples are required. When water-alcohol solvents are employed, the amino acids are dissolved in water and the solutions made to the appropriate volume with the dry organic solvent. A small amount of HCl is added to dissolve tyrosine and cystine. Blank solutions for each solvent mixture are prepared at the same time.

Procedure

In the case of standard solutions of amino acids, triplicate samples of the blank and the standards are pipetted into a series of photometer tubes. When the effluent from a chromatogram is being analyzed, a single sample is pipetted from each fraction. If an automatic fraction collector (1) has delivered the appropriate size of sample directly into the photometer tubes, the pipetting step is eliminated.

If the solutions for analysis are acidic, they should be neutralized (methyl red) to within ±0.1 cc. of 0.1 N NaOH. For a series of tubes from a chromatogram, a preliminary titration is made on a blank sample. Subsequently, 1.0 N NaOH is appropriately diluted so that 1 or 2 drops (0.05 to 0.1 cc.) from a burette will bring the sample within the desired limits. The prescribed number of drops of the alkali of adjusted concentration is added to each tube of the series prior to analysis. Acidic solutions will usually pick up a trace of NH_3 from the air. Since as little as 0.1 γ of NH_3 can be detected by the present procedure, it is important that the blanks and the analytical samples be handled under strictly parallel conditions.

The pipetting machine for the ninhydrin reagent is checked for delivery by running five aliquots into a 5 cc. volumetric flask. This is done daily before use of the machine, and also serves to discard the solution which has been standing in the Neoprene tubing. The delivery should be within ±0.5 per cent of the set value. One or two aliquots of the ninhydrin solution (1 cc. for 0.1 and 0.2 cc. samples; 2 cc. for 0.5 cc. samples) are run into the photometer tubes. With samples in water or in solvents miscible with water, 1 cc. of ninhydrin solution can be used for 0.5 cc. samples if accuracy to the last few per cent is not important. Aluminum caps are placed on the tubes and the tubes are shaken to mix the reagent and sample. If the shaking is done by machine, the rack can be placed for 30 seconds on a reciprocal shaker[9] operating at about 240 excursions per minute.

The rack of tubes is heated for 20 minutes in a vigorously boiling water bath. The pipetting machine for the water-propanol diluent is checked for delivery with a 25 cc. volumetric flask. 5 cc. (±0.03 cc.) of the diluent are added to each tube. The stream is directed into the center of the solution to give good mixing. The tubes are wiped and transferred to a dry rack. The tubes are shaken by hand or by machine (1 minute). Readings are taken on the spectrophotometer starting at about 15 minutes after removal from the bath. Readings are essentially constant for 1 hour after completion of the reaction. The analyses should be run in groups of not more than 50 tubes to permit the readings to be completed within this time.

The tubes are read on the Coleman junior spectrophotometer, model 6-A, at 570 mμ. The blanks are read against a reference tube of 1:1 propanol-water. The average blank tube is chosen, and the rest of the series read with the instrument set on the blank determination as zero. The blank reading should be about 0.08 to 0.10 on the optical density scale for 1 cc. of reagent and 0.15 to 0.20 for 2 cc. of reagent. Proline and hydroxyproline are read at 440 mμ.

For tubes which read near to or above 1.00, the solutions and the blank are diluted with additional 5 cc. samples of the propanol-water solution. When the volume in the tube reaches 16 to 17 cc., shaking by machine is not satisfactory, and inversion of the Neoprene-stoppered tubes is required. If the readings are still off the scale (above 1.00), samples are pipetted into other photometer tubes for further dilution.

When an analysis is completed, the tubes are rinsed with water, a monel wire screen or a perforated aluminum cover being used to permit the draining of a full rack of 50 tubes in one operation. The tubes are half filled with 0.2 per cent aqueous solution of soap flakes. The rack of tubes is heated for 20 minutes in a boiling water bath. The tubes are rinsed three times with water and dried in an oven at 110°. The boiling soap solution is

[9] Catalogue No. 5835, Precision Scientific Company, Chicago, Illinois.

required to remove the band of material that is deposited on the walls of the tubes when volatile solvents are used.

Calculations

A standard curve is plotted for 0.1 cc. aqueous samples of leucine at six concentrations varying from 0.5 to 2.0 mM. Before being plotted, the average values are divided by the pipette calibration factor for water to give corrected readings for 0.100 cc. samples. From the graph, a table is prepared giving the millimolar concentrations corresponding to optical density readings from 0.01 to 1.00, in steps of 0.01 unit. The concentrations are multiplied by 11.1/6.1 and 16.1/6.1 to give concentrations corresponding to the readings obtained after dilution of the 6.1 cc. with one or

TABLE I

Relationship of Optical Density to Leucine Concentration (Condensed Table of Leucine Equivalents)

Determined on 0.100 cc. aqueous samples in photometer tubes of 16.25 mm. inner diameter.

Spectrophotometer reading, optical density × 100	Leucine concentration, mM per liter			Spectrophotometer reading, optical density × 100	Leucine concentration, mM per liter		
	Volume of diluent added				Volume of diluent added		
	5 cc.	10 cc.	15 cc.		5 cc.	10 cc.	15 cc.
10	0.196	0.357	0.518	60	1.18	2.15	3.11
20	0.392	0.714	1.03	70	1.39	2.53	3.67
30	0.588	1.07	1.55	80	1.61	2.93	4.25
40	0.784	1.43	2.07	90	1.83	3.33	4.83
50	0.980	1.78	2.59	100	2.05	3.73	5.42

two additional 5 cc. aliquots of the diluent. A condensed format of the standard table obtained with tubes possessing an inner diameter of 16.25 mm. is given in Table I, which covers concentrations up to 5.4 mM. The procedure can be extended by manual dilutions to much higher concentrations. The curve follows Beer's law through readings up to an optical density of about 0.50. There is a deviation of 4 per cent from the straight line relationship at an optical density of 1.0.

For the other amino acids and related compounds, the color yields per mole can be expressed relative to the leucine value as 1.00. For 0.1 cc. aqueous samples, the millimolar concentrations read from Table I, corrected for pipette delivery, were divided by the millimolar concentrations of the standard solutions to give the yields summarized in Table II. Each amino acid and peptide listed in Table II was checked for correct elementary analysis and, whenever possible, for optical rotation (*cf.* (1)). The other sub-

stances were obtained from commercial sources and were not purified before analysis.

For other than 0.1 cc. aqueous samples, the values given in Table I for millimolar concentration corresponding to a given spectrophotometer reading require correction for the changes in volume involved. Before

TABLE II

Color Yields from Amino Acids and Other Compounds on Molar Basis Relative to Leucine

Determined on 0.1 cc. aqueous samples of 2.0 mM solutions; heating time, 20 minutes; read at 570 mμ.

Compound	Color yield	Compound	Color yield
Alanine	1.01	Glutathione	0.76
Arginine	1.00	Glycine ethyl ester	1.00
Aspartic acid	0.88	Glycyltyrosine	0.88
Citrulline	1.03	Glycylphenylalanine	1.04
Glutamic acid	1.05	Glycylglycine	0.89
Glycine	1.01	Glycylleucine	1.05
Histidine	1.04	Leucylglycine	0.92
Isoleucine	1.00	Phenylalanylglycine	0.97
Leucine	1.00	Phenylalanine ethyl ester	0.98
Lysine	1.12	Histamine	0.65
Methionine	1.00	Taurine	0.97
Phenylalanine	0.88	Tyramine	0.64
Serine	0.94	Sarcosine	0.84 *Ca.*
Threonine	0.92	Glucosamine	1.00
Tyrosine	0.88	Creatine	0.03
Valine	1.02	Creatinine	0.03
Cysteine	0.15 *Ca.*	Dibenzylamine	0.04
Half-cystine	0.54	Glycine anhydride	0.01
Tryptophan	0.72 *Ca.*	Urea	0.03
Proline	0.05	Adenine	0.00
Hydroxyproline	0.03	*p*-Aminobenzoic acid	0.00
Ammonia	0.98 *Ca.*	Diethylbarbituric acid	0.00
Asparagine	0.94	Glucose	0.00
Glutamine	0.99	Uric acid	0.00

calculating the correction factors to be applied to the analysis of samples containing volatile organic solvents, it is necessary to determine gravimetrically the amount of evaporation during the analysis by weighing tubes before and after heating the reaction mixture for 20 minutes under the experimental conditions employed in an actual determination. With butanol-water and propanol-water samples, essentially the entire 0.1 to 0.5 cc. sample of solvent evaporates during the heating process. If acidic

samples are neutralized before analysis, the volume of NaOH solution added must be included in the calculation. When each term is expressed in cc., the correction factors (F) are calculated as follows:

$$F = \frac{\text{(Sample volume + neutralizing solution + ninhydrin solution + diluent) − (loss by evaporation)}}{\text{1.1 + diluent}} \times \frac{0.100}{\text{sample volume}}$$

Representative factors for two organic solvents are given in Table III. As a first approximation, these factors, used in conjunction with the rela-

TABLE III

Factors for Different Sample Sizes and Solvent Systems

Factors by which the millimolar concentrations from Table I are to be multiplied to give corrected leucine equivalents.

Solvent	Sample size	Ninhydrin solution	Loss by evaporation		Factor†		
					Volume of diluent added		
					5 cc.	10 cc.	15 cc.
	cc.	cc.	mg.	cc.*			
Water	0.1	1	(14)‡		1.000	1.000	1.000
	0.2	1	(19)		0.508	0.504	0.502
	0.5	2	(19)		0.246	0.225	0.217
Butanol-water§	0.1	1	94	0.10	0.984	0.991	0.993
	0.2	1	194	0.20	0.492	0.495	0.496
	0.5	2	395	0.45	0.231	0.217	0.212
Butanol-benzyl alcohol-water‖	0.1	1	41	0.03	0.996	0.998	0.998
	0.2	1	62	0.05	0.504	0.503	0.502
	0.5	2	79	0.07	0.244	0.224	0.216

* Approximate.

† To be divided by the calibration factor of the pipette.

‡ The small loss from water samples is subtracted from the loss with organic solvent samples in the calculation of approximate volume change.

§ Butanol-water containing 150 cc. of water per liter (d^{25} = 0.838).

‖ Butanol-benzyl alcohol-water 1:1:0.288 by volume (d^{25} = 0.936).

tive yields of color listed in Table II, give satisfactory results for most of the amino acids in these solvents, if an accuracy greater than ±5 per cent is not required. The color yields given in Table II and the factors listed in Table III may also be used for approximate results with other solvents that may be tried with starch chromatograms. Under such circumstances, the factors given in Table III for water or the butanol-benzyl alcohol solvent may be used for relatively non-volatile solvents, and the butanol factors for volatile solvents.

However, for accurate quantitative work with those solvents which are

selected as optimum for chromatographic analyses, the exact degree of evaporation must be determined experimentally, and the color yields obtained from a given amino acid must be checked by the user with standard solutions made up in the organic solvent. As may be seen from Table IV, the yields thus obtained may differ by a few per cent from the values obtained with aqueous samples given in Table II. The yields should be determined on the same size of sample being used in the chromatographic experiments, although no differences have been observed between 0.1 cc. and 0.5 cc. samples in the solvents studied thus far. For accurate work with solvents other than water, therefore, the concentration of amino acid given in Table I is multiplied by the appropriate factor from Table III and divided by the appropriate color yield from Table IV.

TABLE IV

Color Yields from Amino Acids in Organic Solvent Solutions on Molar Basis Relative to Leucine in Water

Determined on 0.1 cc. to 0.5 cc. samples; heating time, 20 minutes.

Amino acid	Color yield	
	Butanol-water solvent	Butanol-benzyl alcohol-water solvent
Leucine	0.99	1.01
Isoleucine	1.00	1.03
Phenylalanine	0.85	0.86
Tyrosine	0.86	0.87
Methionine	1.00	1.01
Valine	1.01	1.03

The factors given in Table III and the color yields listed in Table IV are fully reproducible when once determined under given experimental conditions. Except with NH_3 and tryptophan, it is not necessary to run controls with each batch of analyses. If this were not the case, the application to chromatography would be rendered unduly complicated by a need for repeated controls. An occasional check on the recovery of leucine from a known solution serves to confirm the reproducibility of the procedure.

The calculation of the recovery of leucine from a synthetic mixture which has been subjected to chromatographic analysis is given in Table V. Suitable data sheets are mimeographed to facilitate the handling of the results. In this example, the automatic fraction collector has been used with a column 0.9 cm. in diameter to deliver approximately 0.5 cc. samples directly to photometer tubes.

When aliquots are pipetted for analysis from larger effluent fractions, the summations of the uncorrected millimolar concentrations are multi-

TABLE V

Data Sheet, Determination of Leucine

Solvent, 1:1:0.288 butanol-benzyl alcohol-water; ninhydrin solution, 2 cc.; fraction collector, 25 drops = 0.504 cc.; entire fraction analyzed; wave-length, 570 mμ; readings recorded as optical density × 100.

Fraction No.	Volume of diluent, 5 cc.		Volume of diluent, 10 cc.	Volume of diluent, 15 cc.	Uncorrected amino acid concentration†
	Read against 1:1 water-propanol	Read against blank fraction*			mM
11	22.0	0	0	0	
13	22.2	0			
15	21.1	−1			
35	22.0	0			
36	22.0	0			0.00
37	23.5	1.5			0.03
38	29.0	7.0			0.14
39		24			0.47
40		57			1.12
41		100	62		2.22
42		140	90		3.33
43		150	102	81	4.30
44		130	84		3.08
45		77			1.54
46		28			0.55
47	28.5	6.5			0.13
48	22.0	0			0.00
49	22.5	0.5			
50	22.0	0			

Integration: Fraction 43. 4.30 × 0.216 × 0.5‡ = 0.464
Sum of Fractions 41, 42, and 44. 8.63 × 0.224 × 0.5 = 0.967
" " " 37-42, and 46-47. 3.98 × 0.244 × 0.5 = 0.486
Total = 1.917 micromoles.

Correction for color yield from leucine in this solvent, 1.917/1.01 = 1.898 micromoles = 0.249 mg. of leucine.

Leucine added, standard aqueous solution of a mixture of amino acids 40 mM with respect to leucine; 0.405 cc. (0.5 cc. pipette, delivery 99 per cent) diluted to 5.00 cc. and 0.485 cc. (0.5 cc. pipette, delivery 97 per cent) placed on the column in butanol-benzyl alcohol. Theoretical yield = 1.920 micromoles = 0.252 mg.; recovery = 98.8 per cent.

* Fraction 11.

† From the expanded form of Table I.

‡ If 0.5 cc. samples are pipetted, the factor becomes 0.216 × (volume of effluent fraction)/(pipette calibration factor).

plied by the appropriate factors from Table III and by the exact volume of an effluent fraction to give micromoles of amino acid. When the whole

fraction is analyzed, as in this example, the sample volume is equal to the volume of an effluent fraction, and the two terms cancel out. At large effluent volumes, when the peaks cover twenty or more fractions, integrations can be obtained from the analysis of every second fraction.

In order to obtain a graphic picture of the fractionation, the uncorrected millimolar concentrations are used directly for preliminary plotting of effluent concentration curves. The graph gives information on the symmetry of the curves and the degree of fractionation. The curves are plotted before decision is made on the division of the peaks for integration. For publication, the curves in the preceding paper (1) have been replotted, each point being corrected to "leucine equivalents" by means of Tables I and III. When the peaks are completely separated, the curves could also be corrected for color yield, but in the case of incomplete separation of the components, this is not possible. In the preceding paper (1) the method is given for the calculation of the amounts of each component in a series of partially overlapping peaks.

Accuracy—When the calibrations have been carefully made both for the pipettes and the photometer tubes, the readings on 0.2 micromole of an α-NH_2 acid can invariably be reproduced to within 0.02 optical density unit, corresponding to an accuracy of about 2 per cent. In the chromatographic analyses, it is necessary to work, in part, below this optimum concentration range. In a large series of chromatograms, integration of the effluent curves has given recoveries of 100 ± 3 per cent, under favorable conditions, and 100 ± 5 per cent for peaks markedly below the optimum average concentration (1).

Reaction with Proline and Hydroxyproline—The preceding method can be used for the determination of proline and hydroxyproline by measuring the yellowish red products of the reaction at their absorption maximum of 440 mμ. However, the optical density readings are only one-fourth and one-seventh, respectively, of those obtained with equimolar solutions of the α-NH_2 acids. The color development is only 80 to 90 per cent complete in 20 minutes at 100°. Standard curves can be prepared for proline and hydroxyproline with a 30 or 40 minute heating period. In the chromatographic analysis, the tubes are usually heated for only 20 minutes along with the rest of the effluent samples. As a first approximation, millimolar concentrations can be calculated from Tables I and III, just as in the case of readings at 570 mμ, and the values can be converted to proline by multiplying by the factor 3.7 and to hydroxyproline by multiplying by the factor 7.2. The measurement of proline by the ninhydrin reaction is of course possible only when this amino acid is completely separated from the other amino acids, as it frequently is on the starch chromatograms. On the other hand, in view of their low absorption at 570 mμ, small amounts

of proline and hydroxyproline can be present simultaneously with α-NH_2 acids without giving significant interference.

Reaction with NH_3—In the oxidative deamination of amino acids by ninhydrin, 1 equivalent of the reagent is reduced in the course of the formation of diketohydrindylidene-diketohydrindamine (9). If oxygen could be completely eliminated by evacuation of the system, the maximum color yields would be obtained from amino acids without the addition of any further source of reduced ninhydrin. The yield from NH_3 under these conditions would be low, since, of itself, NH_3 does not give rise to the reduced ninhydrin which is essential for the formation of the colored complex. This accounts for the fact that NH_3 does not react positively in a number of the colorimetric ninhydrin procedures that have been used (4, 5, 7). When hydrindantin exists preformed in the reaction mixture, however, as it does in the present procedure, the color yield from NH_3 is in the same range as that from the amino acids. In contrast to the amino acid reaction, which is independent of the concentration of hydrindantin above a certain minimum level, the color yield from NH_3 increases with the hydrindantin concentration. With the reagent solution used in the present procedure, the color yield from NH_3 reaches about 90 per cent of its maximum value. Since different batches of reagent solution may vary somewhat in hydrindantin content, a control determination on a known NH_4Cl solution must be run simultaneously if it is desired to obtain accurate values on NH_3 solutions by the photometric ninhydrin method.

Experiments on Color Development

Isolation of Diketohydrindylidene-Diketohydrindamine—The product of the reaction of ninhydrin with glycine at pH 5 was prepared in order to compare its absorption spectrum with that of the unfractionated reaction mixture obtained in the ninhydrin analysis.

The amino acid (75 mg.), dissolved in 10 cc. of water, was heated for 20 minutes at 100° with 700 mg. of ninhydrin dissolved in 20 cc. of citrate buffer, pH 5 (0.2 M). The product (245 mg.) which crystallized from the cooled solution corresponded to 75 per cent of the theoretical yield of the sodium salt of diketohydrindylidene-diketohydrindamine. The same procedure carried out with the peptide leucylglycine (188 mg.) gave the same product in 40 per cent yield. For analysis the sodium salt (50 mg.) was recrystallized from about 15 cc. of hot 1:1 water-*n*-propanol.

$C_{18}H_8O_4NNa$ (325.2).	Calculated.	C 66.5, H 2.5, N 4.3, Na 7.08
Prepared from glycine.	Found.	" 66.2, " 2.6, " 4.4, " 7.07
" " leucylglycine.	"	" 66.6, " 2.6, " 4.3, " 6.94

Absorption Spectra—The absorption curves have been determined with 1.1 cc. samples of 2 mM aqueous amino acid solutions. The final vol-

ume of the reaction mixture was 6.10 cc. in photometer tubes of 16.25 mm. inner diameter. In Fig. 2 the curves for leucine, serine, and ammonia are compared with the absorption spectrum obtained from an equimolar solution of the crystalline sodium salt of diketohydrindylidene-diketohydrindamine. The solution was prepared by dissolving 1.065 mg. of the sodium salt in 100 cc. of a mixture of the ninhydrin solution and the propanol-water diluent in the proportions of 1:10 and was read against this solvent as the blank. The absorption spectra support the conclusion that the α-NH_2 acids and ammonia yield diketohydrindylidene-diketohydrindamine

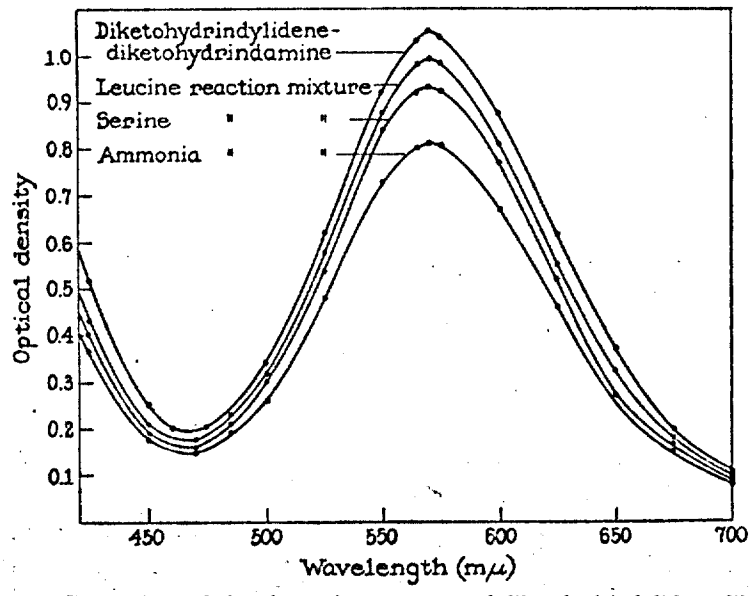


FIG. 2. Comparison of the absorption spectrum of diketohydrindylidene-diketohydrindamine with the spectra obtained after the reaction of ninhydrin with equimolar amounts of leucine, serine, and ammonia.

under the conditions of the determination. The curves obtained with the other α-NH_2 acids, except cysteine, and with peptides, are similar to those shown in Fig. 2. The major end-product is the same in all cases, exhibiting an absorption maximum at 570 mμ. The differences in the color intensities obtained with the individual amino acids arise from variations in the yield of this product. Relative to leucine, the reading of the pure sodium salt corresponds to a color yield of 1.07 (Table II). On this basis, leucine yields about 93 per cent of the theoretical amount of this product. The yields for phenylalanine and glutamic acid, for example, are 82 and 98 per cent.

Color development with ninhydrin is not specific for amino acids, since a variety of primary amines and some secondary amines will give significant amounts of color. The structure of the end-products in these cases remains to be determined. The absorption curves obtained with histamine and with the N-methyl-substituted amino acid, sarcosine, are given in

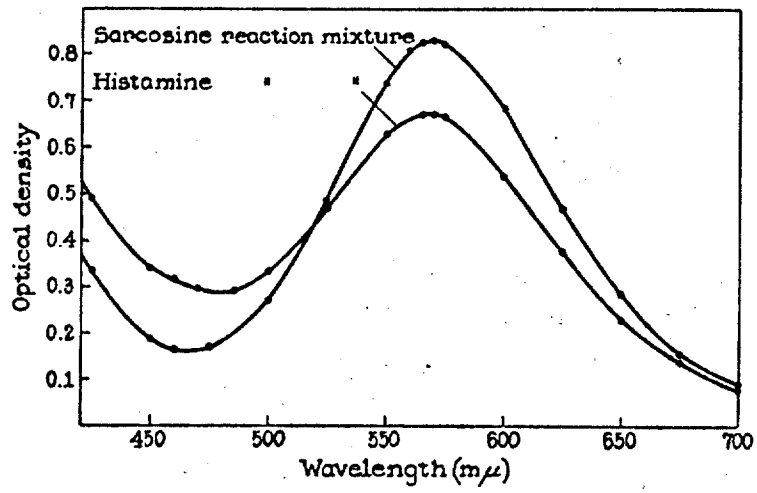


FIG. 3. Absorption spectra obtained after the reaction of ninhydrin with sarcosine and histamine.

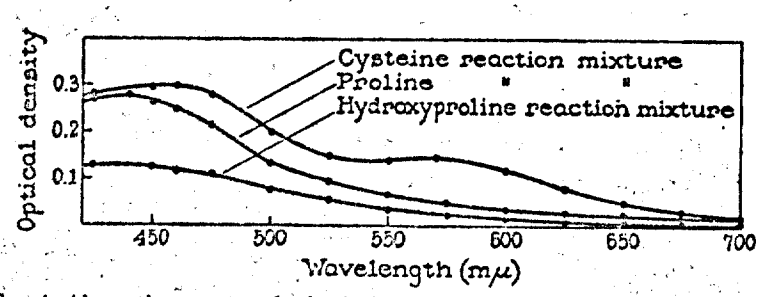


FIG. 4. Absorption spectra obtained after the reaction of ninhydrin with proline, hydroxyproline, and cysteine.

Fig. 3. Both compounds yield products with absorption maxima at 570 mμ.

Cysteine, which has been mentioned as an exception to the general reaction of the α-NH_2 acids, gives an absorption curve which is somewhat similar to that obtained with proline and hydroxyproline (Fig. 4). Neutral cysteine solutions, after standing for 24 to 48 hours, give the same color

yield as cystine, with maximum absorption at 570 mμ. It is possible that the small amount of absorption in the range of 570 mμ observed with fresh solutions of cysteine may be attributed to the presence of some cystine in the reaction mixture. The compounds yielded by the prolines under these conditions presumably correspond to the structures assigned by Grassmann and von Arnim (12).

Variation of pH—The variation of color yield with the pH of the aqueous citrate buffer is shown in Fig. 5. The absorption maximum for the α-NH_2 acids is at 570 mμ over the pH range studied. The maximum color yield from leucine is obtained at pH 5, which has been chosen for the general procedure.

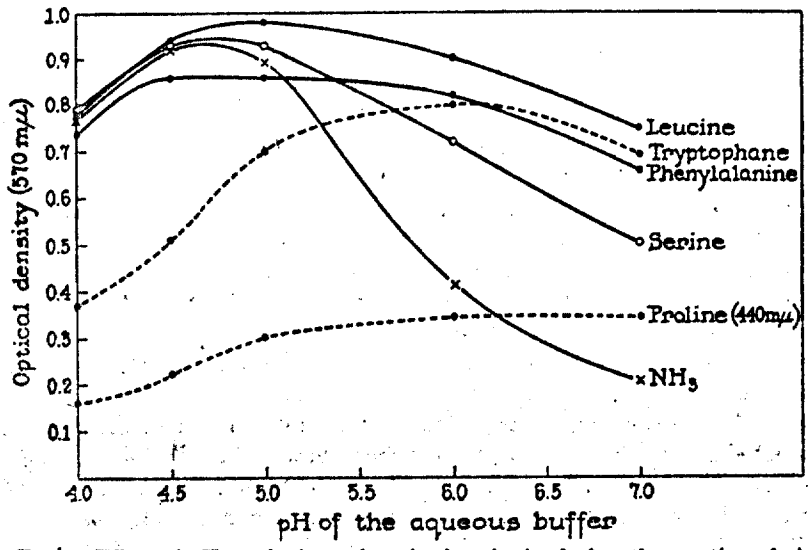


FIG. 5. Effect of pH on the intensity of color obtained after the reaction of ninhydrin with amino acids and ammonia.

The pH optima for the other α-NH_2 acids fall close to this value, with the exception of tryptophan which gives a maximum yield at pH 6. For most of the amino acids, a change in pH of 0.1 unit at pH 5.0 introduces less than a 1 per cent deviation in the optical density reading.

Rate of Reaction—The rates of color development have been determined for representative α-NH_2 acids over a range of pH from 4 to 7. In all cases, at 100° the reaction was complete in less than 20 minutes. The color yields were unaltered by an increase in the ninhydrin concentration. This result parallels the observations on the heating periods required for the complete liberation of CO_2 from amino acids at a ninhydrin concentration of 20 mg. per cc. in the gasometric method of Van Slyke, Dillon, Mac-

Fadyen, and Hamilton (13). A more detailed study of the rate of color development has been made at pH 5. Constant readings are obtained with leucine in 5 minutes and with alanine in 10 minutes. The reaction with glycylphenylalanine and glycylleucine is complete in 20 minutes. On the other hand, phenylalanylglycine and leucylglycine reach only 90 per cent of completion in this time. Primary amines, such as ethylamine and ethanolamine, react still more slowly. The time of 20 minutes has been chosen as a heating period which gives constant readings with all of the α-NH_2 acids and can be expected to give reasonably high readings with most peptides possessing a free NH_2 group.

Temperature—The color yields are lower if the reaction is carried out at temperatures below 100°. For leucine, isoleucine, tyrosine, and phenylalanine, the optical densities were 4 per cent lower when the analysis was carried to completion in a water bath maintained at 95°.

Stability of Color—The rate of fading of the blue color is illustrated by the following average readings obtained on leucine samples at the specified times after removal of the photometer tubes from the heating bath: 15 minutes 0.835, 30 minutes 0.830, 45 minutes 0.835, 60 minutes 0.835, 1½ hours 0.815, 2½ hours 0.810, 4 hours 0.795, 5½ hours 0.785, and 22 hours 0.710. Thus, the color is stable for about 60 minutes, after which time there is a gradual fading, averaging approximately 1 per cent per hour. The end-product is not highly sensitive to oxidation by air, whereas, as noted below, an intermediate in the reaction appears to possess much greater sensitivity.

In early experiments, water was used as the diluent and marked fading was noted as a result of precipitation of the sodium salt of diketohydrindylidene-diketohydrindamine. The use of 1:1 water-*n*-propanol as the diluent serves to keep the relatively insoluble reaction product in solution.

Effect of Stannous Chloride—When 2 mM leucine samples are analyzed with a ninhydrin solution from which the stannous chloride has been omitted, the color yield is about half that obtained in its presence. At lower leucine concentrations the percentage decrease in yield is greater. By carrying out the reaction in vessels evacuated to 20 to 30 mm., as is done in the gasometric ninhydrin method (13), the color yield from 2.0 mM leucine solutions can be raised almost to the maximum value. At a leucine concentration of 0.05 mM, however, the results still run about 10 per cent low.

The first trials on the blocking of the oxidative side reaction by the addition of a reducing agent to the ninhydrin solution were made with hydrindantin. Consistent results were obtained at a hydrindantin concentration of 1 mg. per cc. The color yields were unaltered by a 4-fold increase in this concentration. Since hydrindantin can be prepared by the action

of stannous chloride on ninhydrin, the addition of stannous chloride directly to the reagent solution was tried and found to give the same results. However, the presence of hydrindantin, which is highly insoluble in water, required the addition of an organic solvent which would keep this compound in solution during the course of the reaction and during storage of the reagent solution. Among the solvents tested, methyl cellosolve had the highest solvent power for hydrindantin. The solvent mixture chosen (1:1 water-methyl cellosolve) does not evaporate in the water bath at 100° and does not precipitate sodium citrate from the buffer.

SUMMARY

The reaction of ninhydrin with NH_2 groups to give diketohydrindylidene-diketohydrindamine has been utilized as the basis for a photometric determination of amino acids and related compounds in effluent samples from starch chromatograms. The color yields have been rendered fully reproducible by the incorporation of hydrindantin or stannous chloride in the reagent solution to eliminate oxidative side reactions. Although the color yield from a given amino acid is constant, the different amino acids do not all give the same percentage yield of the blue product. This fact does not prevent the accurate use of the method in chromatographic work in those cases in which the individual amino acids are separated from one another by the fractionation process.

Color development is obtained with a variety of compounds containing NH_2 groups, including amino acids, peptides, primary amines, and ammonia. For chromatographic work, the generality of the reaction extends its usefulness. For work with unfractionated biological material, the lack of specificity is a disadvantage.

The reaction is carried out at pH 5 and 100°. The absorption maximum of the blue product is at 570 mμ. On individual amino acids the accuracy is 2 per cent for samples in the range of 2.5 γ of α-NH_2 nitrogen. The mechanics of the procedure have been developed to permit the analysis of a large number of samples on a routine basis.

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Monosodium Glutamate and the Chinese Restaurant Syndrome

It has been suggested[1-4] that monosodium glutamate (MSG) is responsible for the "Chinese restaurant syndrome"—a burning sensation in the back of the neck spreading to the forearms and to the anterior thorax, accompanied by a feeling of infraorbital pressure, tightness and substernal discomfort. But no study of this phenomenon involved a double blind technique, or any other experimental condition which can be used to assess the significance of subjective reactions.

We have carried out a study on twenty-four healthy volunteers (seventeen males and seven females) aged between 18 and 34 (mean 25), using double blind and cross-over techniques. MSG was administered at lunch time in doses of 3 g/subject in 150 ml. of beef broth. This was followed by other dishes (meat, vegetables and fruit).

Subjective and objective evaluations were carried out every 20 min during the following 3 h. The subjects, randomly divided into two groups, were told they were going to receive three different kinds of broth, one of which would contain MSG (the L isomer). Observations were carried out in two experimental sessions. In the first, eight subjects received MSG in the broth and sixteen had normal beef broth—in the second session, two days later, the eight subjects who had previously received MSG were given normal beef broth, and the other sixteen were given MSG in the broth.

The subjects were asked to specify every twenty minutes any particular feeling or sensation they were experiencing. They were supplied with a form which listed possible symptoms (headache, burning feeling, flushing, facial pressure, chest pressure, weeping, gastric distress, nausea, perspiration, tiredness), and there was space to record additional symptoms. The symptoms had to be scored as mild, moderate or severe. These parameters were considered to be subjective symptoms. At the same time, arterial blood pressure (max and min), pulse and respiration rate were recorded. These parameters were considered to be objective symptoms. The data were subjected to the χ^2 test, Bartlett-box test and variance analysis.

No differences in subjective symptoms were observed between the subjects given MSG and those who had normal beef broth (Tables 1 and 2). There was also no significant difference in the number of times each single symptom occurred in the 3 h period (Table 1) nor in the number of subjects who experienced symptoms at each time (Table 2). There was a tendency in both groups to experience more symptoms during the first hour.

It should be emphasized that nobody, either in the control or in the MSG group, experienced the burning feeling which is typical of the Chinese restaurant syndrome. Also, there was no difference in the intensity of the symptoms experienced by each group. Only one subject, a female 24 years old, experienced a panic-like syndrome after being given MSG. This lasted for about 30 min, but it was not associated with any significant modification of objective parameters such as arterial pressure, pulse or respiration rate.

There was no significant variation in objective parameters ($P > 0{\cdot}05$). There was some tendency towards an

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Table 1. INCIDENCE OF THE VARIOUS SUBJECTIVE SYMPTOMS IN THE 3 H OBSERVATION PERIOD

Symptoms	Number of subjects experiencing the symptom		Number of times (observations) in which the symptom was experienced in the three hours	
	Control	MSG	Control	MSG
Headache	1	2	3	5
Burning feeling	0	0	0	0
Flushing	3	1	4	2
Facial pressure	0	1	0	2
Chest pressure	4	2	10	3
Weeping	0	1	0	1
Fainting feeling	1	2	2	4
Gastric distress	2	2	6	3
Nausea	0	1	0	2
Perspiration	1	1	1	2
Tiredness	5	4	15	17
Total	17	17	41	41

No difference between control and MSG groups was statistically significant ($P > 0{\cdot}05$).

Table 2. INCIDENCE OF SYMPTOMS AT VARIOUS TIMES AFTER ADMINISTRATION

Time (min)	Number of subjects experiencing symptoms		Total number of symptoms experienced	
	Control	MSG	Control	MSG
20	5	6	7	14
40	6	5	7	7
60	5	4	7	5
80	4	1	6	1
100	4	1	4	1
120	2	2	3	1
140	2	1	1	1
160	1	0	2	0
180	1	0	1	0
		Total	38	30

No difference between control and MSG groups was statistically significant ($P > 0{\cdot}05$).

increase in maximum blood pressure in those given MSG, but there was no variation in minimum values (Table 3). In fact, by variance analysis, there was a significant difference ($P < 0{\cdot}01$) in the overall variation. It should be noted that all the blood pressure variations are within the physiological modifications which take place during a normal digestive process. Previous authors[2] have reported that MSG is responsible for the Chinese restaurant syndrome in a very small number of subjects. The same authors later studied the acute pharmacology of MSG using an experimental procedure which was not fully described[1]. No double blind trials or statistical analysis were reported. The high dose used and the route of administration were also very questionable.

From our observations, there is no evidence that Chinese restaurant syndrome follows ingestion of MSG in a beef broth. The only subject who described a panic-like syndrome did not experience any burning feeling. Moreover, many of the subjective symptoms considered to be part of the Chinese restaurant syndrome may also be present during a normal digestive process. We therefore believe that MSG administered orally in relatively high doses does not provoke any symptoms of Chinese restaurant syndrome.

We thank Dr S. Paglialunga for the statistical

Table 3. CHANGES IN BLOOD PRESSURE AFTER MSG

Time (min)	Maximum values		Minimum values	
	Control	MSG	Control	MSG
20	$-1{\cdot}54 \pm 1{\cdot}50$	$+0{\cdot}67 \pm 1{\cdot}61$	$+2{\cdot}96 \pm 1{\cdot}92$	$+1{\cdot}71 \pm 2{\cdot}18$
40	$-3{\cdot}29 \pm 1{\cdot}90$	$+1{\cdot}08 \pm 2{\cdot}02$	$+1{\cdot}58 \pm 2{\cdot}49$	$+4{\cdot}00 \pm 2{\cdot}82$
60	$-5{\cdot}71 \pm 2{\cdot}34$	$-0{\cdot}87 \pm 2{\cdot}01$	$+0{\cdot}79 \pm 2{\cdot}58$	$+0{\cdot}96 \pm 3{\cdot}22$
80	$-4{\cdot}38 \pm 2{\cdot}38$	$-1{\cdot}42 \pm 1{\cdot}83$	$-3{\cdot}67 \pm 2{\cdot}52$	$-1{\cdot}04 \pm 2{\cdot}92$
100	$-5{\cdot}29 \pm 2{\cdot}45$	$-2{\cdot}29 \pm 2{\cdot}15$	$-6{\cdot}83 \pm 2{\cdot}56$	$-0{\cdot}67 \pm 3{\cdot}06$
120	$-5{\cdot}25 \pm 1{\cdot}91$	$-0{\cdot}37 \pm 2{\cdot}16$	$-6{\cdot}08 \pm 2{\cdot}36$	$-3{\cdot}12 \pm 2{\cdot}96$
140	$-3{\cdot}79 \pm 2{\cdot}15$	$-1{\cdot}54 \pm 2{\cdot}48$	$-4{\cdot}79 \pm 1{\cdot}98$	$-3{\cdot}83 \pm 3{\cdot}04$
160	$-3{\cdot}75 \pm 2{\cdot}22$	$-3{\cdot}92 \pm 1{\cdot}62$	$-0{\cdot}17 \pm 2{\cdot}03$	$-0{\cdot}67 \pm 3{\cdot}30$
180	$-4{\cdot}83 \pm 1{\cdot}82$	$-0{\cdot}79 \pm 1{\cdot}72$	$-2{\cdot}50 \pm 2{\cdot}24$	$-1{\cdot}04 \pm 3{\cdot}05$

The figures show the mean differences (mm Hg) ± s.e. from the basal values of blood pressure. Basal values in control session = 114 ± 2 (max); 67 ± 1 (min); basal values in MSG session = 111 ± 2 (max); 69 ± 2 (min).

evaluation of the results. MSG was kindly supplied by COFAG (Paris).

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ON THE FREE GLUTAMIC ACID OCCURRING IN FOODSTUFFS

The content of numerous foodstuffs in albumin-bound glutamic acid is known and can be found listed in corresponding tabulations. For example, a high glutamic acid content is found in wheat protein (29%), corn (25%) and milk (20%) [1].

Concerning unbound (free) glutamic acid, scarcely a mention is found in the literature. The quantitative analysis of unbound glutamic acid is attended by experimental difficulties, because this form appears only in low concentrations. L. Hac and co-workers [2] investigated the content of unbound glutamic acid in different vegetables and its dependence on various factors: for example, ripeness, storage time, and storage conditions, with the help of a microbiological method of analysis (Lactobacillus arabinosus). The application of an analytical method of a microbiological nature to the determination of low amounts of free glutamic acid demands a meticulous, lengthy separation of the sample and is, besides, very time consuming.

With the help of paper electrophoresis, it is, on the contrary, easily possible to separate the small quantity of free glutamic acid from the great mass of similar substances and to measure it quantitatively.

The method of analysis developed by the authors [3, 4], which has for years been used for the analysis of glutamic acid in hydrolyzed albumin, will be used in the investigation, hereafter described, of the free glutamic acid content of various foodstuffs. Because of the great number of foods of both animal and plant origin only a sampling was studied from each one to the next. Only when an extremely high or low value for glutamic acid was found were additional samples analyzed.

The observed contents can only be approximately accurate, because, for example with fruits and vegetables, the quantity of soluble glutamic acid was influenced by the brand, growth conditions, storage time, and storage conditions; and in the case of meat and

fish through changes after death and in cheeses through size and the depth of aging (maturity).

Experimental.

1. Quantitative analysis of glutamic acid.

The glutamic acid was separated from the interfering materials by paper electrophoresis, on an electrophoresis strip colored with ninhydrin with subsequent elution and photometric analysis of the colored matter [4].

This method does not make it possible to distinguish between the D- and L- forms of glutamic acid. As it is known that only L- glutamic acid is contained in the living systems studied, the scope of these investigations did not require differentiation between the two optical isomers.

2. Preparation of the samples for analysis.

2.1. Dried herbs and vegetables.

Each time, 10 g. of the finely ground substance was heated with water under reflux for 1 hr. and afterwards cooled and diluted to 200 ml. 0.04 to 0.08 ml of the filtrate (2 to 4 mg of the substance studied) was used for the electrophonetic separation.

2.2. Oranges, grapefruit, and apples.

Oranges, grapefruit, and apples were reduced to very fine pieces in a fast acting boiler with the addition of water. The mush so formed was transferred with a self filling micropipet (0.02 ml) onto the electrophoresis strip.

2.3. Juices, beaten egg, and milk.

Juices, beaten egg, and milk were used untreated for the electrophoretic separation.

2.4. Fresh vegetables.

Each 50 g. of fine chopped vegetable was heated under reflux with 100 ml water for 1 hr. and the slush was used for analysis.

2.5. Fish and Meat.

Each 10 g. of finely ground sample was heated under reflux with 100 ml of water for 1 hr. and the glutamic acid in 0.04 to 0.08 ml of the suspension was analyzed.

2.6. Bread and rolled oats.

Samples of 60 to 80 g were pulverized with the addition of water and diluted to 300 ml, from which 0.04 to 0.06 ml was used for analysis.

2.7. Cheese.

Each 10 g was ground until homogeneous in a mortar, mixed with water warmed to 50°C, and diluted to 40 ml; the glutamic acid in 0.01 to 0.04 ml was measured.

Results.

The average content of unbound glutamic acid in the foodstuffs studied are collected in Tables 1-7. Since the pH of most

(For Tables 1-7 refer to end of text)

of the nutriments is weakly acid to neutral, the unbound glutamic acid in them exists as a salt. In the tables, therefore, the content is given as monosodium glutamate monohydrate obtained from the commercial base (CB) and as the dry substance (DS).

1. Fresh vegetables (Table 1).

The content, obtained from the usual commercial product, fell at under 0.1% for the samples studied. Measured as the dried substance, deep frozen peas reach the highest value of this group with 0.37%.

2. Dried Vegetables (Table 2).

This table contains only values obtained from the ordinary commercial product. The glutamate content fluctuates within the limits 0.05 to 1.7%.

The glutamate content of the tomato flakes, 1.5%, corresponds to the high content in tomato juice (Table 4).

The two examples of mushrooms studied showed strongly differing glutamate contents (0.22 and 1.7%).

3. Dried Herbs (Spices) (Table 3).

Most of the dried herbs studied have a lower glutamate content than the dried vegetables.

4. Fruit and Juices (Table 4).

Tomato juice has a high proportion of glutamate with 0.3% of the CB and 5.5% of the DS. The expected likely outcome was confirmed for currants, and both kinds of apples--only a trace of glutamate. Essentially higher content is found in oranges and grapefruits.

5. Fish and Meat (Table 5).

In all the meat and fish varieties studied, less than 0.01% of glutamate was found. The meat extract contained 0.25% by CB.

6. The glutamate contents given in Table 6 for regular commercial staples are all very low.

7. Cheese.

As was expected, the glutamate content varied not only in the individual types of cheeses but also among varieties within the types. Volume and depth of maturity should be responsible for these variations.

Summary.

With the aid of a paper electrophoresis method previously developed by the author, the content of free glutamic acid in numerous staples and foodstuffs was estimated. Because of the great number of nutriments in question, of plant and animal origin, only one sample of most could be studied inside the scope of this work.

Fish and meat contain less than 0.01% glutamate (obtained from the CB); in herbs 0.01 to 0.15% was found from the CB. The highest glutamate content was found in cheese (0.02 to 2.17% of the CB) tomato flakes (1.5% of the CB) and dried mushrooms (1.7% of the CB).

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Table 1. Fresh Vegetables

No.	Designation	% Glutamate of the CB	% DS	% Glutamate of the DS
1	Beans, fresh	0.002	8.8	0.02
2	Beans, frozen	0.008	8.6	0.09
3	Peas, frozen	0.076	20.6	0.37
4	Carrots, frozen	0.013	9.6	0.14
5	Leeks, frozen	0.016	8.8	0.18
6	Potatoes	0.016	25.3	0.24

Table 2. Dried Vegetables

No.	Designation	% Glutamate by CB
1	Mushroom Powder	0.18
2	Lump mushrooms	0.20
3	Mushroom	0.22
4	Mushroom	1.7
5	Paprika, red	0.06
6	Paprika, green	0.05
7	Carrots	0.07
8	Carrot juice	0.12
9	Peas	0.33
10	Beans	0.11
11	Leeks, green	0.08
12	Leeks, powdered	0.11
13	Leeks, white	0.12
14	Celery (ground bulbs)	0.29
15	Celery leaves	0.05
16	Diced onions	0.10
17	Tomato flakes	1.50
18	Dried potatoes	0.13

Table 3. Dried Herbs

No.	Designation	% Glutamate by CB
1	Rose paprika	0.15
2	Paprika, precious sweet (edelsuss)	0.10
3	Cayenne pepper	0.12
4	White pepper	0.01
5	Black pepper	0.04
6	Marjoram	0.03
7	Garlic, dried	0.13
8	Onion juice	0.03
9	Curry	0.03
10	Parsley	0.06
11	Thyme	0.04
12	Rosemary	<0.01
13	Pimento	<0.01
14	Bay leaves	<0.01
15	Lovestock (Liebstockel)	0.07
16	Carroway	0.11
17	Coriander	0.07
18	Nutmeg	<0.01
19	Clove	<0.01
20	Curcuma*	<0.01
21	Celery seed	0.04
22	Macis*	<0.01

* In German - Ed.

Table 4. Fruits and Juices

No.	Designation	% Glutamate of the CB	% DS	% Glutamate of the DS
1	Orange	0.023	6.5	0.355
2	Grapefruit	0.036	11.0	0.327
3	Apple, "Cox-Orange"	0.004	14.7	0.027
4	Apple, "Boskop"	0.003	16.8	0.015
5	Currant, black	0.003	13.7	0.022
6	Tomato juice	0.27	5.3	5.1
7	Tomato juice, concentrated	0.37	6.3	5.9

Table 5. Meat and Fish

No.	Designation	% Glutamate of the CB
1	Calf, raw	<0.01
2	Calf, cooked	<0.01
3	Beef, raw	<0.01
4	Beef, cooked	<0.01
5	Pork, raw	<0.01
6	Pork, cooked	<0.01
7	Meat extract	0.25
8	Fresh herring	<0.01
9	Cod fish filet, frozen	<0.01

Table 6. Various Staples of Life

No.	Designation	% Glutamate of the CB	% DS	% Glutamate of the DS
1	White bread	0.002	65.2	0.003
2	Whole corn bread	0.009	58.5	0.016
3	Rye bread	0.025	61.7	0.041
4	Whole corn-oat flakes	0.014	90.5	0.016
5	Poultry egg	0.023	25.1	0.092
6	Fresh milk	0.004	13.9	0.027
7	Condensed milk	0.014	33.4	0.042

Table 7. Cheese

No.	Designation	*)	% Fat	% Glutamate of the CB	% DS	% Glutamate of the DS
1	Cottage (skim curd)	1	20	0.015	18.3	0.081
2	Fresh cheese	3	70	0.004-0.006	41.4-47.1	0.008-0.014
3	Melted cheese (cheese spread)	4	60	0.046-0.193	47.6-48.5	0.089-0.405
4	Melted cheese	3	45	0.033-0.257	45.6-51.1	0.072-0.503
5	Melted cheese	2	30	0.190-0.204	35.7-36.8	0.516-0.571
	Parmesan	3	32-35	1.803-2.17	85.7-86.8	2.14 -2.43
	Gouda	7	—	0.113-0.979	54.9-58.0	0.206-1.75
8	Emmentaler	3	—	0.792-0.908	63.3-67.8	1.18 -1.39
9	Edam	1	—	0.075	53.3	0.141
10	Tilsiter	1		0.322	61.1	0.527

* Number of samples studied

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40023

Über das Vorkommen freier Glutaminsäure in Lebensmitteln

Von H. MÜLLER

Mit 7 Tabellen

(Eingegangen am 6. März 1970)

Der Gehalt zahlreicher Lebensmittel an im Eiweiß gebundener Glutaminsäure ist bekannt und kann entsprechenden Tabellenwerken entnommen werden.

Hohe Glutaminsäuregehalte haben z. B. Weizen- (29 %), Mais- (25 %) und Milchprotein (20 %). (1)

Über die Gehalte der Lebensmittel an nicht gebundener Glutaminsäure findet man in der Literatur kaum Angaben.

Die quantitative Bestimmung der nicht gebundenen Glutaminsäure ist – weil diese nur in geringen Konzentrationen vorkommt – mit experimentellen Schwierigkeiten verbunden.

L. HAC und Mitarb. (2) untersuchten mit Hilfe einer mikrobiologischen Bestimmungsmethode (Lactobacillus arabinosus) den Gehalt verschiedener Gemüse an nicht gebundener Glutaminsäure und dessen Abhängigkeit von verschiedenen Faktoren wie z. B. Reifegrad, Lagerdauer und Lagerbedingungen.

Die Anwendung derartiger mikrobiologischer Bestimmungsmethoden zur Erfassung geringer Gehalte an freier Glutaminsäure erfordert eine sorgfältige, langwierige Aufbereitung der Probe und ist außerdem sehr zeitaufwendig.

Mit Hilfe der Papierelektrophorese ist es dagegen leicht möglich, geringe Mengen freier Glutaminsäure von großen Mengen von Begleitsubstanzen zu trennen und quantitativ zu bestimmen.

Die vom Verfasser entwickelte Analysenmethode (3, 4), die sich seit Jahren bei der Bestimmung von Glutaminsäure in Eiweißhydrolysaten bewährt hat, wurde für die im folgenden beschriebenen Untersuchungen verschiedener Lebensmittel auf ihre Gehalte an freier Glutaminsäure eingesetzt.

Wegen der großen Zahl der in Frage kommenden Lebensmittel tierischer und pflanzlicher Herkunft wurde von jedem zunächst nur ein Muster untersucht. Nur wenn extrem hohe oder extrem niedrige Glutaminsäurewerte gefunden wurden, wurden zusätzliche Proben analysiert.

Die gefundenen Gehalte können deshalb nur Richtwerte sein, weil z. B. bei Obst und Gemüse die Menge an löslicher Glutaminsäure durch Sorte, Vegetationsbedingungen, Lagerzeit und Lagerbedingungen, beim Fleisch und Fisch durch postmortale Veränderungen, beim Käse durch Umfang und Tiefe der Reifung beeinflußt werden.

Experimentelles

1. *Quantitative Bestimmung der Glutaminsäure*

Die Glutaminsäure wird papierelektrophoretisch von den bei der quantitativen Bestimmung störenden Stoffen abgetrennt, auf dem Elektrophoresestreifen mit Ninhydrin angefärbt und der Farbstoff eluiert und photometrisch ausgewertet (4).

Diese Methode ermöglicht nicht die Unterscheidung zwischen der D- und der L-Form der Glutaminsäure. Da bekannt ist, daß in den untersuchten Lebensmitteln nur L-Glutaminsäure enthalten ist, war die Differenzierung zwischen den beiden optischen Antipoden im Rahmen dieser Untersuchungen nicht erforderlich.

2. *Aufbereitung der Proben für die Analyse*

2.1. *Trockengewürze und Trockengemüse*

Jeweils 10 g der feingemahlenen Substanzen werden mit 100 ml Wasser 1 Stunde lang am Rückfluß gekocht, nach dem Abkühlen auf 200 ml aufgefüllt. 0,04—0,08 ml des Filtrates (2–4 mg der zu untersuchenden Substanz) wurden für die elektrophoretische Trennung eingesetzt.

2.2. Orangen, Pampelmusen und Äpfel wurden in einer schnell-laufenden Küchenmaschine unter Zugabe von Wasser sehr fein zerkleinert. Die so entstandene Aufschlämmung wurde mit einer selbstfüllenden Mikropipette (0,02 ml) auf die Elektrophoresestreifen übertragen.

2.3. *Säfte, Milch und verrührtes Ei* wurden unverdünnt für die elektrophoretische Trennung eingesetzt.

2.4. *Frischgemüse*

Je 50 g feinzerkleinertes Gemüse wurden mit 100 ml Wasser 1 Stunde lang am Rückfluß gekocht und die Aufschlämmung für die Analyse benutzt.

2.5. *Fisch und Fleisch*

Je 10 g der feingemahlenen Probe wurden mit 100 ml Wasser 1 Stunde lang am Rückfluß gekocht, in 0,04–0,08 ml der Suspension wurde die Glutaminsäure bestimmt.

2.6. *Brot und Haferflocken*

60–80 g der Proben wurden unter Zugabe von Wasser zerkleinert und auf 300 ml aufgefüllt, davon 0,04–0,06 ml für die Analyse eingesetzt.

2.7. *Käse*

Je 10 g wurden in einer Reibschale bis zur Homogenität verrieben, portionsweise mit 50 °C warmem Wasser gemischt und zu 40 ml aufgefüllt; in 0,01–0,04 ml wurde die Glutaminsäure bestimmt.

Ergebnisse

Die ermittelten Gehalte der untersuchten Lebensmittel an nicht gebundener Glutaminsäure sind in den Tabellen 1–7 zusammengefaßt. Da der pH-Wert der meisten Lebensmittel „schwach sauer" bis „neutral" ist, liegt in ihnen die nicht gebundene Glutaminsäure als Salz vor. In den Tabellen sind darum die Gehalte als Mononatriumglutamat · 1 H_2O – bezogen auf Handelsbasis (HB) und auf Trockensubstanz (TS) – angegeben.

1. *Frischgemüse* (Tab. 1)

Die Gehalte an Glutamat – auf die handelsüblichen Produkte bezogen – lagen bei den untersuchten Proben unter 0,1 %.

Auf Trockensubstanz bezogen erreichten tiefgefrorene Erbsen mit 0,37 % den höchsten Wert dieser Gruppe.

Tab. 1. Frischgemüse

Nr.	Bezeichnung	% Glutamat a. HB	% TS	% Glutamat a. TS
1	Bohnen, frisch	0,002	8,8	0,02
2	Bohnen, gefroren	0,008	8,6	0,09
3	Erbsen, gefroren	0,076	20,6	0,37
4	Karotten, gefroren	0,013	9,6	0,14
5	Lauch, gefroren	0,016	8,8	0,18
6	Kartoffeln	0,061	25,3	0,24

2. *Trockengemüse* (Tab. 2)

Diese Tabelle enthält nur Werte, die auf die handelsüblichen Produkte bezogen sind. Die Glutamatgehalte schwanken innerhalb weiter Grenzen (0,05–1,7 %).

Der Glutamatgehalt der Tomatenflocken mit 1,5 % korrespondiert mit den hohen Glutamatgehalten der Tomatensäfte (Tab. 4).

Die beiden untersuchten Champignonmuster weisen stark unterschiedliche Glutamatgehalte auf (0,22 und 1,7 %).

Tab. 2. Trockengemüse

Nr.	Bezeichnung	% Glutamat a. HB
1	Pilzmehl	0,18
2	Steinpilze	0,20
3	Champignon	0,22
4	Champignon	1,7
5	Paprika, rot	0,06
6	Paprika, grün	0,05
7	Karotten	0,07
8	Karottengrieß	0,12
9	Erbsen	0,33
10	Bohnen	0,11
11	Lauch, grün	0,08
12	Lauchstaub	0,11
13	Lauch, weiß	0,12
14	Sellerie (Knollenmehl)	0,29
15	Sellerielaub	0,05
16	Zwiebelscheiben	0,10
17	Tomatenflocken	1,50
18	Trockenkartoffeln	0,13

3. *Trockengewürze* (Tab. 3)

Die meisten der untersuchten Trockengewürze haben geringere Glutamatgehalte als Trockengemüse.

Tab. 3. Trockengewürze

Nr.	Bezeichnung	% Glutamat a. HB
1	Rosenpaprika	0,15
2	Paprika, edelsüß	0,10
3	Cayenne-Pfeffer	0,12
4	weißer Pfeffer	0,01
5	schwarzer Pfeffer	0,04
6	Majoran	0,03
7	Knoblauch, getrocknet	0,13
8	Zwiebelgrieß	0,03
9	Curry	0,03
10	Petersilie	0,06
11	Thymian	0,04
12	Rosmarin	< 0,01
13	Piment	< 0,01
14	Lorbeerblätter	< 0,01
15	Liebstöckel	0,07
16	Kümmel	0,11
17	Koriander	0,07
18	Muskatnuß	< 0,01
19	Nelken	< 0,01
20	Curcuma	< 0,01
21	Selleriesamen	0,04
22	Macis	< 0,01

4. *Obst und Säfte* (Tab. 4)

Tomatensaft hat mit 0,3 % a. HB und 5,5 % a. TS einen hohen Anteil an Glutamat.

Erwartungsgemäß wurden im Johannisbeersaft und in den beiden Apfelsorten nur Spuren von Glutamat nachgewiesen.

Wesentlich höher ist dessen Gehalt in Orangen und Pampelmusen.

Tab. 4. Obst und Säfte

Nr.	Bezeichnung	% Glutamat a. HB	% TS	% Glutamat a. TS
1	Orange	0,023	6,5	0,355
2	Pampelmuse	0,036	11,0	0,327
3	Apfel „Cox-Orange"	0,004	14,7	0,027
4	Apfel „Boskop"	0,003	16,8	0,015
5	Johannisbeersaft „schwarz"	0,003	13,7	0,022
6	Tomatensaft	0,27	5,3	5,1
7	Tomatensaft kons.	0,37	6,3	5,9

5. *Fisch und Fleisch* (Tab. 5)

In allen untersuchten Fleisch- und Fischsorten waren weniger als 0,01 % Glutamat nachweisbar. Der Fleischextrakt enthielt 0,25 % a. HB.

Tab. 5. Fleisch und Fisch

Nr.	Bezeichnung	% Glutamat a. HB
1	Kalbfleisch, roh	< 0,01
2	Kalbfleisch, gebraten	< 0,01
3	Rindfleisch, roh	< 0,01
4	Rindfleisch, gebraten	< 0,01
5	Schweinefleisch, roh	< 0,01
6	Schweinefleisch, gebraten	< 0,01
7	Fleischextrakt	0,25
8	Hering, frisch	< 0,01
9	Kabeljau-Filet, gefroren	< 0,01

6. Die in *Tabelle 6* enthaltenen Glutamatgehalte verschiedener handelsüblicher Lebensmittel sind alle sehr gering.

Tab. 6. Verschiedene Lebensmittel

Nr.	Bezeichnung	% Glutamat a. HB	% TS	% Glutamat a. TS
1	Weißbrot	0,002	65,2	0,003
2	Vollkornbrot	0,009	58,5	0,016
3	Roggenbrot	0,025	61,7	0,041
4	Vollkorn-Haferflocken	0,014	90,5	0,016
5	Hühnerei	0,023	25,1	0,092
6	Frischmilch	0,004	13,9	0,027
7	Kondensmilch	0,014	33,4	0,042

7. *Käse*

Wie zu erwarten war, unterscheiden sich nicht nur die Glutamatgehalte der einzelnen Käsesorten, sondern auch diejenigen innerhalb der Sorten. Umfang und Tiefe der Reifung dürften für diese Unterschiede verantwortlich sein.

Tab. 7. Käse

Nr.	Bezeichnung	*)	% Fett i. T.	% Glutamat a. HB	% TS	% Glutamat a. TS
1	Magerquark	1	20	0,015	18,3	0,081
2	Frischkäse	3	70	0,004–0,006	41,4–47,1	0,008–0,014
3	Schmelzkäse	4	60	0,046–0,193	47,6–48,5	0,089–0,405
4	Schmelzkäse	3	45	0,033–0,257	45,6–51,1	0,072–0,503
5	Schmelzkäse	2	30	0,190–0,204	35,7–36,8	0,516–0,571
6	Parmesan	3	32–35	1,830–2,17	85,7–86,8	2,14–2,43
7	Gouda	3	—	0,113–0,979	54,9–58,0	0,206–1,75
8	Emmentaler	3	—	0,792–0,908	63,3–67,8	1,18–1,39
9	Edamer	1	—	0,075	53,3	0,141
10	Tilsiter	1	—	0,322	61,1	0,527

*) Zahl der untersuchten Proben

Zusammenfassung

Mit Hilfe einer vom Verfasser früher entwickelten papierelektrophoretischen Methode wurden die Gehalte zahlreicher Lebensmittel an freier Glutaminsäure ermittelt.

Wegen der großen Zahl der in Frage kommenden Lebensmittel pflanzlicher und tierischer Herkunft konnte im Rahmen dieser Arbeit von den meisten nur eine Probe untersucht werden.

Fisch und Fleisch enthielten weniger als 0,01 % Glutamat (bezogen a. HB), in Gewürzen wurden 0,01–0,15 % a. HB gefunden. Die höchsten Glutamatgehalte wurden in Käse (0,02 bis 2,17 % a. HB), Tomatenflocken (1,5 % a. HB) und getrockneten Champignons (1,7 % a. HB) ermittelt.

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THE TRANSAMINATION OF GLUTAMIC AND ASPARTIC ACIDS DURING ABSORPTION BY THE SMALL INTESTINE OF THE DOG *IN VIVO*

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The fate of ingested protein was re-investigated by Dent & Schilling in 1949, and they concluded that heterologous protein is absorbed almost entirely as free amino acids and that the concentration of the amino acids in the portal blood after eating casein is largely what one would expect from a knowledge of the amino acid content of that protein. Glutamic acid, however, is exceptional in that its concentration in the portal blood is much lower than would be expected in view of the high glutamic acid content of casein. In 1953 Wiseman investigated the ability of the isolated small intestine of the rat to transport various amino acids against a concentration gradient, and found that although the L-forms of alanine, phenylalanine, methionine, histidine and isoleucine are absorbed against a concentration gradient, those of glutamic and aspartic acids are not. He also found that part of the glutamic and aspartic acids disappears from the experimental system as a whole. Matthews & Wiseman (1953), using an *in vitro* technique (Wiseman, 1953), then showed that when either glutamic acid or aspartic acid is introduced into the intestinal lumen, alanine appears in the fluid bathing the serosal surface of the intestine. They concluded that transamination occurs during the absorption of glutamic or aspartic acid and results in the appearance of alanine in the portal blood. The following experiments, investigating this process *in vivo*, confirm their hypothesis, and also give the quantitative relationship between these amino acids during absorption from the dog's small intestine.

METHODS

Experimental procedure. Dogs, weighing between 12 and 20 kg, were used and were allowed only water during the 24 hr before the experiment. Intravenous pentobarbitone (approximately 30 mg/kg body weight), intraperitoneal hexobarbitone (approximately 50 mg/kg body weight), or ether was used for induction of anaesthesia, and then surgical anaesthesia was maintained by intravenous pentobarbitone. A femoral vein was exposed and used for any intravenous injections required (pentobarbitone and heparin). A carotid artery was exposed and cannulated with a T-shaped glass cannula and the arterial blood pressure recorded from time to time. The side arm

of the cannula was used for collecting arterial blood samples at intervals during the experiments; before each sample was taken, enough blood was drawn out to clear the dead space.

The preparation of a segment of small intestine with the mesenteric vein draining it was similar to that described by Matthews & Smyth (1954): with this technique none of the blood from the experimental segment of intestine returns to the circulation. The site and length of the segment were chosen not by position along the small intestine but for suitability for cannulation of the venous drainage. The segment was kept warm throughout the experiment by saline packs and the temperature noted from time to time. The vein draining it was cannulated by a small glass cannula attached to a length of polythene tubing (Portex size 52: bore 1 mm, wall 0·25 mm) through which the blood was collected. The arterial blood pressure gradually fell from about 100 mm Hg at the beginning to about 35 mm Hg at the end of the experimental period. As the blood pressure fell the polythene tubing was shortened in order to maintain a satisfactory rate of blood flow. After the preparation of the intestinal segment and cannulation of its vein, resting blood samples were collected and then 10 ml. of the appropriate amino acid or control saline solution was introduced into the lumen.

Amino acid solutions. The L-glutamic and L-aspartic acids were commercial samples (L. Light and Co., Ltd.) used without further purification. They were dissolved in distilled water with the aid of NaOH and the final solution was adjusted to near pH 6 using chlorophenol red as indicator. Where an amino acid solution would have been hypotonic, NaCl solution was added to render it isotonic. In control experiments 10 ml. isotonic saline were used instead of an amino acid solution.

Treatment of blood samples. The blood samples were collected into graduated measuring tubes immersed in an ice-bath, the final volume of each sample being between 30 and 40 ml. A 20 ml. sample from this was then frozen at −20° C for several hours. After slow thawing the venous blood was usually found to be haemolysed by this process, but arterial blood often had to be frozen once again. The haemolysed blood was then deproteinized by the use of 30% trichloroacetic acid, using enough to give a final concentration of 5%. The treated blood was then stirred vigorously several times until the original rather firm consistency had become more fluid, and was then filtered on a Büchner funnel with strong suction. A measured amount (7–8 ml.) of the filtrate was then brought to about pH 7·5 by the addition of N-NaOH (mixed with chlorophenol red as indicator) and allowed to stand for 3 min. This procedure was found to convert enough of the trichloroacetic acid to chloroform to allow the use of the analytical methods described below. (We have found, however, that this procedure is not satisfactory when pyruvic carboxylase is to be used. If the filtrate is made strongly alkaline the trichloroacetic acid is converted to formic acid and not to chloroform.) A known amount of N-HCl (usually about 0·1 ml.) was then added to the filtrate to bring the pH to about 4 and the chloroform removed by heating to 80° C for 1 min.

Recovery of residual amino acids from intestinal lumen. At the end of each experiment the lumen of the experimental portion of intestine was washed out and the washings collected in a volumetric flask (25, 50 or 100 ml., depending on the yield of amino acid expected). This was then deproteinized and estimated for amino acids in the same manner as the blood samples.

Estimation of amino acids. L-glutamic acid was estimated by the manometric method of Gale (1945), and aspartic acid by the manometric method of Krebs (1950). Alanine was estimated by the colorimetric method of Alexander & Seligman (1945) as modified by Christensen, Riggs & Ray (1952).

It was found that an equimolecular concentration of alanine did not interfere with the estimation of either glutamic acid or aspartic acid. Equimolecular amounts of glutamic acid did not interfere with the alanine estimation, and aspartic acid only interfered to a very slight extent.

RESULTS

The results show that the concentration of alanine increases in the venous blood draining the experimental portion of intestine during the absorption of glutamic acid, and the increase depends on the concentration of glutamic acid in the

intestinal lumen. The concentrations of glutamic acid and alanine in this venous blood during the absorption of glutamic acid are shown in Tables 1–4, and the concentrations of glutamic acid, alanine and aspartic acid in this venous blood during absorption of aspartic acid are shown in Tables 5 and 6.

TABLE 1. Blood glutamic acid and alanine concentrations after introduction of 10% glutamic acid solution into intestinal lumen*

Sample	Collected at (min)	Volume (ml.)	Glutamic acid concn. (μmole/100 ml.)	Alanine concn. (μmole/100 ml.)
Arterial	5	25	104	35
	19	25	103	41
	42	25	127	49
	67	25	137	47
	95	25	150	95
Venous	0–5	30	60	43
	5–16	60	108	44
	16–27	60	205	68
	27–40	65	246	76
	40–53	60	311	124
	53–73	60	570	157
	73–99	63	680	190

* In all tables arterial samples from carotid artery; venous samples from vein draining experimental portion of small intestine. Experimental solutions introduced into intestinal lumen after collection of first venous sample.

TABLE 2. Blood glutamic acid and alanine concentrations after introduction of 2% glutamic acid solution into intestinal lumen

Sample	Collected at (min)	Volume (ml.)	Glutamic acid concn. (μmole/100 ml.)	Alanine concn. (μmole/100 ml.)
Arterial	5	25	82	71
	31	25	86	74
	73	25	77	92
	101	25	84	97
Venous	0–7	30	62	121
	7–15	30	65	119
	15–23	30	212	147
	23–30	30	435	159
	30–37	30	460	165
	37–49	40	489	204
	49–59	30	420	217
	59–71	30	409	216
	71–85	30	359	202
	85–102	30	265	229

The tables also give the concentrations in the arterial blood. Table 7 gives the concentrations of glutamic acid and alanine during the absorption of 10 ml. of isotonic saline. Each table refers to an individual experiment.

The increase of venous glutamic acid is more rapid with a 2% glutamic acid solution (Table 2) than with a 10% solution (Table 1), suggesting that the hypertonic 10% solution delays the absorption of glutamic acid to some extent. When 10%, 2% or 0·5% glutamic acid solutions are used the venous

glutamic acid concentrations increase during the experimental period, but when 0·15% glutamic acid is introduced into the intestinal lumen no increase of the venous glutamic acid occurs.

In all the experiments the concentration of alanine increased in the venous blood draining the experimental portion of intestine. As the resting alanine concentration varies from animal to animal the exact effect of different

TABLE 3. Blood glutamic acid and alanine concentrations after introduction of 0·5% glutamic acid solution into intestinal lumen

Sample	Collected at (min)	Volume (ml.)	Glutamic acid concn. (μmole/100 ml.)	Alanine concn. (μmole/100 ml.)
Arterial	4	25	87	59
	65	25	92	91
	91	25	102	136
Venous	0–8	30	84	73
	8–21	30	102	103
	21–34	30	186	169
	34–48	30	161	160
	48–65	30	145	186
	65–95	30	130	189

TABLE 4. Blood glutamic acid and alanine concentrations after introduction of 0·15% glutamic acid solution into intestinal lumen

Sample	Collected at (min)	Volume (ml.)	Glutamic acid concn. (μmole/100 ml.)	Alanine concn. (μmole/100 ml.)
Arterial	2	25	120	110
	51	25	102	123
	90	25	127	197
Venous	0–8	30	99	119
	8–17	30	99	112
	17–26	30	99	139
	26–36	30	98	153
	36–45	30	—	—
	45–55	30	85	153
	55–64	30	69	182
	64–76	30	87	177
	76–90	30	76	182

concentrations of glutamic acid cannot easily be ascertained, but the results clearly indicate that the increase of the venous alanine is greater when higher concentrations of glutamic acid are used. It should be noted that the absolute venous alanine concentration never rose above about 200μmole/100 ml.

Tables 5 and 6 show the venous aspartic acid, glutamic acid and alanine concentrations when 0·5 and 0·15% aspartic acid solutions are introduced into the intestinal lumen. With both these concentrations there is an increase of the venous aspartic acid concentration, more marked with the 0·5% than with the 0·15% solution. The increase of venous alanine concentration is much the same as that of venous aspartic acid, and is comparable with the increase of

TABLE 5. Blood aspartic acid, glutamic acid and alanine concentrations after introduction of 0·5% aspartic acid solution into intestinal lumen

Sample	Collected at (min)	Volume (ml.)	Aspartic acid concn. (μmole/100 ml.)	Glutamic acid concn. (μmole/100 ml.)	Alanine concn. (μmole/100 ml.)
Arterial	5	25	5	108	79
	47	25	20	114	87
	77	25	12	99	102
Venous	0–9	30	0	93	82
	9–17	30	38	91	92
	17–25	30	21	84	123
	25–33	30	100	86	125
	33–41	30	83	96	151
	41–51	30	35	81	164
	51–64	30	42	92	181
	64–80	30	26	79	174

TABLE 6. Blood aspartic acid, glutamic acid and alanine concentrations after introduction of 0·15% aspartic acid solution into intestinal lumen

Sample	Collected at (min)	Volume (ml.)	Aspartic acid concn. (μmole/100 ml.)	Glutamic acid concn. (μmole/100 ml.)	Alanine concn. (μmole/100 ml.)
Arterial	4	25	0	83	74
	53	25	1	78	102
	82	25	7	83	118
Venous	0–6	30	0	65	92
	6–14	30	8	65	95
	14–22	30	25	71	124
	22–31	30	39	74	132
	31–40	30	26	71	135
	40–50	30	0	56	138
	50–62	30	6	53	138
	62–69	30	0	25	121
	69–76	30	6	31	117
	76–84	30	0	38	135

TABLE 7. Blood glutamic acid and alanine concentrations after introduction of isotonic saline solution into intestinal lumen

Sample	Collected at (min)	Volume (ml.)	Glutamic acid concn. (μmole/100 ml.)	Alanine concn. (μmole/100 ml.)
Arterial	5	25	101	56
	48	25	91	84
	73	25	91	98
	87	25	98	136
Venous	0–7	30	98	99
	7–18	45	91	86
	18–30	45	72	73
	30–45	45	77	88
	45–61	30	63	100
	61–87	30	69	118

the alanine concentration when corresponding concentrations of glutamic acid are used. There is little change in the venous glutamic acid concentration when these concentrations of aspartic acid are being absorbed. In control experiments with 10 ml. of isotonic saline in the intestinal lumen (Table 7), the venous glutamic acid and alanine concentrations both decrease temporarily.

It was found that about 95% of the amino acid introduced into the intestinal lumen disappeared during the course of the experiment, except in the case of 10% glutamic acid, when about 35% disappeared.

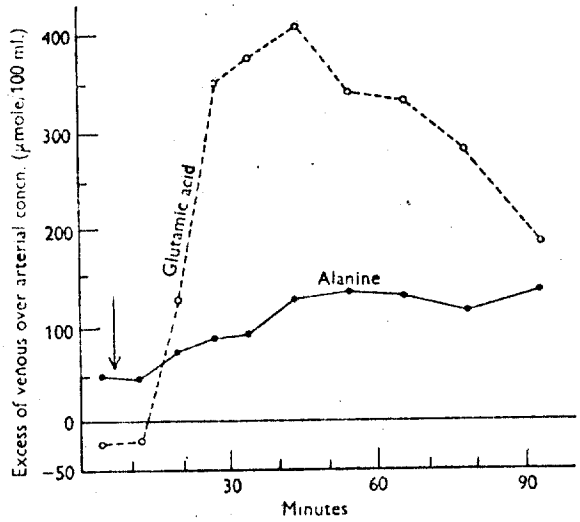

Fig. 1. 10 ml. 2% glutamic acid solution introduced into lumen of experimental portion of intestine at the time indicated by the arrow. Above zero on the ordinate indicates by what amount the amino acid concentration in the venous sample from the experimental portion of intestine was greater than that in the arterial; values below zero on the ordinate arise when the venous sample has a lower concentration than the arterial.

As the concentrations of the amino acids (particularly that of alanine) alter in the arterial blood, it is essential to examine their concentrations from the point of view of any changes which may occur between artery and vein, and thus to obtain figures which are proportional to the amount of amino acid being absorbed. The actual increase or decrease of amino acid concentration in the blood passing through the intestine (i.e. venous concentration minus arterial concentration) and how this increase or decrease varies during the absorption of the amino acid solution is shown in Figs. 1 and 2 for a 2 and 0·15% glutamic acid solution respectively, and in Fig. 3 for a 0·5% aspartic acid solution: each figure represents one experiment.

When a 10% glutamic acid solution is used the difference in glutamic acid

concentration between artery and vein continues to increase during the experimental period of 90 min and rises to a value of over 500 μmole/100 ml.; the difference in alanine concentration between artery and vein is almost exactly as that shown in Fig. 1. During the absorption of a 0·5% glutamic acid solution the arterio-venous differences in glutamic acid and alanine concentrations are between those found during the absorption of a 2% and a 0·15% glutamic acid solution. The arterio-venous differences in glutamic

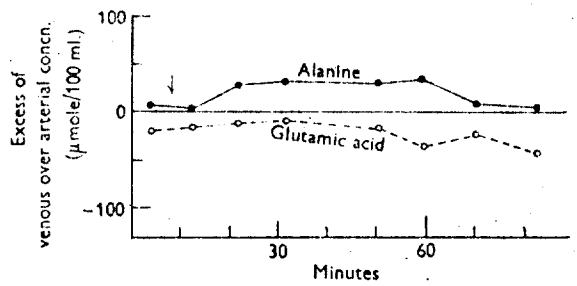

Fig. 2. 10 ml. 0·15% glutamic acid solution introduced into lumen of experimental portion of intestine at the time indicated by the arrow.

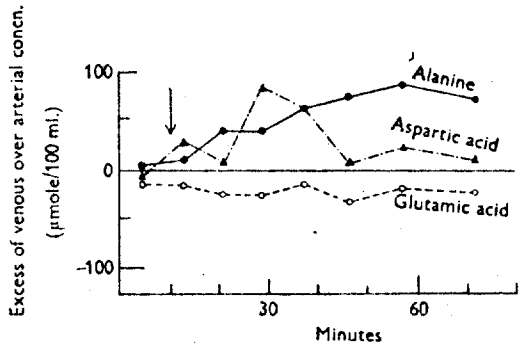

Fig. 3. 10 ml. 0·5% aspartic acid solution introduced into lumen of experimental portion of intestine at the time indicated by the arrow.

acid and alanine concentrations during the absorption of 10 ml. isotonic saline show a fall, and throughout the experiment the venous alanine concentration remains above the arterial, while the venous glutamic acid concentration remains below. Fig. 2 shows that during the absorption of a 0·15% glutamic acid solution, and as was found during the absorption of isotonic saline, the venous alanine concentration remains above, and the venous glutamic acid concentration below, the respective arterial concentrations. Fig. 3 shows the arterio-venous differences in the concentrations of aspartic acid, glutamic acid and alanine when a 0·5% aspartic acid solution is being absorbed.

DISCUSSION

The results indicate that when glutamic acid is absorbed from the small intestine of the dog, glutamic acid appears in the mesenteric venous blood together with an increased concentration of alanine. The concentration of alanine in the mesenteric venous blood seems to have a maximum value in the region of 200 μmole/100 ml. (Tables 1–4). This finding of a maximum concentration agrees well with a similar observation of Klingmüller, Gayer & Barmstedt (1955). They found that after intravenous injection of glutamate into rabbits the blood concentration of alanine rose, and reached a maximum which did not depend on the amount of glutamate administered.

With the higher concentrations of glutamic acid (i.e. 2% or greater) in the intestinal lumen, most of the glutamic acid is absorbed as such, and the alanine production is relatively low. However, when lower concentrations of glutamic acid are introduced into the intestinal lumen the glutamic acid increase in the venous blood is then considerably lower and the alanine production is more obvious.

During the absorption of aspartic acid the change in venous alanine concentration is comparable with that which occurs when glutamic acid is being absorbed. But even when 0·15% aspartic acid solution is used an appreciable amount of aspartic acid is absorbed as such.

It would appear from the above findings that when proteins are being digested and amino acids liberated in relatively low concentrations in the intestinal lumen the transamination of almost all the glutamic and aspartic acids results in an increase in the concentration of alanine in the mesenteric veins.

Lastly, it is interesting to note that during the short period before introducing the glutamic acid into the lumen, the glutamic acid concentration is lower in the vein than in the artery, while the alanine concentration is higher, suggesting that the intestine transaminates not only the glutamic acid being absorbed from the lumen, but also that in the blood perfusing it.

SUMMARY

1. The absorption of glutamic acid and aspartic acid by the small intestine of the dog has been studied *in vivo*.

2. Glutamic and aspartic acids are partly absorbed as such and partly transaminated during absorption, resulting in the appearance of alanine in the mesenteric venous blood.

3. When high concentrations of glutamic acid are present in the intestinal lumen most of it enters the blood as glutamic acid; with low concentrations most of it is transaminated before reaching the blood.

4. With low concentrations of aspartic acid more is absorbed as such than is the case with glutamic acid.

5. The small intestine transaminates the glutamic acid in the blood passing through it.

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THE ALANINE AND OXO ACID CONCENTRATIONS IN MESENTERIC BLOOD DURING THE ABSORPTION OF L-GLUTAMIC ACID BY THE SMALL INTESTINE OF THE DOG, CAT AND RABBIT *IN VIVO*

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It has been shown by Neame & Wiseman (1957) that the absorption of L-glutamic acid and aspartic acid by the dog's small intestine *in vivo* results in an increased alanine concentration in the blood passing through the intestine, and their findings suggest that when protein is being digested and glutamic acid liberated in relatively low concentration in the intestinal lumen almost all the glutamic acid is involved in the transamination reaction. These observations explain the finding of a lower level of glutamic acid than was expected in the portal blood after feeding casein to dogs (Dent & Schilling, 1949) and an examination of their chromatogram shows an unexpected increase in blood alanine.

The study of transamination by the small intestine *in vivo* has now been carried further and the experiments described below show that the phenomenon also occurs in the cat and rabbit. The mesenteric blood concentrations of pyruvate and α-oxoglutarate have also been investigated during the absorption of glutamic acid by the dog, cat and rabbit, but no significant relationship was found between any changes in their concentrations and the transamination that was occurring.

METHODS

Experimental procedure

Dogs weighing between 8 and 22 kg and cats and rabbits weighing between 1·6 and 3·0 kg were used, and were allowed only water during the 24 hr before the experiment. Pentobarbitone sodium B.P. was used as the anaesthetic, the dogs receiving about 50 mg/kg body weight (intravenously), the cats about 50 mg/kg body weight (intraperitoneally), and the rabbits about 60 mg/kg body weight (intravenously). Artificial respiration via an intratracheal cannula was maintained throughout all experiments by means of an 'Ideal' pump (C. F. Palmer, Ltd., London) and amounted to about 0·5 l./kg body weight/min in the case of dogs, about 0·6 l./kg body weight/min in the case of cats, and about 0·7 l./kg body weight/min for rabbits. The artificial respiration was begun as soon as possible after anaesthesia had been induced.

The abdomen was opened by a mid-line incision, and in readiness for the taking of blood from the mesenteric venous trunk the latter was located and carefully cleared distal to its junction with the gastrosplenic vein. The mesenteric artery was then cleared and a loose thread ligature

placed around it ready for ligation towards the end of the experiment (see below). The abdominal aorta was next cleared ready for the taking of an arterial blood sample. Then the small intestine was ligated in all three animals at about the middle of the duodenum, this being the approximate dividing line between blood draining into the gastrosplenic vein and blood draining into the superior mesenteric vein. The small intestine was further ligated just proximal to the ileocaecal junction in the case of the dog and cat, and in the case of the rabbit at a point level with the tip of the appendix, which lies in conjunction with the lower end of the ileum in this animal. A cannula was introduced into each end of the experimental portion of the small intestine (the part between the ligatures) and isotonic saline (at 37° C) was used to wash out the intestinal lumen. Any residual saline was then gently expelled from the intestinal lumen, a ligature was tied around each end of the experimental portion of intestine so as to exclude the cannulae, and the cannulae were then removed.

In the dog and cat the mesenteric venous blood collected comes from the small and large intestine, though mainly from the small intestine. In the rabbit, however, a large part of this mixed blood is derived from the large intestine, which, in this animal, has a relatively large bulk, and therefore the blood from the experimental portion of intestine may be considerably diluted by blood from the large intestine. In view of this, two series of experiments were carried out on the rabbit. In one series both the large and small intestine were left *in situ*, while in the other series that part of the intestine distal to the experimental portion of small intestine was removed immediately after opening the abdomen. In practice it was found necessary to leave *in situ* a short length of the colon, some of which has a mesentery in common with part of the small intestine.

In all except control experiments a neutralized 2% L-glutamic acid solution (6–10 ml./kg body weight) at 37° C was introduced into the upper end of the lumen of the experimental portion of intestine and gently massaged down the intestine so as to distribute it evenly. The abdominal wall was then closed after a thermometer had been inserted, and the animal left undisturbed for 30 min. At the end of this period the abdomen was reopened, the closed tip of a pair of Doyen's scissors passed under the portion of mesenteric vein previously cleared, and the vein raised on the blunt edge of the scissors so as to obstruct any back-flow of blood along the mesenteric vein during collection of the venous sample. The blood was drawn from the mesenteric vein into a syringe moistened with heparin solution. When sufficient had been obtained (10–20 ml.) the vein was clamped and the ligature round the mesenteric artery tied to prevent loss of blood into the intestinal vessels (although this was not necessary in the case of the dog). About 1 ml. of the blood taken (to be used for oxo acid estimation) was transferred rapidly into each of three 15 ml. weighed centrifuge tubes, each containing 3 ml. of distilled water and 4 ml. of 10% trichloroacetic acid. The exact volume of blood added to each tube was determined by weighing. The remainder of the venous sample (to be used for glutamic acid and alanine estimations) was not deproteinized until after an arterial blood sample had been taken from the abdominal aorta, and after the experimental portion of intestine had been removed and drained of its contents. The time interval between the taking of the mesenteric venous sample and the arterial sample was usually 1·5–2 min and never more than 2·5 min. In view of the instability of pyruvate in freshly drawn blood (Bueding & Wortis, 1940; Long, 1941) the exact time interval between the venepuncture and the deproteinization of the venous blood was noted; the time between the taking and the deproteinization of the arterial blood was made to correspond with this interval in order to compensate for any losses in the pyruvate between the drawing of the blood and its deproteinization. This time interval was usually between 1 and 1·5 min and never more than 2 min.

In control experiments the same procedure was carried out, except that the small intestine was left empty after the initial washing out with saline.

Chemical methods

Amino acid solutions. The L-glutamic acid was a commercial sample (L. Light and Co., Ltd.) used without further purification, and gave the theoretical yield of gas on decarboxylation. It was

dissolved in distilled water with the aid of NaOH and the final solution was adjusted to about pH 6 (chlorophenol red as indicator).

Treatment of blood samples. The initial treatment of the blood samples for the estimation of oxo acids has been described above. After weighing the tubes to which the blood had been added, the deproteinized blood in each tube was thoroughly mixed, centrifuged at 2500 r.p.m. for 10 min and the supernatant filtered. Blood for glutamic acid and alanine estimation was well stirred and deproteinized as described by Neame & Wiseman (1957).

Recovery of residual amino acids from the intestinal lumen. At the end of each experiment the experimental portion of intestine was drained and washed out with isotonic saline. A sample was deproteinized and its glutamic acid content estimated as described by Neame & Wiseman (1957).

Estimation of amino acids. L-Glutamic acid was estimated by the manometric method of Gale (1945) and alanine by the colorimetric method of Alexander & Seligman (1945) as modified by Christensen, Riggs & Ray (1952).

Estimation of oxo acids. Pyruvic acid and α-oxoglutaric acid were estimated by the method of Goodwin & Williams (1952) with certain modifications. 6 ml. of each blood filtrate was pipetted into a 50 ml. separating funnel, which was then brought to 25° C in a water-bath (10 min being needed for this purpose). 1 ml. of 0·1% (w/v) 2:4-dinitrophenylhydrazine in 2 N-HCl was added and the separating funnel allowed to stand at room temperature for 25 min. The 2:4-dinitrophenylhydrazones of the oxo acids were then extracted with 4 ml. of diethyl ether by shaking for 1 min. The two layers were allowed to separate and the aqueous (lower) layer discarded. In some cases the ethereal layer was not clear but, after draining off most of the aqueous layer, clearing could be induced by giving two or three firm shakes to the separating funnel. The ethereal layer was extracted with 4 ml. of 15% (w/v) Na_2CO_3 by shaking for 1 min and the Na_2CO_3 layer transferred to a test-tube by means of a Pasteur pipette. 2 ml. of this was treated with 1 ml. of 3 N-NaOH and the optical density was estimated in a Hilger Uvispek spectrophotometer at 414 mμ (isosbestic point) and at 443 mμ (maximal absorption of pyruvic acid 2:4-dinitrophenylhydrazone). Standard solutions of pyruvate and α-oxoglutarate were taken through this entire procedure and all oxo acid estimations carried out on the same day as the experiment. The values for total oxo acid concentrations obtained from replicate samples were usually within 1%.

Paper chromatography of oxo acids. The remainder of each Na_2CO_3 extract was acidified by the dropwise addition of conc. HCl until effervescence no longer occurred and the yellow colour of the solution had become pale. The 2:4-dinitrophenylhydrazones were then extracted with 4 ml. of diethyl ether and the ethereal layer transferred to a shallow tube, evaporated to dryness *in vacuo* at room temperature, and stored at −20° C until required for chromatography. (Evaporation to dryness is effected more rapidly if the last stages of the acidification are carried out carefully with dilute HCl.) The 2:4-dinitrophenylhydrazones were dissolved in about 0·5 ml. of diethyl ether, and 0·1 ml. applied to Whatman No. 1 filter paper and dried in a stream of warm air. Ascending chromatograms were run for 36 hr using butanol saturated with N-NH_4OH as solvent (Cavallini, Frontali & Toschi, 1949).

Oxo acid standards. Commercial samples (L. Light and Co. Ltd.) of sodium pyruvate and α-oxoglutaric acid were used as standards without further purification. The purity of the sodium pyruvate was found to be 81% by the manometric method described by Umbreit, Burris & Stauffer (1949). The purity of the α-oxoglutaric acid was assessed by the manometric method of Krebs (1950), and found to be 97%.

Identification of parts drained by the vein used for venepuncture

The animals used for this purpose were killed by an overdose of pentobarbitone sodium B.P. administered as described previously. The abdomen was opened, the mesenteric vein identified and a loose ligature tied around it. The vascular system of the intestine was washed out with isotonic saline introduced into the aorta or mesenteric artery (using a pressure of about 140 mm Hg), depending upon the size of the animal. A cannula connected to a pressure bottle containing blue Neoprene Latex (B. B. Chemical Co., Ltd., Leicester) from which all air bubbles were care-

fully excluded, was then inserted into the portal vein as near the liver as possible. A pressure of about 20 mm Hg was then exerted on the Neoprene Latex to induce passage into the intestinal vessels. As soon as the Neoprene Latex had reached the antimesenteric side of the intestine the pressure was released, a ligature tied around the portal vein distal to the tip of the cannula, and the cannula removed. The stomach, intestine and spleen were then removed *in toto* and placed in a 5% solution of formaldehyde acidified with HCl (the pH being about 2). After 2–4 hr the specimen can be dissected and the vessels exposed and examined. The site used for venepuncture for the blood samples during an absorption experiment is easily identified.

RESULTS

The results show that during the absorption of glutamic acid by the small intestine of the dog, cat and rabbit there is an increase in the concentration of alanine in the mesenteric blood as it passes through the small intestine. The phenomenon can be easily demonstrated in the dog and cat even when the large intestine is left *in situ* (as is shown by the results in Tables 1 and 2). In the case of the rabbit, however, when the entire intestine is left *in situ* the difference between the alanine concentration in the arterial and the mesenteric venous blood during the absorption of glutamic acid shows either no change (Table 3; Expts. 1–3 and 6) or a definite rise (Table 3; Expts. 4, 5 and 7). However, when the large intestine of the rabbit is removed before the experimental period has begun the mesenteric venous blood shows a much greater

TABLE 1. Alanine, pyruvate and α-oxoglutarate concentrations in arterial (Art.) and mesenteric venous (Mes.) blood taken 30 min after the introduction of 2% L-glutamic acid solution into the lumen of the small intestine of the dog. (Small and large intestine *in situ*. In the control experiments the small intestine was left empty.)

	Alanine (μmole/100 ml.)		Pyruvate (μmole/100 ml.)		α-Oxoglutarate (μmole/100 ml.)		Glutamic acid absorbed		
Expt. no.	Art.	Mes.	Art.	Mes.	Art.	Mes.	(μmole)	(μmole/kg body wt.)	% of amount introduced
1	133	151	43	41	0	0	4649	580	87
2	56	79	22	22	4	5	11430	820	65
3	79	113	23	23	2	1	9250	840	69
Control 1	75	66	41	37	1	1	—	—	—
Control 2	42	46	9	9	0	0	—	—	—

TABLE 2. Alanine, pyruvate and α-oxoglutarate concentrations in arterial (Art.) and mesenteric venous (Mes.) blood taken 30 min after the introduction of 2% L-glutamic acid solution into the lumen of the small intestine of the cat. (Small and large intestine *in situ*. In the control experiments the small intestine was left empty.)

	Alanine (μmole/100 ml.)		Pyruvate (μmole/100 ml.)		α-Oxoglutarate (μmole/100 ml.)		Glutamic acid absorbed		
Expt. no.	Art.	Mes.	Art.	Mes.	Art.	Mes.	(μmole)	(μmole/kg body wt.)	% of amount introduced
1	78	124	19	26	5	5	1403	830	94
2	108	169	21	24	2	2	2320	800	86
3	25	51	10	11	1	1	1706	1070	95
Control 1	47	40	14	12	1	1	—	—	—
Control 2	64	63	15	16	1	2	—	—	—

alanine concentration than the arterial blood during the absorption of glutamic acid (Table 4). The reason for this variability in the experimental findings in the case of the rabbit when the entire intestine is left *in situ* is that the contribution of venous drainage from the large intestine to the common mesenteric vein is so much greater in the rabbit than in the dog or cat. Determination of the parts of the intestine drained by the vein from which venous blood samples were collected showed that in the dog and cat the vein used for venepuncture drained mainly the small intestine, and only a small amount of blood came from the large intestine. In the rabbit, however, where the large intestine comprises much of the bulk of the total intestine, the venous drainage from the small intestine appeared to be only slightly greater than the venous drainage from the large intestine. In the experiments carried out on the rabbit after the removal of the large intestine, blood from the small intestine was no longer diluted by blood from the large intestine. A rise in alanine concentration in the blood passing through the intestine could then be clearly demonstrated. The results with the rabbit show the importance of determining the area drained by the common mesenteric vein in this type of experiment.

TABLE 3. Alanine, pyruvate and α-oxoglutarate concentrations in arterial (Art.) and mesenteric venous (Mes.) blood taken 30 min after the introduction of 2% L-glutamic acid solution into the lumen of the small intestine of the rabbit. (Small and large intestine *in situ*. In the control experiment the small intestine was left empty)

	Alanine (μmole/100 ml.)		Pyruvate (μmole/100 ml.)		α-Oxoglutarate (μmole/100 ml.)		Glutamic acid absorbed		
Expt. no.	Art.	Mes.	Art.	Mes.	Art.	Mes.	(μmole)	(μmole/kg body wt.)	% of amount introduced
1	126	124	66	34	3	5	1090	450	41
2	119	119	53	46	2	3	998	590	38
3	92	99	37	33	2	2	1450	630	52
4	130	147	50	40	7	6	1440	480	43
5	135	163	65	48	2	3	1600	670	52
6	154	159	62	49	3	5	1260	440	37
7	131	181	68	55	2	2	1690	840	62
Control	99	90	53	37	2	1	—	—	—

TABLE 4. Alanine, pyruvate and α-oxoglutarate concentrations in arterial (Art.) and mesenteric venous (Mes.) blood taken 30 min after the introduction of 2% L-glutamic acid solution into the lumen of the small intestine of the rabbit. (Only small intestine *in situ*. In the control experiments the small intestine was left empty)

	Alanine (μmole/100 ml.)		Pyruvate (μmole/100 ml.)		α-Oxoglutarate (μmole/100 ml.)		Glutamic acid absorbed		
Expt. no.	Art.	Mes.	Art.	Mes.	Art.	Mes.	(μmole)	(μmole/kg body wt.)	% of amount introduced
1	104	153	36	30	10	11	935	580	42
2	217	286	87	57	9	6	2350	940	67
3	208	297	45	25	9	10	1910	710	60
4	153	230	67	41	7	13	1460	660	50
Control 1	127	124	76	53	4	3	—	—	—
Control 2	125	114	40	32	4	4	—	—	—

In control experiments with the small intestine empty there was no appreciable difference in the alanine concentration between the arterial blood and mesenteric venous blood.

In some of the experiments on dogs, cats and rabbits in which the whole intestine was left *in situ*, glutamic acid was determined in both arterial and venous blood at the end of the experimental period. This was not possible in experiments on rabbits with the large intestine removed, owing to insufficient blood. In the dog the mesenteric venous concentration of glutamic acid was from 25 to 83 μmole/100 ml. more than that in the arterial blood. With the cat the mesenteric venous concentration was from 57 to 144 μmole/100 ml. higher than the arterial level. In both these animals the arterial concentration of glutamic acid at the end of the experiments was about 140 μmole/100 ml., compared with a value of about 90 μmole/100 ml. in the control experiments. In the rabbit with the whole intestine *in situ* there was no significant difference between the arterial and mesenteric venous concentrations of glutamic acid at the end of the experimental period. These results show that, with the amounts of glutamic acid introduced into the lumen of the intestine in these experiments, some glutamic acid does appear in the blood passing through the intestine of the dog and cat.

As the glutamic acid left in the experimental portion of intestine at the end of the experimental period was determined, the amount which had disappeared from the lumen during this period could be calculated. In the case of the dog it was found that an average of 74% of the glutamic acid introduced had disappeared by the end of 30 min; in the cat an average of 92% disappeared, and in the rabbit an average of 49% disappeared.

The concentrations of pyruvate and α-oxoglutarate in the mesenteric venous blood from the experimental portion of intestine were not appreciably different from their concentrations in the arterial blood, except in the case of the rabbit, where the arterial pyruvate concentration was higher than the mesenteric venous concentration under both experimental and control conditions.

The oxo acid 2:4-dinitrophenylhydrazones derived from the blood samples collected at the end of the experimental periods were examined by one-dimensional paper chromatography. It was found that only pyruvate and α-oxoglutarate were present in arterial and venous blood collected both from control loops of intestine and from intestine absorbing glutamic acid.

DISCUSSION

The results show that when glutamic acid is absorbed from the small intestine of the dog, cat and rabbit, a rise in the concentration of alanine occurs in the blood passing through the intestine. This is clearly demonstrable in the case of the cat and the dog in spite of dilution of the blood from the small intestine

with blood from the large intestine. In the rabbit, however, the difference between the concentration of alanine in the mesenteric venous blood and in the arterial blood varies considerably from one experiment to another when the whole intestine is left *in situ*. This is probably due to a variable dilution of the blood from the small intestine by the comparatively large volume of blood flowing from the large intestine. During glutamic acid absorption in the rabbit with the large intestine removed the mesenteric venous concentration of alanine was always considerably higher than the arterial concentration and the rise in alanine content of the blood was greater than in the experiments with the dog and cat.

The concentrations of pyruvate and α-oxoglutarate in the blood passing through the intestine are unchanged by the transamination that occurs after introducing glutamic acid into the intestinal lumen. A consideration of the transamination reaction suggests that the rise in alanine concentration in the venous blood would be accompanied by an increase in α-oxoglutarate concentration and a concomitant fall in the pyruvate concentration, but this was not supported by the experimental results. A fall in venous pyruvate concentration occurred in the rabbit only and was unrelated to the rise in alanine concentration, since it occurred even in control experiments when no glutamic acid was introduced into the intestinal lumen.

From these results it would appear that the normal metabolism of the intestinal cells overshadows or compensates for any changes in the oxo acid concentrations of the blood brought about as a result of the transamination of the absorbed glutamic acid. The alanine added to the blood during its passage through the glutamic acid-absorbing intestine is usually considerably more than the amount of pyruvate found in the arterial blood supplying the intestine and must come from the general metabolism of the intestinal cells. If all the α-oxoglutarate produced as a result of the transamination appeared in the mesenteric venous blood its concentration would be increased about tenfold. The site of the transamination in the intestine is as yet undetermined, but the most likely position would be in the mucosal epithelial lining.

It is stimulating to find that a phenomenon first discovered from *in vitro* experiments on the rat intestine (Matthews & Wiseman, 1953) is now shown to occur *in vivo* in the dog (Neame & Wiseman, 1957), cat and rabbit.

SUMMARY

1. The absorption of L-glutamic acid by the small intestine of the dog, cat and rabbit has been studied *in vivo*.

2. The absorption of glutamic acid is accompanied by a rise in the concentration of alanine in the blood passing through the intestine in all three animals. In the rabbit this can only be demonstrated consistently after removal of the large intestine.

3. The concentrations of pyruvate and α-oxoglutarate in the blood passing through the intestine in the dog and cat remain unaffected by the absorption of glutamic acid and the transamination involved.

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OCCULT MECHANISMS OF BRAIN DYSFUNCTION

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It is clear from Dr. Millichap's presentation that important advances in the diagnosis and treatment of seizure disorders and minimal brain dysfunction syndromes have been realized in recent years. Unfortunately, significant progress toward the companion goal of prevention remains seriously hampered by our poor understanding of the etiology and pathogenesis of these conditions. Genetic factors undoubtedly play at least a predispositional role in many cases, but the potential contribution of an adverse environment deserves constant emphasis, particularly in that corrective measures are more easily brought to bear upon the environment than upon the gene. Dr. Nair, in discussing the toxic effects of drugs upon the developing CNS, touched upon an important theme worth reiterating here; that, in contrast to the gross malformations which characteristically result from exposure to toxic agents in early stages of development, exceedingly subtle forms of brain damage capable of defying current methods of detection may occur from exposure in later developmental stages. Dr. Millichap indicated, and I think many would agree, that the more carefully one examines children with minimal brain dysfunction, the more convinced one becomes that some sort of subtle brain damage underlies these conditions. Observations similar to those of Drs. Nair and Millichap led me to begin studying mechanisms which might inconspicuously give rise to brain damage in relatively late stages of development. I shall confine this discussion to certain subtle types of brain damage which we and others have been able to induce in experimental animals by exposing infants or late-gestation fetuses to select chemicals which are not uncommonly encountered by the immature human under ordinary circumstances. Implicit in the title "Occult Mechanisms of Brain Dysfunction" is the suggestion that the neurotoxic mechanisms we have been

examining could underly human brain dysfunction syndromes without our being aware of it; whether they do, in fact, contribute to brain dysfunction in the human is an open question which has not been adequately explored.

The first compound I shall discuss, glutamic acid (Glu), occurs in the ordinary diet as a natural constituent of protein. The sodium salt, monosodium glutamate (MSG) is an extensively used food additive. Fifteen years ago, Lucas and Newhouse (1957) reported that retinal ganglion cells rapidly degenerate following subcutaneous administration of MSG to infant mice - a surprising finding in view of the high concentrations of Glu normally present throughout the mammalian CNS, including the retina.

From a series of more recent investigations, it is now known that certain populations of nerve cells in the developing brain are also destroyed by MSG administration (Olney, 1969; Olney, 1971a; Burde et al., 1971; Abraham et al. 1971; Everly, 1971; Murakami and Inouye, 1971; Johnston and Reynolds, 1972); that oral doses in the range of 0.5 to 1 mg/g body wt are effective in producing such damage (Olney and Ho, 1970; Olney et al., 1972a; Burde et al., 1971; Abraham et al., 1971; Johnston and Reynolds, 1972); that lesion formation is accompanied by selective uptake and accumulation of Glu in the brain areas where neurons are being destroyed (Perez and Olney, 1972); that several animal species, including mice (Olney, 1969), rats (Burde et al., 1971; Everly, 1971), chicks (Snapir, 1971) and rhesus monkeys (Olney and Sharpe, 1969; Olney et al., 1972a) are susceptible to MSG-induced brain damage and, a point to be emphasized for our present purposes, that the neurodegenerative reaction to MSG can occur in the developing brain in the absence of outward signs of distress, yet give rise later in life to

sequelae ranging from obesity and various neuroendocrine disturbances (Olney, 1969; Knittle and Ginsberg-Feller, 1970; Matsuyan, 1970; Redding et al., 1971) to deficits in discrimination learning (Pradhan and Lynch, 1972). Further, unless the brain is examined in the acute stages of the degeneration process it is exceedingly difficult to establish whether brain damage even occurred (Olney, 1971b). Destroyed neurons are phagocytized and degeneration products disposed of so efficiently that there are few traces of the tissue reaction, other than a decreased number of neurons, if examination of the brain is delayed even for a few days (Olney, 1971a).

One of the very intriguing biological properties of Glu is its ability to excite neurons when introduced into their surroundings by the delicate procedure of microelectrophoresis. David R. Curtis and colleagues (Curtis and Watkins, 1960; 1963) clarified the molecular specificity of this effect by demonstrating that a select group of acidic amino acids which are fundamentally similar to Glu in molecular structure, also excite neurons when administered by microelectrophoresis. These compounds (glutamic, aspartic, cysteic, cysteine sulfinic, homocysteic acids and certain of their substituted synthetic congeners) were designated the "neuroexcitatory amino acids." In recent experiments we have found that the glutamate type of neurotoxic reaction can be reproduced in the infant mouse hypothalamus by subcutaneous administration of any one of the "neuroexcitatory amino acids" but not by a variety of other amino acids or non-amino acid compounds (Olney, Ho, Rhee, 1971). This suggests that a similar mechanism may be responsible for both the neuroexcitatory and neurotoxic properties of Glu. When neuroexcitatory amino acids are administered in combination with one another the toxic

reaction is augmented in an additive manner (Fig. 1).

One of the amino acids tested for neurotoxic potential, L-cysteine, requires separate consideration. Doses of L-cysteine in the range of 1 mg/g do not produce the MSG type lesion; instead, a slightly delayed and much more widespread neurodegenerative reaction affecting the thalamus, hippocampus, amygdala and cerebral cortex develops over the course of 24-48 hr. (Olney et al., 1972b). Rats sustaining this type of brain damage from a single dose of L-cysteine in infancy appear grossly normal as adults but perform learning tasks very poorly (Sharpe, Olney, Olendorf and Zimmerman, unpublished). Both cysteine (Olney et al., 1972b) and MSG (Murakami and Inouye, 1971) produce their respective types of brain damage in fetal rodents when the amino acids are administered to the pregnant dam in late gestation. Susceptibility of the primate fetus to transplacentally-induced brain damage has not been studied for either compound.

In brief, available evidence indicates that there are two basic brain damage syndromes (MSG-type and cysteine-type) which can be induced by administering single amino acid loads to immature animals. The acidic (excitatory) amino acids such as glutamic, aspartic, cysteine sulfinic, cysteic and homocysteic acids tend to produce a focal pattern of brain damage (MSG-type) restricted to midline tissue zones bordering on the roof and floor of the third ventricle (Fig. 2). Cysteine, which is neither an excitatory nor acidic amino acid, produces a more diffuse and extensive pattern of brain damage (Fig. 3) which curiously tends to spare the diencephalic periventricular zones affected by acidic amino acids.

The fact that Glu is found in very high concentrations in brain under normal circumstances makes it both enigmatic and of considerable interest

that ingestion of this amino acid can rapidly destroy CNS neurons. An important key to understanding this phenomenon may lie in distinguishing between intracellular and extracellular brain Glu concentrations. It has been shown in microelectrophoretic studies that it is the extracellular and not intracellular application of Glu which excites neurons (Coombs et al., 1955). The same may be true for the neurotoxicity of Glu. While a high intracellular content of Glu is the normal state of health for the central nervous system, relatively slight extracellular concentrations may be a state of emergency - leading first to neuronal excitation (depolarization) and finally, if the extracellular milieu is not rapidly corrected, to neuronal necrosis. Blood brain barriers and homeostatic mechanisms within brain are probably geared toward maintaining the extracellular environment of central neurons relatively free from Glu ion. Assuming that these barriers and mechanisms are underdeveloped in the immature organism, this might explain the greater vulnerability of immature animals to brain damage from exogenous loads of Glu.

In the absence of regulatory restrictions on the use of MSG in foods, children in the United States, including those with seizure disorders and minimal brain dysfunction syndromes, routinely consume commercially processed foods containing added MSG. Some foods intended specifically for infants are supplemented with free amino acid mixtures (protein hydrolysates) which include several of the compounds (glutamic, aspartic and cysteic acids) shown to have additive brain damaging effects on infant animals. The majority of human infants raised in the United States over the past twenty years were weaned on foods containing up to 0.6% added MSG. One small jar (130 g) of baby food supplemented with 0.6% added MSG would expose a three month old

infant weighing 6 kg to an exogenous load of 0.13 g/kg or about 1/4 the oral dose (0.5 g/kg) required to destroy hypothalamic neurons in the infant animal brain. The fact that human infants and children typically exhibit no signs of distress from MSG intake could be dangerously misleading in that infant monkeys, while sustaining brain damage from the ingestion of MSG, also manifest no signs of acute distress (Olney et al., 1972a). It has also been demonstrated in infant monkeys that it requires only a transient and moderate elevation of blood Glu to produce brain damage (Olney et al., 1972a) and in infant mice that vulnerable brain regions rapidly accumulate Glu within minutes after blood concentrations begin to rise (Perez and Olney, 1972). Whether the average human infant can metabolize Glu efficiently enough to prevent dangerous levels from developing in local brain regions following ingestion of MSG-supplemented foods is not known nor is it known to what extent the Glu metabolizing capabilities of some infants or children deviate from the average. Blood Glu curves monitored in human adults following an ingested MSG load (Himwich and Peterson, 1954) are characterized by marked individual variation with some individuals reaching much higher peak values than others. Whether similar studies, if conducted on infants and children, would reveal even more extreme variability and a tendency of some individuals to develop very high peak values from a modest MSG load warrants serious consideration.

Menkes et al. (1962) reported on a family in which five male children, although normal at birth, soon developed severe seizures followed by spastic paraplegia and death by 3 years of age with autopsy findings of

cerebral and cerebellar degeneration. Blood Glu concentrations, although only investigated in two infants, were repeatedly (moderately) elevated. Yoshida et al. (1964) described a child, apparently normal at birth, who soon developed severe mental retardation, cortical atrophy and, despite only slightly elevated blood Glu concentrations, had CSF levels of Glu 4 times higher than normal. Various relatives died in infancy from a similar syndrome but were not examined for blood or CSF abnormalities. Assuming that these were Glu-mediated neurodegenerative syndromes, (no other plausible mechanism was uncovered), it is of interest that a substantial CSF elevation of Glu, when looked for, was found even though resting blood Glu levels were not markedly elevated. This would be consistent with a genetic error expressed primarily as a disturbance within the CNS, perhaps involving an excessive CNS build-up of extracellular Glu with consequent brain degeneration. Worth contemplating is the possibility that such a pathological process occurs more commonly in mild form - for example, manifesting as seizure or minimal brain dysfunction syndromes - but is not identified as a Glu-related process because Glu-tolerance curves and CSF assays for Glu are not obtained on such patients. Adding MSG to the diet, in such cases, might be detrimental.

Years ago Price et al. (1943) reported that oral administration of MSG was beneficial in some cases of epilepsy; however, they described one child who had an increase to approximately three times his ordinary seizure frequency each time he was given MSG. Recently, Dr. M. G. Stemmerman (personal communication) notified me of a young child in his pediatric practice with frequent seizures which respond poorly to ordinary anticonvulsants

but can be controlled by avoiding foods containing added MSG. It is instructive that the connection between MSG and seizures in this patient was only discovered because Dr. Stemmerman was persistent in testing the hypothesis that such a connection might exist. The fact that a Glu mediated mechanism has not been implicated more frequently in the pathogenesis of seizure or minimal brain dysfunction syndromes may signify that such a connection is of rare occurrence. It would be unwise, however, to ignore the alternative possibility that the connection is a common but subtle and cryptic one which, unless suspected and looked for, will simply not be found.

The ability of L-cysteine to produce an extensive pattern of brain damage is of particular interest in that cysteine is a pivotal compound in sulphur amino acid metabolism and errors of amino acid metabolism are frequently associated with neuropsychiatric disturbances. It is not clear whether L-cysteine has neurotoxic properties in its own right or damages brain by intracerebral conversion to another compound; for example, to one of its acidic analogues. The acidic analogues of both cysteine and homocysteine have excitatory and neurotoxic properties (Olney et al. 1972b) so that an accumulation of such analogues in the CNS, particularly in the extracellular compartment, might be expected to produce neuropsychiatric disturbances. Homocystinuria, the second most common error of amino acid metabolism known, may be a case in point. In this condition a block in the enzymatic condensation of homocysteine with serine to form cystathionine results in a deficiency of brain cystathionine and probably an accumulation of homocysteine and related metabolites. The latter, although poorly studied, may be important in the pathogenesis of the neuropsychiatric disturbances

(mental retardation, seizures and schizophrenia-like syndromes) which are common among homocystinurics. Wong et al., (1972), postulating that the deficiency of brain cystathionine gives rise to these disturbances, have advocated treating homocystinurics with massive doses of cysteine (to stimulate alternate pathways of cystathionine synthesis). We would suggest, however, that restricting dietary intake of the homocysteine precursor, methionine, while avoiding massive doses of any sulphur amino acid, may be more rational therapy since there is at least evidence in experimental animals that exogenous loads of cysteine itself and acidic metabolites of cysteine and homocysteine induce brain damage but no evidence that cystathionine deficiency is toxic or requires correction. In that the several acidic (excitatory) amino acids are synergistic in neurotoxic action (Fig. 1), it must also be kept in mind that an endogenous disturbance giving rise to abnormal extracellular accumulations of one such compound in the CNS (for example, an acidic sulphur amino acid accumulating in homocystinuria) might be aggravated by exogenous loads of another such compound (for example, MSG being added to foods).

A brief work should be included about an additional compound, hexachlorophene - an antibacterial soap which is absorbed readily through the skin and for years has been used routinely in 3% solution for total body bathing of human infants. It was recently reported that when mean blood levels of hexachlorophene reach 1.2 μg/ml in rats it produces cystic degeneration of axon tracts in the CNS (Gaines and Kimbrough, 1971); that blood concentrations exceeding 1/2 this level were detected in the human infant after only 5 daily washings with 3% hexachlorophene (Curley et al.,

1971) and that when infant monkeys were bathed daily for 90 days with 3% hexachlorophene they developed mean blood levels of 2.3 μg/ml and CNS examination revealed well developed cystic degeneration up and down the neuroaxis with spinal cord, brain stem, cerebellar and cerebral white matter areas all being involved (Winthrop Laboratories, unpublished; cited in FDA Drug Bulletin, Dec. 1971). Over the spring and summer months of this year (1972) approximately 40 human infants died in France from skin exposure to a talcum powder which, by accident, contained 6% hexachlorophene.

We have begun studying the effects of hexachlorophene on infant rodents and have encountered rather widespread edematous degeneration of the developing myelin sheath (the degree of axonal involvement is uncertain) in various CNS regions, including the developing optic pathways, corticospinal tracts at various CNS levels and the fimbria of the hippocampus (Fig. 4). The CNS changes are sometimes detectable for days or weeks before clinical symptoms appear. This suggests the need for studies to determine whether hexachlorophene exposure in infancy, at a time when myelination and synaptogenesis are in progress has detrimental effects on the axon which, although asymptomatic at time of exposure, might become clinically manifest in later years; for example, in the form of a hyperkinetic syndrome.

Occult processes underly many of the brain dysfunction syndromes encountered in pediatric medicine today. I have drawn attention to several common constituents of the chemical milieu of the developing CNS which have the potential to function as occult neurotoxins in order to emphasize the need for research aimed at clarifying what role, if any, such agents play in the pathogenesis of human brain dysfunction syndromes.

Renee: most recent supergiant demonstration 5482

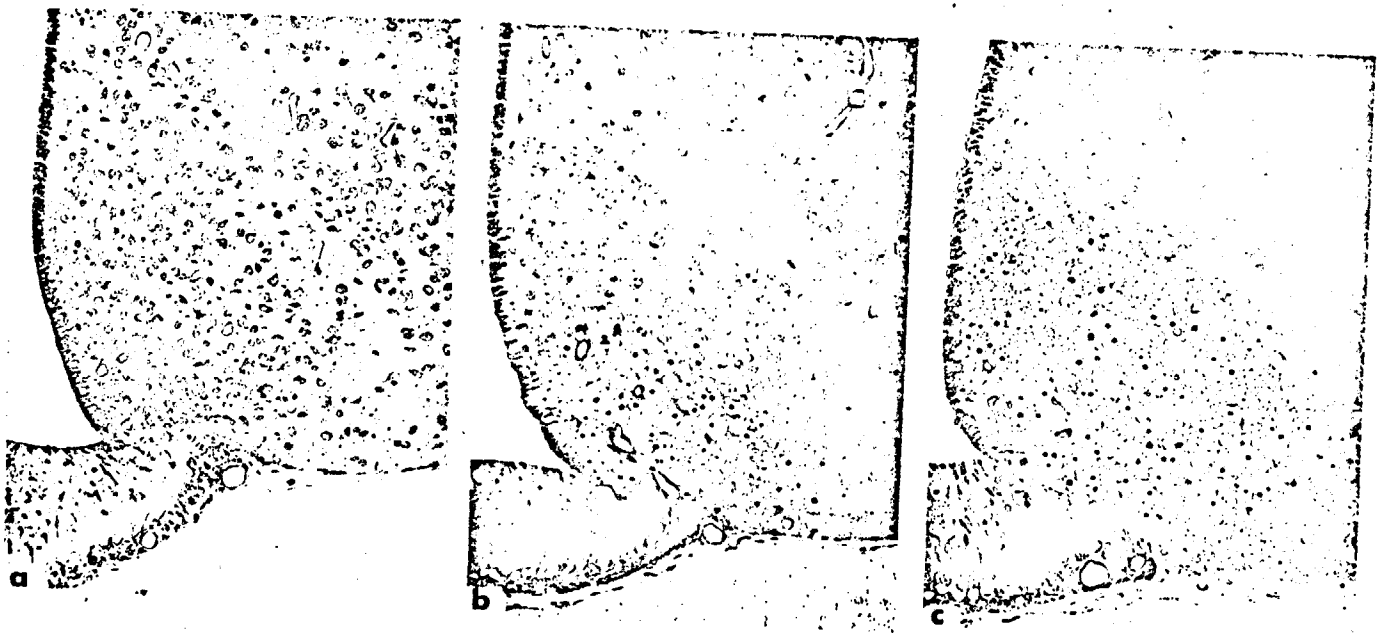
Fig. 1 - Sections through the arcuate nucleus of the 10 day old mouse hypothalamus, 3 hours following different treatment regimens, all given subcutaneously. a. (1) L-MSG, 0.75 mmol/kg. The arcuate nucleus appears normal. When the sodium salts of (2) L-aspartic, (3) cysteic or (4) cysteine sulfinic acids are given in the same dosage (0.75 mmol/kg) there is also no reaction in the arcuate nucleus. b. A combination of the above 4 agents, each at a dose of 0.75 mmol/kg (total dose of acidic amino acids = 3.0 mmol/kg). The small lesion in the arcuate nucleus is approximately the same size any of the 4 agents given separately at 3.0 mmol/kg would produce. c. A combination of the above 4 agents each at a dose of 3 mmol/kg (total dose of acidic amino acids = 12 mmol/kg). The large lesion involving essentially the entire arcuate nucleus is similar in size to the lesion which any of the agents would produce if given separately at 12 mmol/kg. Note that doses are expressed in mmol/kg for purposes of the comparisons being made in this experiment rather than mg/g as is used elsewhere in this paper and in the literature. Histology as described elsewhere (Olney, 1971a); Mag. about 100X.

Fig. 2 - Coronal section of 10 day old mouse brain 3 hours following a large subcutaneous dose (4 mg/g) of cysteine sulfinic acid. Areas surrounding the roof and floor of the third ventricle stain lightly because of the edematous lesion selectively affecting these regions. This is the pattern of damage produced by any of the neuroexcitatory amino acids. When smaller doses are given the lesion tends to affect only the arcuate nucleus (See Figs. 1b and c). This is a maximal MSG-type lesion. Infant mice often convulse and die when the lesion begins spreading in this fashion to include all midline areas bordering on the roof of the third ventricle between the psalterium rostrally and the superior colliculus caudally. Histological procedures as described in Olney (1971a); Mag. about 10X.

Fig. 3 - Coronal section of a 9 day old mouse brain 24 hours following a 1 mg/g dose of L-cysteine. Light areas about the amygdala, cerebral cortex, hippocampus and thalamus are those typically affected by L-cysteine. Although the infant survived treatment for 24 hours while the lesion was developing, this is a severe degree of brain damage from which death might have ensued later. Some infants treated with similar doses of L-cysteine, particularly younger infants, sustain milder degrees of brain damage but the pattern is the same. Neither the habenular nor arcuate nuclei at the roof and floor of the third ventricle respectively is affected, in contrast to the MSG-type lesion (Fig. 2) which localizes about these regions. Histological procedures as described in Olney (1971a); Mag. about 10X.

Fig. 4 - a. Fimbria of hippocampus from 19 day old control mouse following 8 daily subcutaneous injections of olive oil (the vehicle used to deliver hexachlorophene in Fig. 4b). There are no pathological changes. b. Fimbria of hippocampus from 19 day old mouse following 8 daily subcutaneous injections of hexachlorophene (25 mg/kg). The animal was asymptomatic despite rather marked edematous changes in structures of the fimbria which preliminary electron microscopic examination revealed to be grossly dilated myelin-bound compartments, the contents of which require further study to determine the extent of axonal involvement. Histology as described elsewhere in Olney (1971a); Mag. about 100X.

Drugs and developing brain
occult mechanisms of brain dysfunction
John W. Olney
Fig. 1



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Drugs and Developing Brain
Occult Mechanisms of Brain Dysfunction

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Fig 2

Drugs and Developing Brain
Occult Mechanisms of Brain Dysfunction

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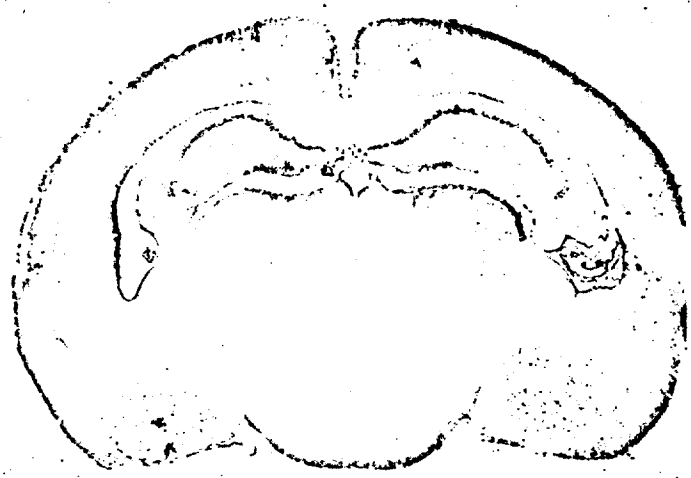
Fig 3



Drugs and the Developing Brain
Occult Mechanisms of Brain Dysfunction
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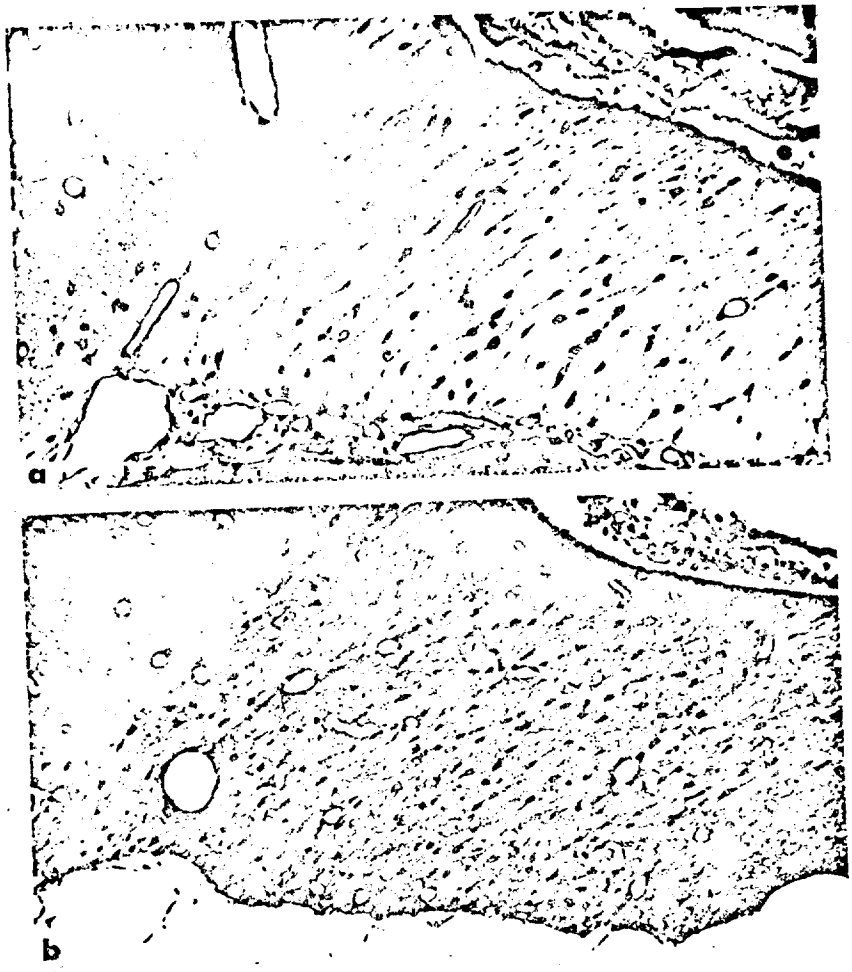
Fig 4



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THE EFFECTS OF LARGE AMOUNTS OF GLUTAMIC ACID UPON SERUM LIPIDS AND STEROL METABOLISM IN MAN

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It is now well established that diet can alter the postabsorptive concentration of serum lipids and lipoproteins in man. Most investigators who have dealt with this problem, however, have studied the effects of variation in the amount and kind of dietary fat, sterols, and carbohydrate.[1-5] The contribution of dietary protein to the regulation of serum lipid concentrations and fat transport has been relatively neglected.[6]

The marked hypocholesterolemic effect of an isocaloric, isofat, low protein diet in adult males was first reported by us from the University of Pittsburgh in 1958.[7] In these studies, the feeding of diets containing 25 g/day of rice or mixed vegetable protein and 36% of calories from butter fat caused a fall of some 20% in serum cholesterol with lesser reductions in the concentration of phospholipids and negligible effects upon triglycerides. Ultracentrifugal measurement of serum β-lipoprotein concentration suggested that the reduction of serum lipids was accounted for by a fall in serum or low-density β-lipoproteins. Table 1 shows the effect upon serum cholesterol of low protein feeding of adult American men (0.1 g of protein per kg/day) measured against control values observed in the same subject when they were fed adequate diets containing more than 1 g of protein per kg/day. These effects are contrasted with the [illegible] demonstrated that children with protein deficiency before and after [illegible] on diets adequate in protein. These children were studied [illegible] of a general investigation of the biochemical aspects of protein-calorie malnutrition being conducted in the Anemia and Malnutrition [illegible] Center of Chiang Mai Medical College and St. Louis University School of Medicine. This Center constitutes a clinical research center [illegible] school. It has been established in protein-calorie malnutrition that the response in serum lipids to diet therapy is due to [illegible] other components of the diet.[8] It seems likely that [illegible] the result of producing a deficit in the biosynthesis of the [illegible] lipoproteins.[9] It appears, therefore, that the hypo-

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TABLE 1. EFFECT OF DIETARY PROTEIN ON SERUM CHOLESTEROL IN HUMAN SUBJECTS

Subjects	N	Serum Cholesterol: Low Protein (0.4 g/kg/day)	Serum Cholesterol: Adequate Protein (>1.0 g/kg/day)
		mg/100 ml	mg/100 ml
Thai children*	19	78 ± 7	182 ± 10
American men†	9	215 ± 14	269 ± 18

*Thai children with protein-calorie malnutrition ranged in age from one to four years and were admitted to the Malnutrition Research Ward of Chiang Mai Medical College for study and treatment.

†American men were initially well nourished subjects 25 to 51 years of age who were fed low protein diets containing 25 g of vegetable protein per day.

cholesterolemia of protein malnutrition is visible both in children with severe deficiency disease and in adult subjects maintained on diets containing 0.4 g/kg or less per day. Other studies carried out by us at the University of Pittsburgh established that the hypocholesterolemia seen in low protein feeding of adults was not modified by changes in sterol intake or by alteration in the composition of dietary fat.[11] It was, in fact, the result of a reduction in the quantity and/or quality of the protein ingested.

It soon became evident to us that an attempt to determine the amino acid deficits most responsible for the hypolipoproteinemia associated with low protein feeding would depend upon the use of amino acid formulas. The relative inflexibility of amino acid composition achieved by combining various naturally occurring proteins prevented a suitable design. Diets containing amounts of nitrogen equivalent to that present in our standard house control diet were, therefore, formulated using crystalline amino acids as the primary source of nitrogen. This communication reports the results of studies of two such formula diets containing quantitatively adequate amounts of the eight essential amino acids required by adult human beings but differing in their content of nonessential nitrogen. Experiments designed to elucidate the nature of the hypocholesterolemic response to glutamate as a source of nonessential nitrogen are also reported.

MATERIALS AND METHODS

[illegible]

TABLE 2. ESSENTIAL AMINO ACIDS FED TO ADULT MEN

Amino Acid	*g/Day*
L-Lysine	2.40
L-Methionine	3.30
L-Isoleucine	2.10
L-Threonine	1.50
L-Leucine	3.30
L-Valine	2.40
L-Phenylalanine	3.30
L-Tryptophan	0.75

period of three to six weeks on this control diet, each subject was fed a formula diet containing the eight essential amino acid levels of three times the minimum daily requirement[12] as shown in Table 2. The caloric value and fat content and quality of these diets was the same as that of control diets. In order to keep the formula diets isonitrogenous (16 g of nitrogen per day), given nonessential amino acids were added to reach the nitrogen levels of the control diet. In determining the essential amino acid requirements of man, Rose and Wixom[12] had used glycine and ammonium ions as a source of nonessential nitrogen. We employed this mixture in one formula which we called AAF. We believed, however, that glutamic acid might be a superior source of nonessential nitrogen because of its direct convertibility to α-ketoglutarate, a Krebs cycle [illegible]. For these reasons, a second experimental diet was com[illegible] which glutamate provided all of the nonessential nitrogen. [illegible] called AAFG. Carbohydrate was adjusted for the larger [illegible] content of glutamate, and cholesterol was added in amounts of [illegible] to simulate the house diet. Carbohydrate was supplied [illegible] sugars mainly in fruit juices in the ratio 1:2. Com[illegible] of these two formula diets is shown in Table 3. Minerals and [illegible] added in sufficient amounts.

TABLE 3. COMPOSITION OF EXPERIMENTAL DIETS[a]

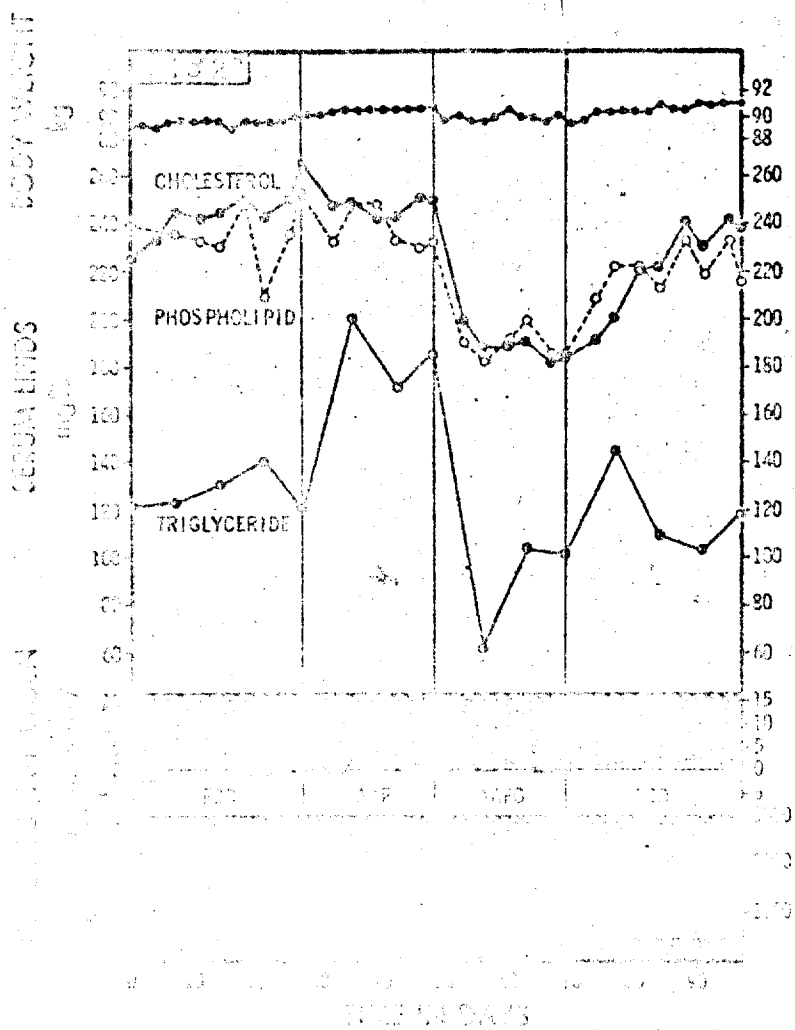
[illegible]	AAF	AAFG
	g/day	*g/day*
[illegible] acids	19.1	19.1
[illegible]	33.4	
	[illegible]	
		[illegible]
	[illegible]	[illegible]
	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
	[illegible]	

In one case, a low protein diet containing 25 g of protein (4 g of nitrogen) was fed as a basal diet to which glutamate was added to provide 12 g of nitrogen. In another case, graded amounts of glutamic acid were added to the normal control diet. In this latter case, the total nitrogen intake was increased in proportion to added glutamate.

Throughout the study, serum lipids were measured twice weekly and serum lipoproteins every other week. Total serum cholesterol determinations were made by the method of Abell *et al.*,[13] serum lipid phosphorus was determined after the chloroform methanol extraction by the method of Fiske and Subbarow[14] and serum triglycerides were measured by the method of Van Handel and Zilversmit.[15] Serum lipoproteins were measured by the technique of de Lalla and Gofman.[16] Nitrogen balances were also measured throughout the study. Twenty-four hour urine collections were made for each subject and pooled into three-day and four-day samples. Likewise, stools were collected in three-day and four-day periods each week. Total urinary and fecal nitrogen was measured by the Kjeldahl procedure.

The analyses for the determination of fecal bile acids and sterols were determined basically by the techniques of Grundy, Ahrens, and Miettinen,[17] Miettinen, Ahrens, and Grundy,[18] and Grundy and Ahrens.[19] Losses during the isolation of fecal sterols and bile acids were corrected by including an internal standard. In the case of sterols, it was cholesterol-1,2-^{3}H; in the case of bile acids, cholanic acid was used and measured by gas liquid chromatography. A model 630 F&M flame ionization gas chromatograph was employed in the analysis of sterols and bile acids. In the [illegible] acetate-1-^{14}C was injected into ven[illegible] in other experiments [illegible] measured by [illegible] to two [illegible] and [illegible] Noble.[illegible]

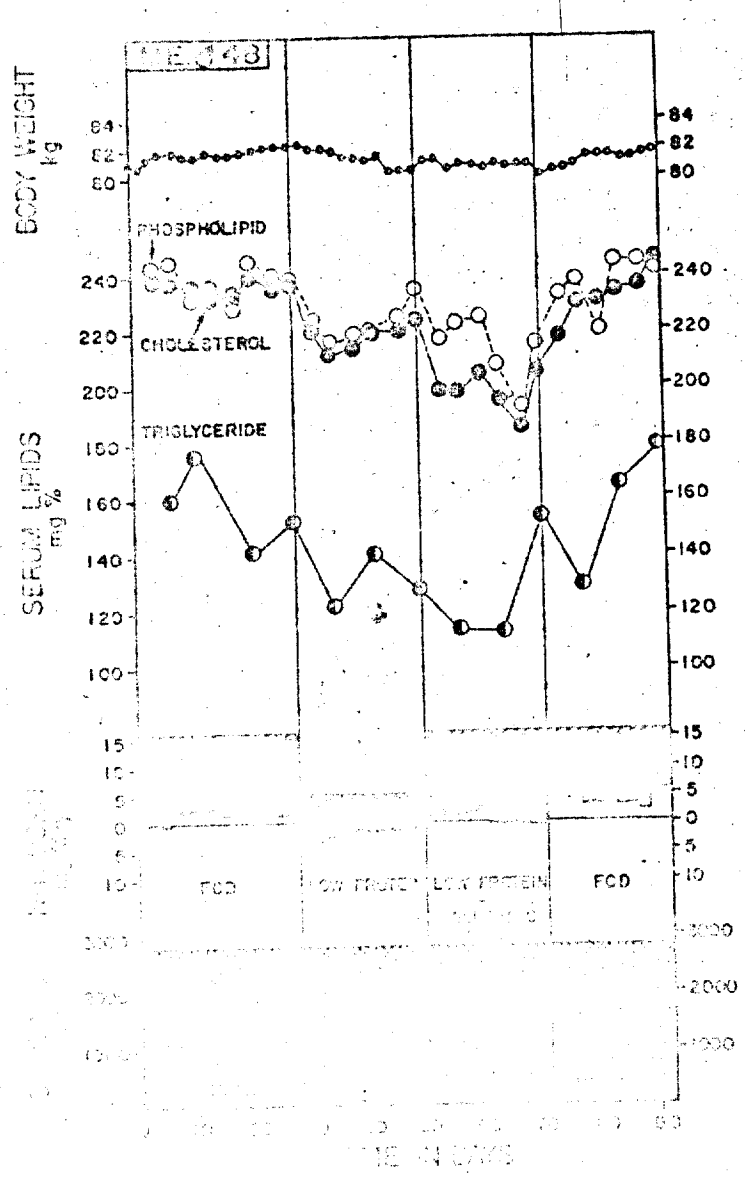
[illegible]


CHOLESTEROL
PHOSPHOLIPID
TRIGLYCERIDE
92
90
88
260
240
220
200
180
160
140
120
100
80
60
15
10
5
0

lipoprotein values returned to normal over a period of four weeks and triglycerides remained at control values. Contrary to expectations, the dramatic effect of AAFG on the concentration of $S_{f0\text{-}20}$ low-density lipoprotein suggested that the source of nonessential nitrogen played a crucial role in the regulation of β-lipoprotein levels. Substitution of a mixture of nonessential amino acids simulating that found in the control diet was neutral, *i.e.*, like the AAF diet, it had no hypocholesterolemic effect. The hypocholesterolemic effect of substituting AAFG for AAF formula diets has been confirmed in 10 additional patients showing similar responses to that of this subject.

In view of this marked effect of glutamate in a formula diet containing only essential amino acids, the question arose, "Does glutamate effect serum lipid levels when added to ordinary diets?" The first experiment was conducted with patient M.E. (Fig. 2), who was fed a low protein diet containing 25 g of protein and who had a small fall in serum cholesterol, considerably less than that observed in other patients (Table 1). The addition of glutamate to provide 12 g of nitrogen (126 g of glutamate) induced an additional hypocholesterolemic effect equivalent to a 20 mg/100 ml fall in serum cholesterol. This was accompanied by a proportional change in low-density β-lipoprotein. The additional nitrogen supplied by glutamate changed the nitrogen balance from negative to positive. The effect of adding 126 g of glutamate to a mixture of amino acids in which the essential amino acids comprised 7 g/day and nonessential 15 g/day was not as effective as adding 187 g of glutamate to a mixture of amino acids in the AAFG formula which provided 19 g of essential amino acids/day.

[illegible]


BODY WEIGHT
kg
PHOSPHOLIPID
CHOLESTEROL
TRIGLYCERIDE
SERUM LIPIDS
mg %
FCD
FCD

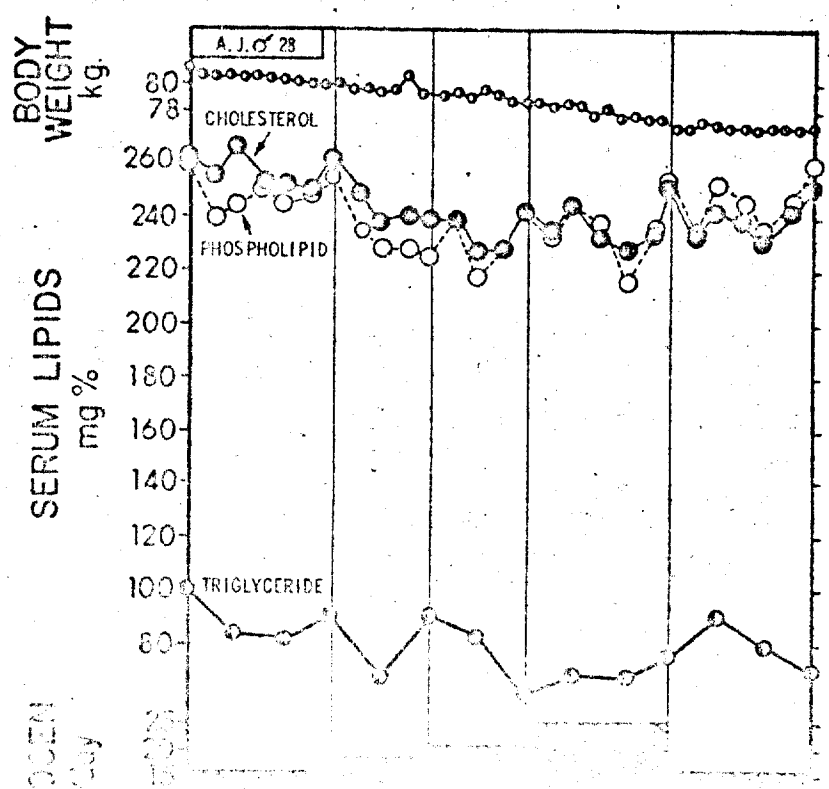

A. J. ♂ 28
BODY WEIGHT kg.
80
78
CHOLESTEROL
260
240
PHOSPHOLIPID
220
200
180
160
140
120
100
TRIGLYCERIDE
80
SERUM LIPIDS mg %

TABLE 4. DATA FOR CALCULATION OF RATES OF APPEARANCE AND DISAPPEARANCE OF SERUM CHOLESTEROL FROM ACETATE-1-^{14}C EXPERIMENT (AFTER WRENSHALL[22])

Period	Serum Cholesterol	λ	N_t	K	dN/dt	R_a	R_d
AAF (Steady state)	224	.062	26.9	—	—	1.67	1.67
AAFG (Transition state)	172	.053	25.3	.051	.27	1.34	1.61
	155	.045	24.8	.051	.25	1.12	1.37
(Steady state)	153	.045	24.7	—	—	1.11	1.11

λ = Fractional turnover coefficient for plasma cholesterol pool.
N_t = Pool size of plasma cholesterol at given time.
K = First order constant for change in cholesterol concentration with therapy.
R_a = Rate of appearance of cholesterol molecules in the plasma pool in grams per day.
R_d = Rate of departure of cholesterol molecules from the plasma pool in grams per day.

isotopic cholesterol was calculated by the method of least squares. The rates of the appearance and disappearance of molecules from the plasma pool were calculated from the turnover constants and the changing pool size according to Wrenshall.[22] These data are presented in Table 4.

The rates of arrival and departure of cholesterol molecules to and from the plasma space are shown in Figure 4. According to these calculations [illegible] hypocholesterolemia is caused by a primary fall in entry of [illegible] molecules into the plasma pool, followed by a reduction in [illegible] of molecules until a new steady state is reached at a [illegible] turnover rate. The actual turnover rate changed from 1.7 to 1.1 [illegible]

[illegible] was also determined for W.B. during the two experi- [illegible] in Table 5, the glutamate diet (AAFG) caused [illegible] excretion. [illegible] distribution of [illegible] experiment to experiment [illegible] AAFG feeding. [illegible]

[illegible]

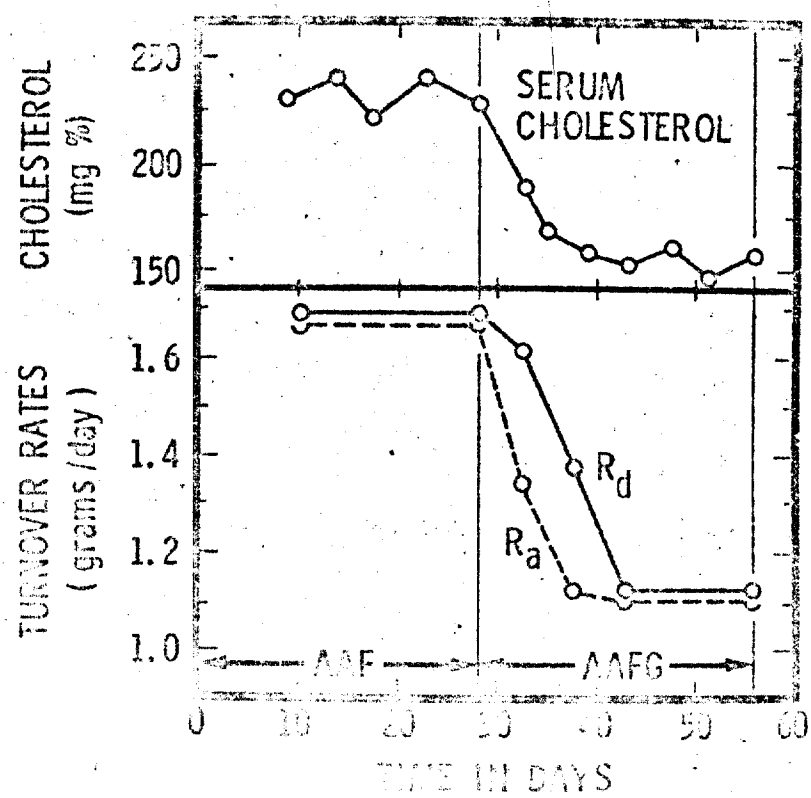
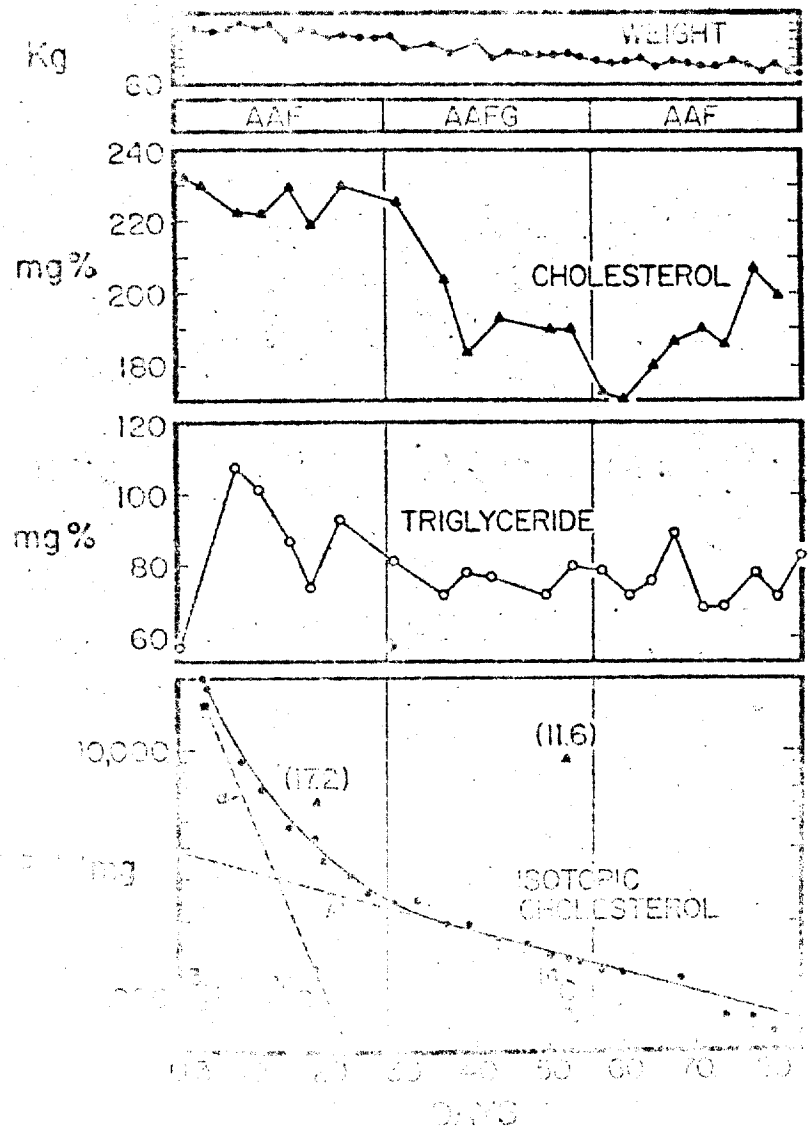


FIG. 4. Serum cholesterol concentration and turnover rates in subject W.B. on [illegible] AAF and AAFG. [illegible] the plasma pool; R_a, rate of departure of cholesterol [illegible] plasma pool.

TABLE 5. [illegible] IN W.B. [illegible]

Study Period	AAF	AAFG	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]


Kg
WEIGHT
AAF
AAFG
AAF
mg%
CHOLESTEROL
mg%
TRIGLYCERIDE
(11.6)
(17.2)
ISOTOPIC
CHOLESTEROL

full decay curves for the cholesterol-4-^{14}C are not shown in Figure 5, the α and β-slopes were also calculated from these curves. Serum cholesterol fell about 40 mg/100 ml upon change of diet from AAF to AAFG. Triglycerides remained constant. The cholesterol pool size diminished from 17 g (mean of 2 measurements) to 11.6 g at the end of the AAFG period (Table 6). Although the α-line slope changed somewhat in the two periods, the β-line slope computed from both the ^{3}H and ^{14}C experiments remained constant. This finding suggests that the changes in kinetics of cholesterol turnover are confined to the labile plasma pool. The production rate for cholesterol (PRA), *i.e.*, the rate of entry of cholesterol molecules into the plasma pool, decreased from 1.1 g/day on diet AAF to 0.7 g/day on diet AAFG.

All of the data obtained on cholesterol turnover during the switch from AAF to AAFG using acetate-1-^{14}C, cholesterol-1,2-^{3}H, and cholesterol-4-^{14}C as isotopic markers, and the study of sterol balance under these conditions, support the view that the event which causes the fall in serum cholesterol, and its associated low-density lipoprotein, is a decrease in the entry of cholesterol molecules into the plasma pool.

The precise biochemical mechanism is not yet clear. The action of glutamate could be exerted at some step in cholesterol biosynthesis, possibly at the HMG-CoA step due to some interference with the reduction of this compound to mevalonate by glutamate or a glutamate metabolite. It could equally well result from an effect upon the synthesis or release of the apoprotein of the low-density lipoproteins from the liver cell. We have recently shown that the gerbil is an experimental animal in which glutamate feeding has the same effect upon serum lipids as in [illegible]. The way is thus open in this experimental model for [illegible] turnover rate of intermediates in [illegible]

[illegible]

and [illegible] that parenteral administration of glutamate to newborn mice and monkeys caused hypothalamic lesions, growth failure, and obesity. This may be due to the immaturity in newborn animals of the blood-brain barrier, which permits toxic levels of glutamate to reach vital neurological centers. None of our adult patients manifested any of these signs or symptoms despite the high doses of glutamate given.

SUMMARY

Glutamic acid, as a sole source of nonessential nitrogen in chemically defined diets fed to adult men, caused a marked fall in plasma cholesterol, phospholipids, and low-density β-lipoproteins. The same amount of glutamic acid added to a normal diet had a negligible effect on serum lipids. The isotopic turnover studies of plasma cholesterol and fecal steroid balances lead to the conclusion that the probable cause for this drop in serum concentration of cholesterol is a reduction in the entry of cholesterol, and possibly other components of β-lipoproteins, into the labile plasma pool. These large doses of oral glutamate (2.2 g/kg) did not cause any detectable hepatic or neurological damage to the adult patients studied.

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DISCUSSION

Dr. [illegible]: Glutamic acid and several [illegible]

[illegible]

[illegible]

[illegible] The largest amount of glutamate given to our adult patients was [illegible] which is about [illegible] g/kg. It is in the range that has been used for [illegible] stimulation of newborn animals with resulting hypothalamic lesions. [illegible] period which we have fed our diet is six weeks. We have [illegible] liver function and neurological function in these patients [illegible] and no defects.

[illegible] say that we have measured the amino acid content of plasma [illegible] state and have found no change in glutamate concentra- [illegible] was kept fairly low, at about 50 μmoles/liter. In other words, [illegible] normally cleared by patients taking these very large doses so that [illegible] plasma glutamates are not elevated.

(5685)

THE EFFECT OF GLUTAMATE ADMINISTRATION ON BLOOD AMMONIA LEVEL AND ITS CORRELATION WITH CLINICAL STATUS IN PATIENTS WITH HEPATIC COMA*

DONALD M. PEARLMAN

Although sodium glutamate has been used sporadically in the treatment of hepatic coma since Walshe[1] reported its successful use in 3 patients, there have been no precise data on its efficacy or pharmacological action. Walshe suggested that the coma of liver failure was due to an increase in intracellular brain ammonia. He administered glutamate, which will bind ammonia to form glutamine.

The relation between ammonia metabolism and hepatic coma still has not been clarified, although it has been studied since 1927.[2] McDermott *et al.*,[3] however, determining blood ammonia levels daily, have recently reported a significant but imperfect correlation between blood ammonia level and clinical status.

In the study to be reported below, blood ammonia determinations were done on a variety of individuals, including some in hepatic coma, as often as every hour. Determinations were carried out before, during, and after administration of glutamate, and the relation of the blood ammonia level to clinical status was determined.

METHOD

Multiple blood ammonia determinations were carried out on 20 normal controls, 15 patients with clinical and laboratory evidence of hepatic dysfunction and no neurological symptoms, and 13 patients who at some time in their hospital course presented the characteristic symptoms of hepatic coma or "pre-coma."

The blood ammonia concentration was determined by a modification of the Conway micro-diffusion technique essentially identical to that described by McDermott and Adams.[4] Determinations were made daily on stable patients and as frequently as hourly on patients who showed any change in clinical status or in any known factor which would influence ammonia metabolism.

Five patients in hepatic coma received intravenous infusions of 25 gm. of sodium glutamate in 1 hour. Three of the 5 received 2 infusions each. The intravenous infusion was prepared by making up a 10 per cent solution of commercial monosodium glutamate in water and autoclaving it. Three of these patients also received glutamic acid, 2 gm. per hour by nasogastric tube.

RESULTS

Normal and Abnormal Blood Ammonia Levels. The range of blood ammonia levels in all patients studied is shown in Table 1.

It is noteworthy that while the blood ammonia nitrogen levels of pa-

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Surg Forum, Proc. 41st Conf. Am. Coll. Surgeons (1955) 364-367

Table 1. 328 Blood Ammonia Determinations on 48 Patients

	RANGE OF BLOOD AMMONIA NITROGEN IN MICROGRAMS PER CENT
No hepatic dysfunction (20 patients, 76 determinations)	40-86
Hepatic dysfunction, no neurological symptoms (15 patients, 92 determinations)	70-180
Hepatic coma or pre-coma (13 patients, 160 determinations)	50-530
Highest level in 24 hours preceding onset of neurological symptoms	190-440

tients in coma or pre-coma ranged from normal to greatly elevated values, there invariably was a significant elevation of blood ammonia preceding the onset of coma. The dynamic relationship between blood ammonia nitrogen level and clinical status will be illustrated by the detailed cases presented below.

Effect of Glutamate on Blood Ammonia Nitrogen Level. The changes in blood ammonia nitrogen concentration accompanying the infusion of 25 gm. of sodium glutamate in 1 hour are shown in Fig. 1. The fall in blood ammonia nitrogen ranged from 80 to 240 μg. per cent; the effect was maximal from 1 to 2 hours after the start of the infusion; the duration of significant effect was at least 4 hours. No deleterious effects were noted

BLOOD AMMONIA NITROGEN AFTER INFUSION OF 25 GM OF SODIUM GLUTAMATE IN ONE HOUR

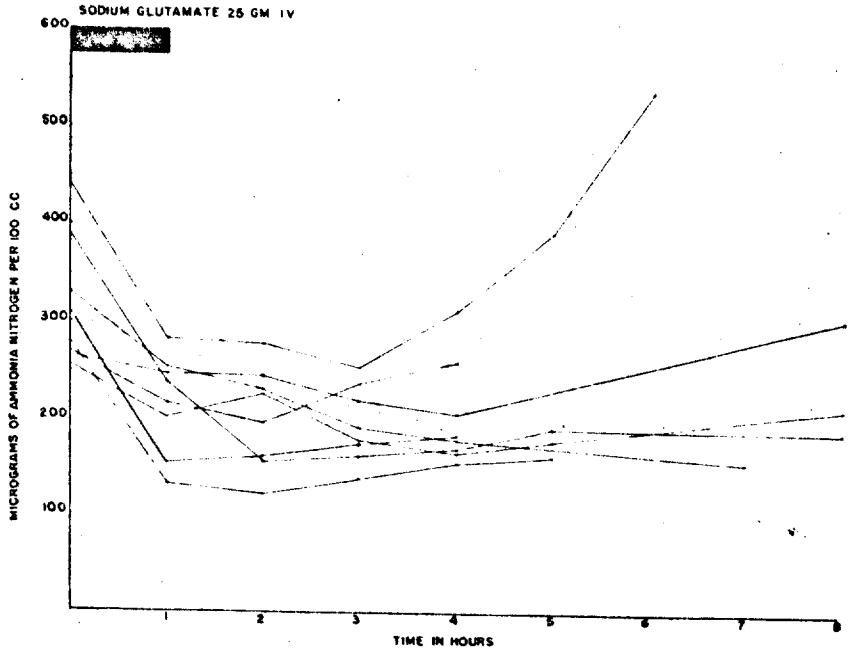

Fig. 1.

from the infusion. The only factor limiting the amount of sodium glutamate which could be administered was the alkalosis and hypernatremia produced.

Clinical Effects of Changes in the Blood Ammonia Concentration. Of the 5 patients treated with glutamate, 2 died without apparent decrease in their coma, 1 improved somewhat but relapsed into deep coma and died, and 2 made full recoveries.

Both of the patients who died without change in their coma were far advanced cirrhotics who gradually decompensated, went into coma, and died. Glutamate infusion produced only transient (1 to 6 hour) drops in blood ammonia concentration, which rapidly rose to even higher levels post infusion. Clinical courses of the 3 patients who demonstrated changes in neurological status during glutamate therapy are charted in Figs. 2, 3, and 4.

Fig. 2 illustrates the course of a 56 year old woman with posthepatitic cirrhosis and bleeding esophageal varices. On admission a Sengstaken-Blakemore tube was inserted and she was given glutamic acid, 2 gm. per hour through the tube.

The patient began to deteriorate, however, and on the fifth hospital day bled from her stomach below the gastric balloon. Here again, transient falls in the blood ammonia level were not associated with change in the depth of coma.

Fig. 3 illustrates the course of a 18 year old female with an alcoholic past who was exposed to carbon tetrachloride 11 days prior to admission. Icterus and oliguria developed and on the day prior to admission the patient became lethargic.

Although the blood ammonia nitrogen fell to 160 μg. per cent immediately following the first infusion of sodium glutamate and to 152 μg.

POST NECROTIC CIRRHOSIS WITH BLEEDING ESOPHAGEAL VARICES

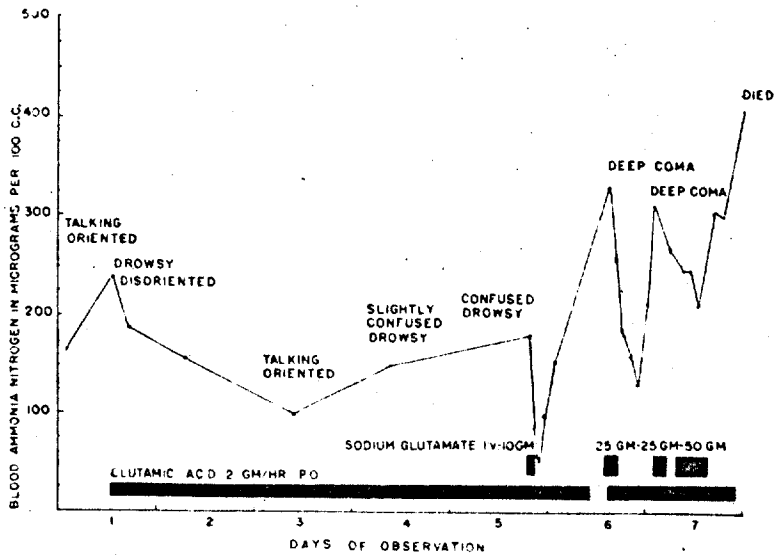

Fig. 2.

LAENNEC'S CIRRHOSIS WITH CARBON TETRACHLORIDE POISONING

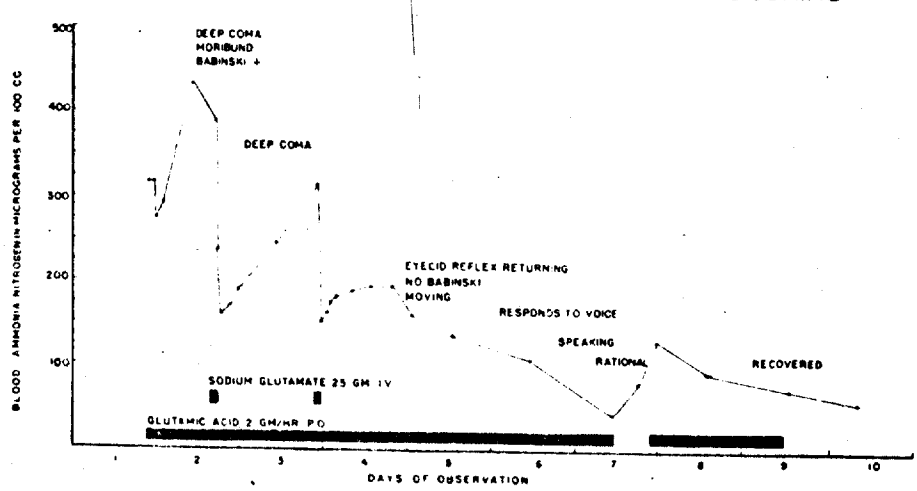

Fig. 3.

PRIMARY BILIARY CIRRHOSIS WITH BLEEDING ESOPHAGEAL VARICES

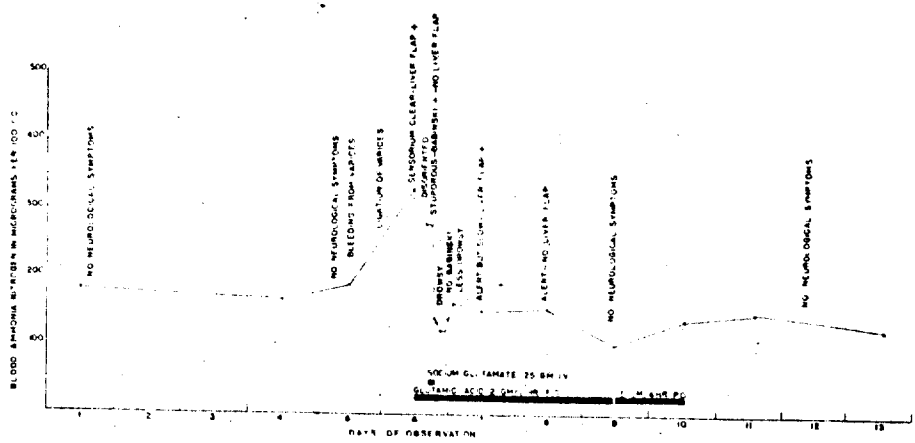

Fig. 4.

per cent following the second infusion, it was not until the blood ammonia nitrogen had been under 200 μg. per cent for over a day that there was any decrease in the depth of the coma.

Fig. 4 illustrates the course of a 17 year old female with primary biliary cirrhosis who entered the hospital for a portacaval shunt because of previous bleeding from esophageal varices. While in the hospital she again bled from her varices, which were then ligated transesophageally.

The sharp rise in blood ammonia concentration followed the accumulation of protein (blood in this case) in her intestine and the insult of her liver of anoxia and anesthesia. Coma did not ensue immediately following the rise in blood ammonia concentration, but 8 or 10 hours later when the level was already falling. In this case, which was characterized

by acute onset of coma and short duration of elevation of blood ammonia, recovery followed the fall in blood ammonia level more quickly than in the case illustrated in Fig. 3. Of interest is the appearance of the peculiar flapping tremor described by Adams and Foley[5] as indicative of impending hepatic coma at the height of the blood ammonia level, its disappearance as coma ensued, and its subsequent reappearance and final disappearance upon recovery.

DISCUSSION AND SUMMARY

In the cases of hepatic coma studied, changes in neurological status occurred only following major changes in blood ammonia level of significant duration. Single or even daily determinations may fail to reveal the relationship of blood ammonia level to clinical status. The data collected are consistent with the hypothesis that the brain does not come into instantaneous equilibrium with the blood as to ammonia content, but rather absorbs ammonia from or discharges ammonia to the blood at a rate so slow that moderate changes in blood ammonia level affect the neurological status of the patient only after many hours. The demonstration by Bessman *et al.*[6] of a significant difference between arterial and jugular bulb blood ammonia levels in cirrhotics in hepatic coma and no difference between these levels in non-comatose cirrhotics tends to support this hypothesis.

Significant drops in the blood ammonia level follow the administration of glutamate. All changes in clinical status following glutamate infusion could be explained by its effect on blood ammonia concentration. Glutamate apparently is of value in treating hepatic coma in cases in which the liver retains some capacity to recompensate.

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Amino Acids, Including Asparagine and Glutamine, in Plasma and Urine of Normal Human Subjects (33859)

J. H. PETERS, S. C. LIN, B. J. BERRIDGE, JR., J. G. CUMMINGS, AND W. R. CHAO
Life Sciences Research, Stanford Research Institute, Menlo Park, California 94025

A recent development in the technology of amino acid assay via ion-exchange column chromatography was the separation of asparagine and glutamine by employing lithium citrate buffer systems (1) instead of the sodium citrate buffer systems usually used for determination of the acidic and neutral amino acids (AN) (2). We recently reported (3) our evaluation and modifications of the use of lithium buffers at flow rates employed in the Beckman/Spinco model 120 amino acid analyzer. Our major changes of the original method of Benson *et al.* (1) were the correction of an inadvertent interchange of the elution positions of citrulline and α-aminoadipic acid, and an improvement in the resolution in the cystine-to-leucine region by lowering the pH of the second buffer of the new, two-stage, lithium buffer system from 4.15 to 3.82.

The present report presents comparative results of the application of both the new and previous techniques for measuring free amino acids in plasma and urine of normal subjects. Also included are results of the determination of total amino acids found after acid hydrolysis of protein-free extracts of plasma and urine. The measurements of free and total aspartic and glutamic acids, as well as their respective amides, provided an opportunity to evaluate the contributions of these acids, their amides, and other conjugates to the total hydrolyzable aspartic and glutamic acids in plasma and urine.

Materials and Methods. Collection of samples for analysis. Fasting (overnight) blood samples (10 ml) were taken by venipuncture at three 6-month intervals from two female and three male, healthy, mature volunteers (wt range, 53–77 kg; age range, 24–42 years). The blood was immediately mixed with dry sodium heparinate (10 units/ml of blood), cooled in an ice bath, and centrifuged at 1500 rpm for 10 min. The plasma was removed and recentrifuged, and a portion was deproteinized immediately. The remainder was stored at −20° for later direct determination of tryptophan and creatinine.

Concurrent with the last two blood samplings, we also collected urine from the volunteers. The collection period was from 6:30 to 10:30 a.m., during which time the overnight fast was continued. Only water *ad libitum* was allowed. At the midpoint of the urine collection time, the blood sample was withdrawn. These conditions were dictated by what was considered to be the most practicable in the clinical situation for studies in uremic patients (4, 5), since the samples from these normal subjects were to serve as bases for comparison. The urine was refrigerated immediately after voiding. Following completion of the collection, a portion was deproteinized and the remainder was stored at −20°.

Deproteinization. Protein-free filtrates of plasma or urine for analysis of amino acids were prepared by the picric acid method (6). Urine was deproteinized in anticipation that samples from uremic patients would require

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this procedure. A 4-ml aliquot of plasma or urine was mixed with 20 ml of 1% picric acid. After centrifugation, the excess picric acid was removed by passing the supernatant liquid through a column (2 × 1.9 cm) of an anion exchange resin, AG-2, X10, chloride form (Bio-Rad Laboratories). The effluent liquid was then lyophilized, reconstituted in 5.0 ml of 0.02 *N* HCl, and stored at −20°. Plasma and urine (0.2 ml) for analysis of tryptophan were deproteinized with 4 vol of 10% aqueous trichloroacetic acid. Plasma and urine for analysis of creatinine were deproteinized with sulfuric acid-sodium tungstate solutions: 0.2 ml of plasma or of a 1:100 dilution of urine was efficiently mixed with 0.2 ml of water, 0.2 ml of 0.67 *N* H_2SO_4, and 0.2 ml of 5% aqueous sodium tungstate solutions; the mixture was then centrifuged.

Analysis for amino acids. Amino acids in the protein-free filtrates of plasma or urine were determined on an automatic analyzer (Beckman/Spinco model 120) which had been upgraded to yield sensitivities comparable to those of currently available models and modified to provide continuous automatic operation after sample addition (3). The accelerated two-column chromatographic method (2, 7) was used. Sodium citrate buffers (7) were used for analysis of basic amino acids (B) and for AN of plasma obtained at the first and second bleedings (Groups 1 and 2) as well as in the urine collected concurrently with the plasma from the second bleeding (Group 2). These buffers were also used for analysis of B of plasma and urine obtained at the third bleeding (Group 3), but lithium citrate buffers were employed for AN in the plasma and urine of this group. The lithium citrate buffer formulations and details of their use were as described previously (3). An amount of plasma or urine corresponding to 0.33 ml of original material was used for each analysis of free amino acids, and the equivalent of 0.10 ml of original material was used for each analysis of hydrolysates. Unknowns from analyses using either lithium or sodium buffers were estimated by reference to corresponding integration constants obtained by running standard solutions of amino acids with appropriate additions (3). These constants were redetermined weekly.

Tryptophan was measured by separate assay, as it is lost during the picric acid deproteinization procedure. Creatinine was also measured directly since amounts of this compound were too small to be analyzed in plasma by the analyzer, and its peak was not well resolved in urine samples.

Analysis for total amino acids after hydrolysis. The total amino acids after hydrolysis of the protein-free plasma or urine were determined on the analyzer, using the standard sodium citrate buffers. Using 0.5 ml of the reconstituted protein-free solution and 0.5 ml of 12 *N* HCl, hydrolysis was carried out in a 10-ml hydrolysis tube (Kontes, K-89685). The mixture was shell-frozen in a dry ice–acetone mixture and the tube was evacuated ($\leq$ 50 μ) for 15 min. The evacuated tube was heated at 110 ± 1° for 22 hr. After cooling, the hydrolysate was transferred quantitatively to a round-bottom flask and evaporated to dryness on a rotary evaporator at a maximum bath temperature of 55° under reduced pressure. A few milliliters of water were then added and evaporated to ensure removal of the excess acid. The sample was reconstituted with 1.0 ml of 0.02 *N* HCl and stored at −20° before analysis. The total amino acids were determined on the analyzer, using the sodium citrate buffers.

When standard calibration mixtures (Beckman/Spinco type 1) containing 18 amino acids were hydrolyzed, all but cystine and methionine gave 90–100% recoveries. Asparagine, citrulline, creatine, creatinine, glutamine, and 3-methylhistidine were hydrolyzed individually with calibration mixtures. Asparagine and glutamine were recovered quantitatively as aspartic and glutamic acids, respectively; 50% of the citrulline was hydrolyzed to ornithine; creatine, which is not ninhydrin-positive, was completely converted to creatinine; and 80% of the creatinine was recovered.

Tryptophan assay. Tryptophan was determined in 0.1-ml aliquots of the protein-free filtrates of plasma or urine, obtained as described above, by modifications of the fluoro-

TABLE I. Acidic and Neutral Amino Acids Measured in the Plasma of Fasting Normal Subjects.[a]

Compound	Group:	Plasma concentration (mg/100 ml; mean ± SD)		
		1 and 2[b]	3[c]	1, 2 and 3[d]
Alanine		2.94 ± 0.48	2.85 ± 0.68	2.91 ± 0.53
α-Amino-n-butyric acid		0.19 ± 0.09	0.19 ± 0.08	0.19 ± 0.08
Asparagine			0.63 ± 0.10	0.63 ± 0.10[c]
Asparagine + glutamine		8.33 ± 1.04		8.33 ± 1.04[b]
Aspartic acid		0.04 ± 0.02	0.05 ± 0.02	0.04 ± 0.02
Citrulline		0.51 ± 0.20	0.50 ± 0.18	0.51 ± 0.19
Cystine, ½		0.80 ± 0.43	1.17 ± 0.10	0.93 ± 0.39
Glutamic acid		0.48 ± 0.18	0.44 ± 0.14	0.46 ± 0.16
Glutamine			8.14 ± 0.72	8.14 ± 0.72[c]
Glycine		1.67 ± 0.42	1.76 ± 0.38	1.70 ± 0.40
Isoleucine		0.73 ± 0.13	0.79 ± 0.11	0.75 ± 0.12
Leucine		1.44 ± 0.24	1.56 ± 0.19	1.48 ± 0.23
Methionine		0.30 ± 0.07	0.37 ± 0.07	0.33 ± 0.07
Phenylalanine		0.80 ± 0.08	0.84 ± 0.03	0.81 ± 0.07
Proline		1.65 ± 0.39	1.98 ± 0.51	1.76 ± 0.45
Serine		1.11 ± 0.16	1.31 ± 0.13	1.18 ± 0.18
Taurine		0.63 ± 0.14	0.58 ± 0.07	0.61 ± 0.12
Threonine		1.70 ± 0.34	1.75 ± 0.31	1.72 ± 0.32
Tryptophan		1.06 ± 0.13[c]	1.13 ± 0.17	1.10 ± 0.15[b]
Tyrosine		0.89 ± 0.11	0.99 ± 0.15	0.92 ± 0.13
Urea		30.8 ± 10.7	29.4 ± 5.6	30.3 ± 9.1
Valine		2.31 ± 0.59	2.83 ± 0.38	2.48 ± 0.58

[a] Comparison of levels found using buffers containing Na^+ (Groups 1 and 2) and Li^+ (Group 3).

[b] Mean of 10 observations in five subjects, unless otherwise indicated.

[c] Mean of single observations in five subjects.

[d] Mean of 15 observations in five subjects, unless otherwise indicated.

metric method of Hess and Udenfriend for analysis of tryptamine (8). The method is based on the conversion of tryptophan to the fluorophore norharman in a two-step reaction: (a) the cyclization of tryptophan to a tetrahydronorharman derivative with 18% formaldehyde by heating at 100° for 20 min; and (b) the subsequent oxidation to norharman with hydrogen peroxide by another heating period of 20 min at 100°.

Fluorescence was measured in an Aminco-Bowman spectrophotofluorometer equipped with enhancing mirrors (9) at 365 and 450 mμ, as excitation and fluorescence wavelength maxima, respectively. The major changes from the original method (8) were: (a) the hydrogen peroxide concentration was reduced from 5 to 3%; (b) the heating times were controlled by cooling the tubes immediately in an ice-water mixture at the end of each heating period; and (c) the fluorescence was read at a constant temperature range of 24 ± 2°. In addition, to correct for inherent and variable quenching of the fluorophore by endogenous plasma and urinary constituents, an internal standard was prepared by adding tryptophan, 0.2 μg for plasma and 1.0 μg for urine, to another aliquot of each of the protein-free filtrates being analyzed. Each experimental sample was then corrected for the extent of quenching observed. Control studies demonstrated that the percentage decrease of fluorescence due to quenching was constant in the range of 0.1–2.5 μg of tryptophan sample. Protein-free filtrates of plasma exhibited a mean quenching of 19.7 ± 3.8% (SD) and similar filtrates of urines, 55.9 ± 6.5% (SD). A complete assay system without

formaldehyde or tryptophan served as a blank.

Creatinine assay. Creatinine in plasma and urine was measured by the alkaline picric acid method of Martinez and Doolan (10). Their procedure was modified by reduction of all volumes of reagents by one-half and by extending the time of color development from 30 to 45 min. Absorbancies were determined at 500 mμ on a Gilford spectrophotometer using 1-ml cuvettes.

Results and Discussion. Table I presents the mean values of the AN found in the first two bleedings (Groups 1 and 2) and the third bleeding (Group 3) of our five volunteers. Since no sex difference was noted, we have combined the data from these bleedings. Comparison of the means of these amino acids obtained using the sodium or lithium buffers shows that both systems gave essentially the same results. In addition, the sum of the means for asparagine and glutamine measured separately in Group 3 (8.77 mg/100 ml) was within the range of the mean ± SD for these two compounds measured together in Groups 1 and 2 (8.33 ± 1.04). In Column 4 all determinations are combined to obtain a normal mean for AN in this study. It is apparent that the lithium buffer systems provide the advantage of measuring asparagine and glutamine separately and still retain the ability to resolve the other AN. The mean values for plasma asparagine and glutamine obtained in the present work agree with those reported by Benson *et al.* (1) for plasma, using similar analytic conditions. Stein and Moore (6) indicated that the concentration of asparagine in normal plasma was less than one-tenth that of glutamine. In the current study, the ratio of asparagine to glutamine was approximately 1:13.

The results of the determination of B in these plasma samples are shown in Table II. Again, Groups 1 and 2, and 3 are reported separately even though they were analyzed by the same system. As in the case of the AN, comparison of mean levels found at the different bleeding times shows little difference and therefore a mean of all available values is presented in Column 4 of Table II. The means of both the AN and B found in these subjects agree closely with those reported previously by other authors using ion-exchange column chromatographic procedures (6, 11, 12).

TABLE II. Basic Amino Acids Measured in the Plasma of Fasting Normal Subjects.

Compound	Plasma concentration (mg/100 ml; mean ± SD)		
	Groups 1 and 2[a]	Group 3[b]	Groups 1, 2 and 3[c]
Arginine	1.38 ± 0.25	1.51 ± 0.14	1.42 ± 0.22
Creatinine	1.4 ± 0.2[b]	1.2 ± 0.3	1.3 ± 0.2[c]
Histidine	1.21 ± 0.14	1.27 ± 0.08	1.23 ± 0.12
Lysine	2.41 ± 0.36	2.58 ± 0.20	2.46 ± 0.32
Ornithine	0.66 ± 0.16	0.84 ± 0.11	0.72 ± 0.17

[a] Mean of 10 observations in five subjects unless otherwise indicated.

[b] Mean of single determinations in five subjects.

[c] Mean of 15 observations in five subjects unless otherwise indicated.

The results of analyses for AN in urine collected concurrently with the second and third bleedings are presented in Table III. Since the total volumes of urine excreted by the subjects were quite variable (92–314 ml), the results are expressed in micrograms of constituent per milligram of creatinine excreted during the 4-hr collection period. The mean excretion of creatinine was 264 ± 88 (SD) mg by Group 2 and 208 ± 67 mg by Group 3.

Comparison of the results of Group 2 (sodium buffers) with Group 3 (lithium buffers) again shows that similar results were obtained, although there was much more variation between individuals in these urines than in the plasma samples. Proline was not found in any urine sample. The sum of the separately determined asparagine and glutamine in Group 3 was 56.3 μg/mg of creatinine, within the range of the mean ± SD of the two amides measured together in Group 2 (39.5–59.1). The ratio of asparagine to glutamine in the urine was 1:7 quite unlike the 3:2 ratio stated by Benson *et al.* (1). However, our estimations of this ratio from the sample chromatogram presented by these authors (Fig. 2 of Ref. 1) indicated a ratio of

TABLE III. Acidic and Neutral Amino Acids Measured in the Urine of Fasting Normal Subjects.[a]

Compound	Urinary excretion (μg/mg of creatinine; mean ± SD)		
Group:	2[b]	3[b]	2 and 3[c]
Alanine	12.8 ± 3.7	12.9 ± 5.6	12.8 ± 4.5
α-Amino-n-butyric acid	2.6 ± 0.6	0.8 ± 0.2[d]	1.8 ± 1.1[e]
β-Amino-i-butyric acid	10.0 ± 3.0	9.9 ± 2.6[f]	10.0 ± 3.7[g]
Asparagine		7.0 ± 3.6	7.0 ± 3.6[b]
Asparagine + glutamine	49.3 ± 9.8		49.3 ± 9.8[b]
Aspartic acid	4.7 ± 0.9	2.1 ± 0.9	3.4 ± 1.6
Citrulline	3.1 ± 1.7	2.0 ± 0.6	2.6 ± 0.9
Cystine, ½	7.6 ± 2.5	9.1 ± 4.0	8.3 ± 3.5
Glutamic acid	2.1 ± 0.4	3.9 ± 1.1	2.9 ± 1.2
Glutamine		49.3 ± 24.9	49.3 ± 24.9[b]
Glycine	71.1 ± 40.5	60.6 ± 21.0	65.9 ± 31.0
Isoleucine	1.0 ± 0.2	2.8 ± 1.6	1.9 ± 1.4
Leucine	5.3 ± 0.9	2.9 ± 1.5	4.1 ± 1.7
Methionine	Not resolved	4.6 ± 1.4	4.6 ± 1.4[b]
Phenylalanine	7.4 ± 1.7	5.7 ± 2.0	6.5 ± 2.0
Serine	23.0 ± 3.4	30.4 ± 15.5	26.7 ± 11.2
Taurine	51.8 ± 23.7[d]	65.1 ± 40.0	59.2 ± 32.6[g]
Threonine	13.1 ± 4.8	16.1 ± 10.0	14.6 ± 9.0
Tryptophan	Not determined	11.1 ± 3.4	11.1 ± 3.4[b]
Tyrosine	11.7 ± 3.1	13.8 ± 7.3	12.8 ± 5.4
Urea	12,000 ± 777	9620 ± 463	10,800 ± 534
Valine	2.7 ± 0.3	4.8 ± 1.4	3.8 ± 1.7

[a] Comparison of levels found using buffers containing Na^+ (Group 2) and Li^+ (Group 3).
[b] Mean of five subjects unless otherwise indicated.
[c] Mean of 10 observations in five subjects unless otherwise indicated.
[d] Mean of four subjects.
[e] Mean of nine observations.
[f] Mean of three subjects.
[g] Mean of eight observations.

asparagine to glutamine of approximately 2:9. In the analysis of Group 2 using sodium buffers, methionine could not be calculated because of poor resolution from unidentified materials. This difficulty was overcome in the lithium system by the spreading out of peaks in the cystine-to-leucine region, as described earlier (3). The values for B in the two groups are shown in Table IV. Again, both urine collections gave very similar values and, therefore, in both cases, means were calculated for all observations (Column 4 in Tables III and IV). These values cannot be compared directly with earlier reports since in the past it was a general practice to collect 24-hr urines from nonfasting subjects (13–15). Soupart (15) reported his data by expressing individual amino acids in molar percent of the total excretion of amino acids in 24 hrs. By treating our 4-hr mean values in the same way, we can compare our results with those of Soupart. On that basis, as shown in Fig. 1, good general agreement between our 4-hr excretions and the 24-hr excretions of amino acids is apparent. Only lysine excretion is markedly different in the two groups of data.

An estimation of the conjugated or bound amino acids in protein-free filtrates of plasma and urine was obtained in this study by measuring the total amino acids produced by hydrolysis. The increases of amino acids over the amounts measured before hydrolysis are interpreted as bound or conjugated amino acids; their mean values are presented in Table V. It is clear that these values were

TABLE IV. Basic Amino Acids Measured in the Urine of Fasting Normal Subjects.

Compound	Urinary excretion (μg/mg of creatinine; mean ± SD)		
Group:	2[a]	3[a]	2 and 3[b]
Arginine	1.5 ± 0.6	2.0 ± 0.8	1.7 ± 0.7
Histidine	91.3 ± 41.1	87.4 ± 39.3	89.3 ± 38.0
Lysine	17.8 ± 11.0	24.3 ± 23.0	21.0 ± 17.3
1-Methylhistidine	16.9 ± 12.1[c]	28.5 ± 12.6[d]	21.8 ± 12.8[e]
3-Methylhistidine	25.1 ± 2.2	28.4 ± 5.5	26.8 ± 4.4
Ornithine	1.2 ± 0.5[c]	1.4 ± 0.5	1.3 ± 0.5[f]

[a] Mean of five subjects unless otherwise indicated.
[b] Mean of 10 observations in five subjects unless otherwise indicated.
[c] Mean of four subjects.
[d] Mean of three subjects.
[e] Mean of 7 observations.
[f] Mean of 9 observations.

extremely variable between individuals, as previously reported by Stein (13). The major share of the total bound amino acids in either plasma or urine was due to only a few amino acids. Thus, 70.6% of the total μmoles of bound amino acids in the plasma was accounted for by the four compounds, glutamic acid (49.7%); glycine (10.6%); aspartic acid (5.2%); and alanine (5.2%). Similarly, in the urine, 78.9% of the total μmoles of bound compounds was due to glycine (35.3%); glutamic acid (24.8%); aspartic acid (11.9%); and proline (6.9%). Other single amino acids in plasma or urine contributed less than 5% to the respective totals.

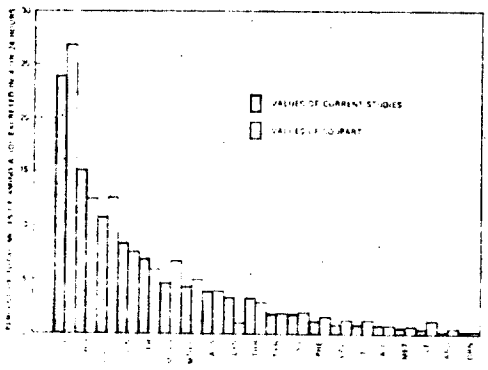

FIG. 1. Comparison of excretion of amino acids in the urine during 4 and 24 hr. Our mean values were from 10 samples of 4-hr fasting urines from 5 normal subjects (2♀, 3♂). The mean values given by Soupart (15) were from 24-hr urines obtained from 15 normal subjects (9♀, 6♂) not being fasted.

The availability of data on the total and free aspartic and glutamic acids and their

TABLE V. Bound or Conjugated Amino Acids Measured in the Plasma and Urine of Fasting Normal Subjects.

Compound	Plasma (mg/100 ml; mean ± SD)[a]	Urine (μg/mg of creatinine; mean ± SD)[b]
Alanine	0.68 ± 0.97[c]	15.9 ± 3.8
α-Amino-n-butyric acid	0.12 ± 0.10[c]	4.4 ± 2.1
Arginine	0.48 ± 0.54[c]	11.8 ± 2.6
Aspartic acid	1.02 ± 0.24[c]	87.1 ± 24.1
Glutamic acid	10.8 ± 3.5	200 ± 58
Glycine	1.18 ± 0.86	145 ± 66
Histidine	0.33 ± 0.41	15.3 ± 7.5[d]
Isoleucine	0.17 ± 0.22	3.7 ± 1.2
Leucine	0.35 ± 0.43	9.4 ± 3.8
Lysine	0.84 ± 0.79	26.6 ± 19.2
Ornithine	0.48 ± 0.30	7.4 ± 1.6[d]
Phenylalanine	0.29 ± 0.25	4.8 ± 2.2
Proline	0.83 ± 0.70	43.6 ± 12.2[e]
Serine	0.37 ± 0.37	15.4 ± 4.2
Taurine	0.17 ± 0.23	2.8 ± 2.2[f]
Threonine	0.50 ± 0.91	14.1 ± 3.6
Tyrosine	0.21 ± 0.26	3.2 ± 1.4[e]
Valine	0.57 ± 0.87[c]	12.2 ± 4.2

[a] Mean of 15 observations in five subjects unless otherwise indicated.
[b] Mean of 10 observations in five subjects unless otherwise indicated.
[c] Mean of 14 observations.
[d] Mean of 9 observations.
[e] Mean of 8 observations.
[f] Mean of 4 observations.

TABLE VI. Contribution of the Respective Amino Acids, Amides, and Unknown Conjugates to the Total Aspartic and Glutamic Acids Measured after Hydrolysis in Plasma and Urine.

Constituents	Plasma* (mg/100 ml)	Urine* (μg/mg of creatinine)
Total aspartic acid after hydrolysis	0.94 ± 0.11	106 ± 25
Aspartic acid before hydrolysis	0.05 ± 0.02	2.1 ± 0.9
Asparagine before hydrolysis	0.63 ± 0.10	7.0 ± 3.6
Net aspartic acid derived from unknown aspartyl conjugates	0.26 ± 0.10	96.8 ± 20.6
Total glutamic acid after hydrolysis	9.47 ± 0.73	230 ± 73
Glutamic acid before hydrolysis	0.44 ± 0.14	3.9 ± 1.1
Glutamine before hydrolysis	8.14 ± 0.72	49.3 ± 24.9
Net glutamic acid derived from unknown glutamyl conjugates	0.89 ± 0.21	177 ± 55

* Values are the means ± SD from five fasting normal subjects. Blood was obtained at the midpoint of the 4-hr urinary collection.

amides now makes possible an estimation of the contributions of the acids and their amides to the total acids measured. The data in Column 2 of Table VI show that in the plasma, 75% of the total aspartic acid found after hydrolysis was due to aspartic acid and its amide, while 25% was derived from unknown aspartyl conjugates. Similarly, 91% of the total glutamic acid of the plasma was due to glutamic acid and its amide; unknown glutamyl conjugates contributed only 9%. In the urine, as shown in Column 3, a markedly different pattern is apparent since 91 and 77% of the total aspartic and glutamic acids were derived from unknown aspartyl and glutamyl conjugates, respectively. It is reasonable to suggest that these unknown conjugates in the urine are β-aspartyl and γ-glutamyl peptides since other workers have reported the isolation of numerous peptides of this type in normal urine (16–18). Their occurrence in plasma has not as yet been demonstrated.

Summary. The use of lithium citrate buffer systems for the analyses of acidic and neutral amino acids in plasma and urine was tested and found superior to the usually employed sodium citrate buffer systems. The lithium systems have the advantage of resolving asparagine and glutamine without loss of resolution of other acidic and neutral amino acids. Normal levels of these amides in plasma and urine were established, and the relative contributions of the aspartic and glutamic acids, their amides, and unknown conjugates (peptides?) to the total measurable acids in protein-free filtrates after hydrolysis were determined. The general pattern of excretion of amino acids in urine collected for 4 hr from fasting individuals was shown to be quite similar to the pattern found in 24-hr urines collected from nonfasting subjects.

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Metabolic Effects of L-Glutamic Acid and of Glycine

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ALTHOUGH much interest was evidenced as early as 1900 (1) in the increased glucose excretion following the administration of various amino acids to phlorizinized or depancreatized animals, the data up until 1922 were interpreted largely in terms of the conversion of amino acids to glucose. With the increase in interest and knowledge of the complex endocrine control of blood sugar, other interpretations were advanced. In 1922 Pollak (2) suggested that amino acids act to raise blood sugar by a sympathetic mobilization of glycogen. He compared this action to that of epinephrine. Weil-Malherbe (3) has recently revived this theory to account for the action of glutamic acid in arousing patients from insulin hypoglycemia. An adrenergic action for this amino acid seems to be indicated by the work of Nord (4) on rabbits as well as Weil-Malherbe's (5) observations of adrenaline-like substances in the blood of patients given glutamic acid. The investigation reported here was planned to compare the effects of glutamic acid and of glycine injected subcutaneously into rabbits on blood sugar, blood pressure, and pupillary diameter as well as the effect of increased plasma glutamic acid on the levels of pyruvic and α-ketoglutaric acids in blood.

METHODS

Adult rabbits weighing from 3.0-4.8 kg were used. Samples of blood were drawn from the common carotid arteries and the blood glucose values determined by the Somogyi method as modified by Nelson (6). In the majority of instances, the animals received the test substance subcutaneously because we found, as had Nord (4), that this was as effective for our purpose as administration by means of stomach tube.

The 'control' animals received a sodium chloride solution adjusted so as to contain the same amount of sodium as the sodium glutamate solution. The 'glutamate' and 'glycine' rabbits received subcutaneously 2 gm/kg of the corresponding amino acid neutralized to pH 7.0 with sodium hydroxide. Only the L-glutamic acid was used and the dosage level was calculated for both amino acids on the basis of the unneutralized acid. To make certain that the results obtained with glycine were not an artifact due either to contamination of the glycine or to an error in preparation of the solution, three separate solutions were made, using in each case glycine from a previously unopened bottle. Similar results were obtained with all three solutions. Blood samples were drawn at zero time, and at 30, 60, 120 and 300 minutes after the administration of the test solution.

In order to study the effects of sodium glutamate and sodium glycine during insulin hypoglycemia, rabbits were given intramuscular injections of insulin in doses from 8-12 U after 24 hours of fasting. One hour after the administration of insulin, the animals received sodium chloride solution, sodium glutamate or sodium glycine at pH 7.0 in the dosage indicated above. Blood samples were drawn 60 minutes after insulin; that is immediately before the administration of the test substance, and 90, 120, 180 and 300 minutes after insulin. The data obtained on these animals were used only if the blood sugar value 60 minutes after insulin was reduced to 66 mg % or less.

A portion of the liver of a number of animals from each group was removed at the termination of the experiment (5 hr. after the injection of insulin or earlier if death appeared imminent) and the amount of glycogen present was determined by the method of Kerr (7), and Kerr and Ghanthus (8).

Photographs of the eyes of nine additional rabbits before and after administration of the test substances or of the control solution were taken with a camera equipped with a stroboscopic flash attachment. Blood samples were not drawn on these animals. The photographs were projected on a screen and the longitudinal and horizontal diameters of the pupil determined.

Blood pressure changes were measured directly from the femoral artery on a kymograph in five rabbits (three given glycine; two, glutamate) from whom blood samples were not drawn. The recording was started before the injection of the test substance, continued during the injection and for 30 minutes thereafter. At the end of this time the blood pressure response was tested with 1 cc of a 1:1000 dilution of epinephrine intramuscularly.

The plasma glutamic acid was determined by the method of Prescott and Waelsch as modified by Himwich and Petersen (9). Pyruvic and α-ketoglutaric acids were measured by the method of Friedemann (10). These determinations were made on a separate group of 12 rabbits receiving 2 gm/kg of glutamic acid at pH 7.0 subcutaneously. The blood samples for this portion of the study were drawn at 0, 60, 90, 120 and 240 minutes.

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TABLE 1. AVERAGE AND RANGE OF BLOOD SUGAR VALUES (MG %) AFTER INJECTION OF SODIUM GLUTAMATE* AND GLYCINE* (PH 7.0)

	Basal	Time, min.			
		30	60	120	300
Control (10)†	80.4 (63–100)	93.2 (72–122)	100.1 (90–112)	93.7 (74–108)	92.2 (70–106)
Glutamate (8)†	93.0 (72–103)	129.0‡ (91–160)	145.6‡ (110–202)	163.6‡ (112–245)	102.7 (45–133)
Glycine (7)†	99.7 (78–106)	137.0‡ (97–202)	177.9‡ (99–285)	207.1‡ (106–405)	150.3 (66–314)

* 2 gm/kg of the unneutralized amino acid.
† No. of animals used.
‡ Significantly different from corresponding control value at 1% level, by analysis of variance.

RESULTS

The subcutaneous administration of glycine or of sodium glutamate at pH 7.0 produced a marked hyperglycemia (table 1). In this table are given only the statistics on those animals who had a basal blood sugar level of 106 mg % or less. Seven 'glutamate' rabbits and eight 'glycine' were discarded on this basis, in order to make the experimental groups more comparable to the control group. Each of the discarded animals, however, in spite of their elevated basal level showed a marked hyperglycemia following the administration of the amino acid. In the case of the 'glycine' animals the rise was evident 15 minutes after the injection of the glycine solution, the average value being 137 mg %; blood samples were not drawn on the 'glutamate' animals at 15 minutes.

Hypoglycemic animals receiving glutamate recovered although the rise in blood sugar was not marked (table 2). In reviewing the data, however, it is evident that the blood sugar levels were higher for these animals than for the comparable 'control' or 'glycine' animals. All the 'glycine' animals, on the other hand, died in less than 5 hours after insulin. This death rate was even greater than that observed in the control animals.

Liver glycogen in the experimental animals with hyperglycemia; i.e., receiving glycine and glutamate but no insulin, was markedly depleted in comparison with that in the control group of animals. The same relative reduction of liver glycogen occurred in the amino acid-treated hypoglycemic animals as in the controls (table 3).

When photographs were taken 10 minu apart under control conditions, the pupill diameters were so variable even in the sa rabbit that no conclusion could be drawn regard to the effects of the amino acids. T pictures leave a strong impression, howev that any changes which occurred could not ascribed to the injection of the neutraliz amino acids.

Significant blood pressure response was n obtained with the injection of the neutraliz amino acids, although the expected rise fo lowed the injection of epinephrine.

The plasma glutamic acid levels after gm/kg of sodium glutamate were highest a hours, (161.0–272.6 mg%) but were still co siderably elevated at 4 hours with a range 75.8–144.5 mg% as compared to the norm plasma value of 2 mg% or less. In 10 of the animals the pyruvic acid fell from the contr level during the first 60 minutes and the tended to rise until at 4 hours it was as high not higher than the basal value. In one of th two atypical animals the fall came at 2 hour while in the other animal the values continue to rise throughout the experiment. In 9 of th 12 animals a marked elevation in α-keto gl taric acid was evident less than 90 minute after the injection of the glutamate. The ris continued throughout the 4-hour period, an resulted in a blood level of α-ketoglut ri acid that was double the basal value in m n animals. The other three animals revealed decrease in α-ketoglutarate with a return t normal values 4 hours after the injection. In n case did the same animal show an atypica response both for pyruvic acid and α-keto

TABLE 2. AVERAGE AND RANGE OF BLOOD SUGA (MG %) IN HYPOGLYCEMIC ANIMALS AFTER INJECTIO OF SODIUM GLUTAMATE* AND GLYCINE* (PH 7.0)*

	Time After Insulin, min.				
	60	90	120	180	300
Control (10)†	38.9 (23–59)	37.4 (15–65)	34.4 (15–51)	32.0 (19–57)	31.6 (13–5[illegible]) 3 dead
Glutamate (10)†	43.5 (19–66)	48.0 (30–67)	45.0 (34–78)	47.2 (27–74)	42.6 (21–8[illegible])
Glycine (6)†	34.9 (21–49)	35.4 (21–49) 1 dead	33.3 (21–44) 2 dead	35.3 (28–40) 6 dead	All dead

* 2 gm/kg of unneutralized amino acid.
† No. of animals used.

TABLE 3. TERMINAL CONCENTRATION OF LIVER GLYCOGEN GM/100 GM

	No.	Glycogen
Control	5	0.879
Glutamate	3	0.116
Glycine	4	0.371
Hypoglycemic		
Control	8	0.034
Glutamate	8	0.008
Glycine	6	0.005

TABLE 4. PYRUVIC AND α-KETOGLUTARIC ACIDS (MG%) IN BLOOD AFTER 2 GM/KG SODIUM GLUTAMATE (PH 7.0)

Time After Injection, min.	Pyruvic Acid	α-Ketoglutaric Acid
0	3.6	3.3
60	2.0	3.5
90	3.5	5.4
120	3.8	5.6

glutaric acid. Data of a typical response are shown in table 4.

DISCUSSION

We found, as has Nord (4), that both glycine and glutamic acid have a marked hyperglycemic effect when injected subcutaneously in doses of 2 gm/kg. The rises in blood sugar shown by our animals receiving 2 gm/kg, however, correspond better to those Nord reported at the 3 gm/kg level than to the values he obtained from animals receiving 2 gm/kg. Perhaps the nutritional state of the animals or the breed of rabbits used would account for this difference.

From the data given here and in the literature (1) it seems evident that an increase in the level of glutamic acid, of glycine or of alanine in the blood of a normal animal disturbs the homeostatic control of blood sugar level. The rise in blood sugar beyond the normal range may be due to stimulation of the adrenal medulla, but equally well from the data available it may be argued that it is caused by an insulin blocking effect, a glucagon stimulating action or a more complex mechanism depending upon stimulation of the pituitary. An adrenergic mechanism was proposed by Nord (4) on the basis that adrenalectomized rabbits failed to respond to glutamic acid or to glycine with a rise in blood sugar. Ginzberg *et al.* (11) on the other hand found adrenalectomy did not influence the protective action of glutamic acid in audiogenic seizures in rats. This discrepancy may be explained either on the basis that glutamic acid has a dual action or that adrenalectomized rats are more physiologically stable than are adrenalectomized rabbits. The failure to obtain a response in terms of blood pressure or of pupillary diameter cannot be considered as evidence against an adrenergic effect since epinephrine is reported to influence metabolism in concentrations below those required to influence blood pressure (12).

Csonka (13) reported that phlorizinized dogs given alanine, glycine or glutamic acid orally excreted nitrogen and glucose in the urine in amounts sufficient to account for the amino acid ingested. From such data it has been argued that these amino acids augment liver glycogen and hence secondarily raise blood sugar. Wilson and Lewis (14) have studied the gluconeogenesis due to amino acids and concluded that *d*-alanine and *dl*-alanine and possibly *d*-glutamic acid but not glycine resulted in a marked deposition of glycogen in rats. These data as well as the rapidity with which hyperglycemia appears after an injection of glycine (15 min.) make it difficult to ascribe the hyperglycemic effect of glycine and of glutamic acid to gluconeogenesis. Moreover, our experiments suggest that, under the conditions here described, a depletion of liver glycogen rather than an increase occurs. Pollak (2) and others (1) have reported that the hyperglycemic effect of amino acids was less marked in undernourished animals.

Glycine and glutamic acid are considered to exert a specific dynamic action (15). However, specific dynamic action has not been shown to occur as early as 15 minutes after a subcutaneous injection. Many early workers in the field of specific dynamic action (1) made comments on the nature of this action which could be interpreted in the light of present knowledge as indicating adrenergic or other endocrine response.

The data obtained on glycine and glutamate with hypoglycemic rabbits is more difficult to explain. It is extremely difficult to compare our animals receiving insulin with those studied by Nord (4) since his rabbits were given a maximum of 0.8 U of insulin while we administered as much as 12 U/animal; also Nord gave insulin and the test substance at the

same time. Our experimental procedure, on the other hand, made it possible to discard any animals that did not react with hypoglycemia by 60 minutes after the injection of insulin. In all, seven animals were discarded on this basis. The observation that glutamate protects animals against hypoglycemia without a marked rise in blood sugar is similar to the data reported by Weil-Malherbe (3) on the arousal of patients in insulin hypoglycemia by glutamic acid as well as that obtained on patients in the same condition by Mayer-Gross and Walker (16). The latter authors showed nearly as good recovery with glycine (50% as compared to 58%) as with glutamic acid; and in a series of only six patients excellent responses were obtained in four subjects given *p*-amino benzoic acid. Mayer-Gross and Walker's (16) results suggest a non-specific action of amino acids in arousing patients from hypoglycemia; Weil-Malherbe, (3) on the other hand, using a casein hydrolysate which would be expected to be rich in glycine as well as other amino acids, did not obtain a response in hypoglycemic patients. Nord (4) under the conditions of his experiments obtained nearly as good results with glycine as with glutamate. He, however, was interested only in preventing the fall of blood sugar caused by a small dose of insulin, while we were attempting to protect the animals against death due to hypoglycemia. Voegtlin, Dunn and Thompson (17) found that alanine but not glycine, nor glutamic acid gave good protection against insulin in rats.

The change of blood pyruvate and α-ketoglutarate after the injection of sodium glutamate was similar to that reported by Klingmüeller and Vogelsang (18) in rabbits although the levels were slightly higher than those obtained by the German workers. It would seem that the rise in α-ketoglutaric acid is due to the deaminization of glutamic acid, while the concomitant fall in pyruvic acid reflects the change of that substance to alanine.

SUMMARY

Rabbits receiving 2 gm/kg subcutaneously of glycine or sodium glutamate in solution at pH 7.0 responded with a marked hyperglycemia. These animals showed a decrease in liver glycogen, but no change in blood pressure. Hypoglycemic rabbits were protected from death by sodium glutamate in doses of 2 gm/kg but not by an equivalent amount of glycine. Rabbits receiving sodium glutamate, 2 gm/kg, responded with a fall in pyruvic acid and a rise in α-ketoglutaric acid in the blood.

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PERMANENT FRACTIONATION OF THE ELECTRORETINOGRAM BY SODIUM GLUTAMATE*

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Although various toxic substances have been reported in the past to affect retinal structures, the retinotoxic properties attributed to sodium l-glutamate are unique. Lucas and Newhouse[1] reported in 1957 that administration of sodium l-glutamate to suckling mice caused degeneration of the ganglion cell layer and failure of formation of the inner nuclear layer of the retina, leaving only the receptor cells intact. No information was given on the electrophysiologic behavior of these eyes. We wished to repeat these experiments in order to study the electroretinogram (ERG) of a retina consisting only of receptor cells. Our results are reported below.

EXPERIMENTAL

White mice (Webster Swiss strain)‡ were injected intraperitoneally with a single daily dose of sodium glutamate beginning on the second day of life. The amount injected was increased slightly each day; and injections

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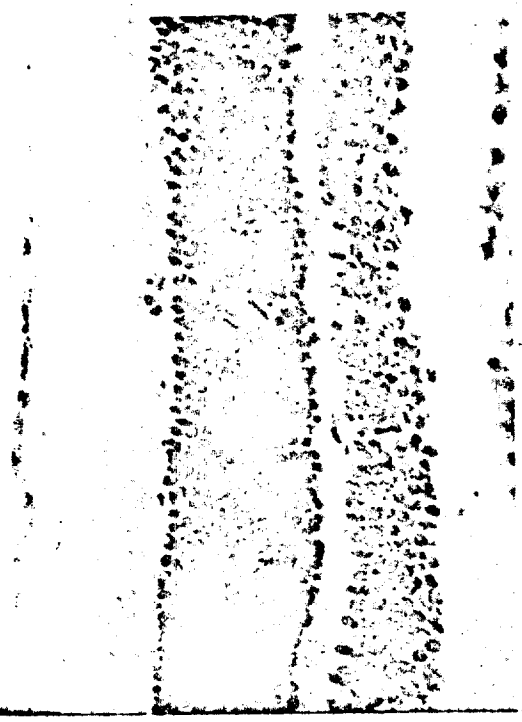
TABLE 1

MOUSE GLUTAMATE DOSAGE

Age (Days)	Dose Mg. per Gm. Body Weight
2	2.2
3	2.5
4	2.8
5	3.2
6	3.4
7	3.6
8	3.8
9	4.0
10	4.2
11	4.4
12	4.6
13	4.8
14	5.0
15	5.2
16	5.4
17	5.6
18	5.8

were continued for from two to 17 days as shown in Table 1. Injection with higher doses caused significant mortality from the procedure.

The eyes of these animals opened on the fourteenth to the sixteenth day after birth. Beginning at the time the eyes opened and



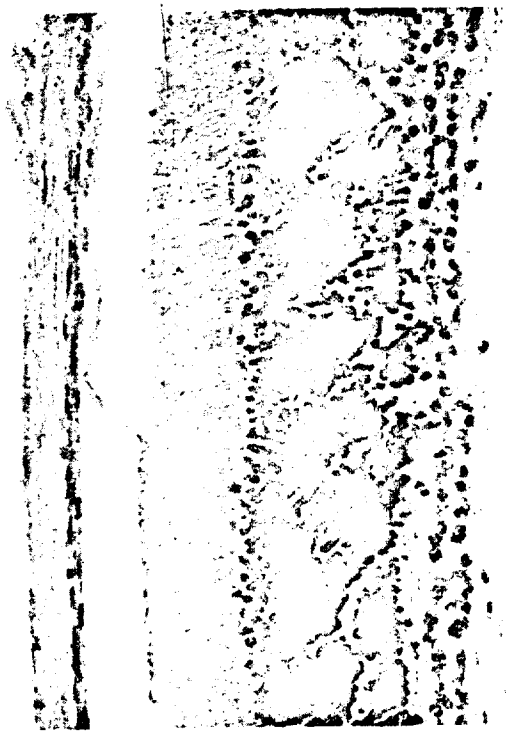


Fig. 2 (Potts, Modrell, Kingsbury). Retina of glutamate treated mouse. (Glutamate 11 days, 54 days old)

at suitable intervals thereafter, the ERG was measured under nembutal anesthesia. Wick electrodes and apparatus were used essentially as described previously by us.[2] Two loops of nickel silver wire appropriately bent and fastened to the supporting board with adhesive tape served at once as speculum and second electrode. At predetermined times an experimental animal and a control animal were killed after measuring the electroretinogram. The eyes were fixed in formalin, imbedded in paraffin, sectioned and stained with hematoxylin and eosin.

In contrast to those of Lucas and Newhouse, our animals were allowed to survive for relatively long periods of time in order to learn how permanent the observed changes in retina and electroretinogram would be.

RESULTS

If injections were continued for seven

TABLE 2

THE EFFECT OF GLUTAMATE ON DEVELOPING RETINAL LAYERS OF THE MOUSE

	Normal Controls							Experimental (All animals 30 or more days old when sacrificed)			
Days After Birth	2	4	6	8	10	Retina Mature 12	Retina Mature 34	Treated with Glutamate for Days: 5	7	9	11
Retinal Layer	Thickness in micra							Thickness in micra			
1) Rod outer segment	—	—	—	—	10	20	30	20	22	22	25
2) Rod inner segment	—	—	—	10	15	20	20	20	20	20	15
3) Outer nuclear layer	20	35	45	55	50	70	70	70	70	70	60
4) Outer reticular layer	—	—	2	10	10	10	10	10	10	10	10
5) Inner nuclear layer		30	65	80	65	80	50	28	15	10	
6) Inner reticular layer	40		20	30	40	60	50	30			
7) Ganglion cell layer		25	30	30	10	10	10	10	15	20	35
8) Nerve fiber layer					15	20	20				

and Newhouse. This consisted of apparently intact receptor cells including the outer nuclear layer. The remainder of the retina was a single or double layer of cells whose nature or function was not ascertainable from the section (figs. 1, 2). Electroretinograms of eyes showing this pattern are uniform in having only an a-wave with no b-wave detectable (fig. 3).

Animals treated for less than seven days showed various degrees of retardation of retinal development. The longer the treatment the more severe the retardation observed. The first portion of Table 2 shows in the mouse as measured with an ocular micrometer on paraffin sections. The absolute measurements are subject to the dimensional changes of fixation and imbedding. The relative measurements from layer to layer and from one animal to the next should be valid. The increase in width and the differentiation of each of the retinal layers may be followed to the twelfth day, at which time development is complete. This may be seen by comparison of the 12 day with the 34 day mouse retina. The second portion of Table 2 shows how development of the inner layers is inhibited by glutamate.

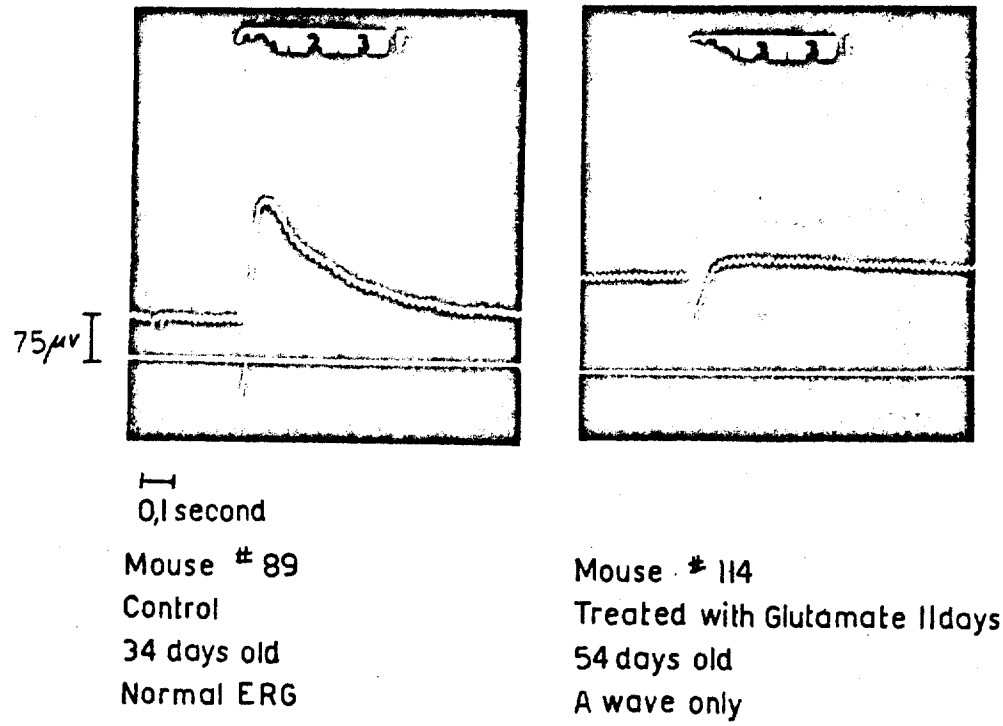


Fig. 3 (Potts, Modrell, Kingsbury). Effect of glutamate on mouse ERG.

[illegible] a normal ERG is found, the inner [illegible] layer is smaller than normal in [illegible] Figures 4 and 5 show the microscopic [illegible]ance and the electroretinographic his- [illegible] one such animal. Although single [illegible]ples are presented here, the experi- [illegible] have been repeated by us many times [illegible] the correlation of the histologic picture [illegible] ERG is uniform.

Discussion

[illegible]here appear to be certain inescapable [illegible]lusions from the findings presented here. [illegible] first of these is that only the initial [illegible]ative a-wave is connected with the physi- [illegible]resence of the receptor cell layer. For [illegible]pearance of the b-wave, the presence [illegible] intermediate cell layers is necessary. [illegible] conceivable, of course, that some por- [illegible] of the receptor cell responsible for the [illegible]ve is damaged concurrently with bipolar [illegible] but that this damage does not result in no experimental evidence to support such an hypothesis.

It is unlikely that the ganglion cells need to be considered in such an analysis, for there is a body of evidence on the integrity of the ERG after optic nerve section and ganglion cell degeneration (for example 3, 4).

There is one previous report which substantiates these conclusions. Noell[3] described the ERG in one eye of a single monkey where the blood supply had been interrupted at the optic nerve and the eye allowed to recover. Degeneration of the inner layers resulted and with this, a negative ERG was observed.

Transient abolition of the b-wave is not infrequently observed following the action of toxic substances on the eye. Granit's analysis of the ERG into three processes was partly based on such a finding.[5] Our earlier studies on the ERG in methanol poisoning[2,6]

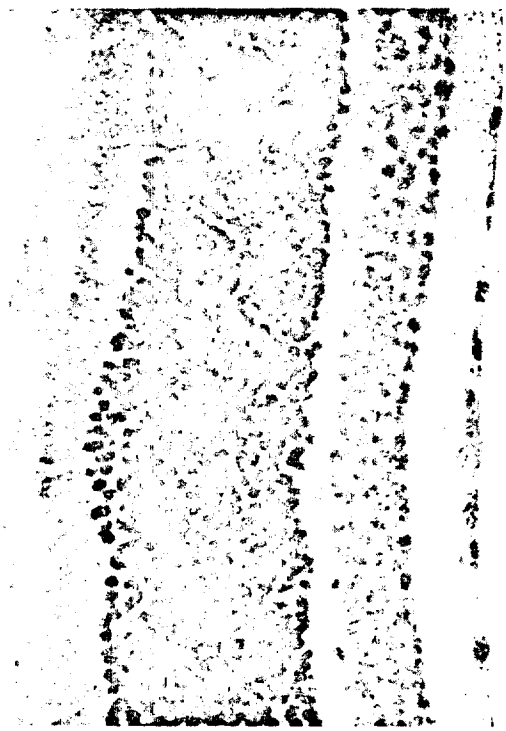
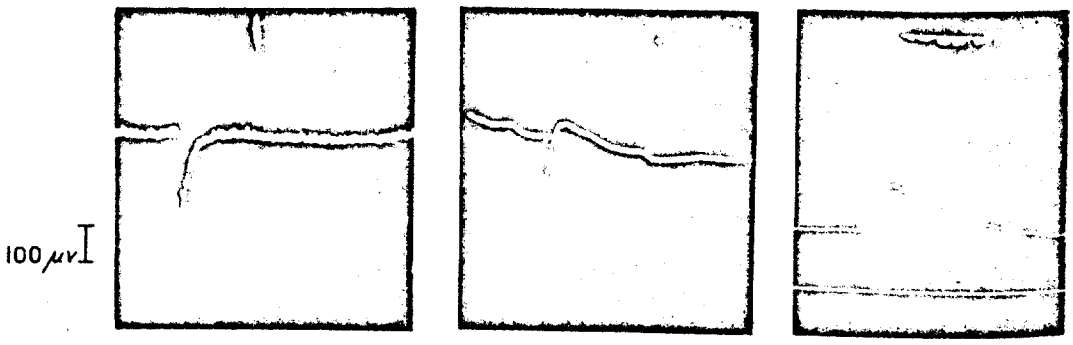


Fig. 4 (Potts, Modrell, Kingsbury). Retina of mouse treated with glutamate five days only. (55 days old)

pounds. It is attractive to hypothesize that these effects represent transient selective inhibition of the bipolar cells while the receptor cells continue to function.

A further question of interest is the mechanism of action of glutamate. It is not an expected physiologic finding that a normal metabolite even in high concentration should have such a destructive effect. The three mechanisms suggested by Lucas and Newhouse all have certain drawbacks voiced by these authors. The suggested indirect effect by adrenal stimulation is unlikely, because other substances such as glycine and p-aminobenzoic acid known to stimulate the adrenal do not affect the retina. The suggestion that the glutamate effect on maintenance of high retinal potassium might play a part is unlikely also. It has never been demonstrated that the presence of glutamate which helps maintain normal levels can cause abnormally high potassium ion concentration in the retina. Further, there is no evidence that abnormally high potassium is selectively deleterious to the inner retinal layers. The suggested interference with carbohydrate metabolism is based on the observed depression of anerobic glycolysis by glutamate which is restored by ATP. An uncoupling effect on oxidation and phosphorylation like that of



Mouse # 110
Treated with Glutamate 5 days

18 days old	26 days old	55 days old
A wave only	Beginning B-wave	Normal ERG

trophenol has also been described. However, dinitrophenol itself will not cause damage to the inner layers of the retina, and ATP will not reverse the glutamate effect.

It seems important to us that the glutamate effect is easily observed only in the developing animal. In the few attempts we made, we were unable to produce the effect in adult mice. In surveying developmental biochemical phenomena, we were impressed by the newer concepts of repression of enzyme formation by products of the specific reac- [illegible] Several glutamic acid enzymes have [illegible] found to increase suddenly in the embryonic chick retina at about the time of hatch- [illegible] and Waelsch has postulated that the glutamate-glutamine system may act as a metabolic buffer.[10] Thus it seems to us that another explanation for the observed effect is most likely—that is: the repression of development of the inner retinal layers by large doses of glutamate given during the period when these layers should be developing. We believe that an enzyme whose reaction product is glutamate (for example glutaminase), [illegible] repressed by the high glutamate level. If [illegible] enzyme is essential to development of [illegible] inner layers because of its participation in a homeostatic system, formation of these layers will be inhibited. If the inhibition is maintained past some critical point, it will be too late for development to occur at all. Experimental investigation of this hypothesis is now under way in the laboratory of one of us (A.M.P.).

Summary

1. Inhibition of the formation of the inner retinal layers in newborn mice by the method of Lucas and Newhouse was repeated by us.

2. Such animals whose retinas are essentially a pure culture of receptor cells show an ERG consisting of a negative a-wave only.

3. In animals receiving inadequate doses of glutamate and showing a normal ERG, the intermediate cell layers are always present.

4. This is considered strong evidence for the origin of the b-wave in the intermediate cell layer.

5. A new hypothesis for the mechanism of glutamate toxicity is proposed.

University of Chicago (37).

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Discussion

Dr. John E. Harris (Minneapolis): This is a particularly easy paper to discuss in certain respects. I have no reason to quibble with the technique. I have no reason to question the results. And it is very fascinating because I can speculate freely as to why glutamic acid is doing this sort of thing. So it has all the attributes of being the perfect paper for a discussant.

As to the interpretation of the electroretinogram, I see no reason to question the conclusion that the beta wave does indeed arise from the bipolar cell layer. I am not particularly knowledgeable in this field, but this suggestion has been made in the past and Dr. Potts' data would seem to confirm it.

I would therefore like to carry on where Dr. Potts left off, and just speculate a bit about the mechanism by which glutamic acid causes these changes in the retina of the developing mouse.

It is a bit surprising to find glutamic acid this toxic. The retina does normally pick up glutamic acid in vitro as shown years ago by Krebs and associates. Indeed these workers found glutamate to be essential for the accumulation of potassium and excretion of sodium by the isolated surviving retina. Whether it plays a similar role in vivo cannot be stated

Among the possibilities I would like to touch on briefly is the question of the potassium concentration raised by Dr. Potts. I am not thinking here that an excessively high potassium concentration may result from administered glutamic acid but rather the reverse, that the potassium may be depleted from the retina. This is a reasonable extension of some of our studies of the lens. For example, we know that glucose or some metabolic residues are required for potassium uptake by the lens in at least certain circumstances. If the glucose concentration is raised, the potassium uptake and sodium excretion may be blocked. In a similar manner an excess of glutamate might block potassium accummulation in the young mouse retina.

However, I don't necessarily believe this is the mechanism by which the toxic effect of glutamic acid is being exerted. There are two other things I would like to bring out. May I ask the projectionist to show Dr. Potts' last slide, please?

(Slide) The first concerns the question of the toxicity of ammonia itself. The body goes to a great deal of trouble to protect itself from ammonia. Ammonia is quite toxic. The toxicity of hepatic coma for example is considered due to an accumulation of ammonia, and if one floods the system with glutamic acid here (pointing) it would be feasible that we would get an accumulation of an excessive amount of ammonia.

Actually, this does belie in a sense the fact that glutamic acid is used to treat hepatic coma. In this case, of course, glutamic acid is presumably removing ammonia by forming glutamine. However, as Dr. Potts has pointed out, if the young mouse it is quite feasible that ammonia toxicity may result.

The second thing that I think is worth while noting is the possibility that an abnormal protein may be formed. This is known to occur as we deplete the diet of an essential amino acid, for example. Contrariwise if we add an excess amount of one amino acid we can form an abnormal protein. This is something that must be kept in mind.

Moreover, where an abnormal protein is being formed, the effects may not be manifest for quite some period of time. Therefore, I am struck with the possibilities of further studies along this line and the significance that they may ultimately have in our understanding of the group of diseases that we call the abiotrophies. In this group of diseases we assume there is a metabolic fault. This metabolic fault could seemingly come from the formation of an abnormal protein or perhaps an abnormal enzyme.

This is the type of study that I think will lead in the future to a much better understanding of these congenital or developmental conditions which may manifest themselves at birth or may not be apparent for several years.

Dr. Werner Noell (Buffalo): This is very fascinating work, and many people wondered, when the paper by Lucas and Newhouse came out, how specific the effect is. Dr. Potts indicated that he believes this is a direct action of glutamate on the retina. I would like to know what evidence he has in support of it.

Glutamate, as Dr. Potts undoubtedly knows, does not go into the brain and probably also not into the retina if injected intravenously or intraperitoneally in the adult animal. It may possibly go in in the young. In the brain it is very well known that no glutamate goes in. All that goes in is glutamine.

In the adult retina we did experiments with glutamate, injecting very high doses intravenously, and found no acute effects on retinal function whereas if you get glutamate directly into the retina or directly in the neighborhood of the ganglion cells you see very striking effects, glutamate acting just like potassium chloride. The same happens if one gives glutamate in the other cerebral ventricle. The animals appear to be very rapidly affected in a similar way as with potassium chloride.

With regard to the biochemical slide, I don't think I would call this transaminase, as Dr. Potts indicated, but glutamic acid dehydrogenase—the reaction of alpha-ketoglutarate to glutamic acid. This particular enzyme is known to lag in development in the rabbit during the early stages of life, and later on, between 30 and 60 days, it increases quite remarkably. I personally feel, for instance, that the increase in glutamic acid dehy-

the late B wave that one sees during development.

DR. A. M. POTTS (closing): I would like to thank the discussers for their cogent remarks. I think you can see from the discussion that our work is cut out for us as long as we care to study this particular problem.

The question of ammonia toxicity and the formation of an abnormal protein are certainly things that have to be investigated.

Concerning the penetration of glutamate into the retina, one has the evidence of Krebs in vitro that, whatever else glutamate does, it does facilitate the penetration of potassium into the retina. Whether it works at the front door or whether it works inside the cell only in vitro, glutamate does affect an aspect of retinal metabolism. We do plan, however, to do a study with labeled glutamate on animals of the age that show this effect, in order to find out exactly how much goes precisely where.

As far as the slide on mechanism is concerned, this was presented not as a hard and fast hypothesis, as we said, but simply as a possible suggestion. In order to inhibit the synthesis of newly formed enzymes one must be dealing with an enzyme of which the inhibiting substance is the product, not the substrate. Even though the dehydrogenase may very well become involved in time, the fact that it operates on glutamate makes it less likely in our mind to be the specific enzyme that is immediately concerned.

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Behavioral Changes in Adult Rats treated with Monosodium Glutamate in the Neonatal Stage

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Abstract—Three doses (1.25, 2.5 and 5 g/kg) of monosodium glutamate (MSG) and water (control) were given to 4 groups of litter mates of Holtzman rats respectively, all in volumes of 10 ml/kg, by stomach tube daily from the 5th to the 10th day after birth. Observed during the first 3 months of age, the gain in body weight in the MSG-pretreated rats appeared to be less compared to controls, the gain being least in the 5 g/kg dose group. The rats of the latter group also showed less spontaneous motor activity and deficiency in discrimination learning as in a maze experiment. Learning of a fixed-ratio food reinforcement schedule was however not affected.

Introduction

Monosodium glutamate (MSG), widely used as a food additive and suggested to be the cause of Chinese restaurant syndrome (1, 2), has been reported to produce some effects on the central nervous system (3). MSG administered to 2 to 9 days old mice induced in adults a syndrome characterized by stunted skeletal development, marked obesity and female sterility, and suggestive of neuroendocrine disturbance (4). MSG has been shown to cause necrosis in certain areas (e.g. floor and roof of the third ventricle, preoptic and arcuate nuclei of the hypothalamus) of the brain in such new born mice (4, 5) as well as in a new born monkey (6). However, other investigators (7–9) failed to demonstrate any hypothalamic lesion in several species of MSG-treated neonatal animals including mice, rats, dogs and monkeys. The present investigation was undertaken to examine any possible behavioral deficit in adult rats treated with MSG during their neonatal stage.

Methods

Eight litter mates from each of ten pregnant Holtzman rats were initially selected for this study and divided into 4 groups consisting of 2 litters from

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each mother. Litters of these groups received water (control) and MSG solution (1.25, 2.5, 5 g/kg) respectively, all in volumes of 10 ml/kg by stomach tube daily from the 5th to 10th day after birth. At the 21st day these weanlings were identified as to their sex and placed in separate cages.

Their body weights were recorded from the 5th day, and food and water consumptions measured from the 31st day onwards daily for 6 days a week until they were about 3 months old. Records for food intake of some rats were found to be incomplete and a number of rats died during this period. Therefore the food intake data (Table I) involve only 30 rats with complete record.

The surviving rats were subjected to three behavioral situations. For technical difficulties it was not possible to handle more than 6 rats from each drug group. However there were only 4 in 2.5 mg/kg group, thus making a total of 22 rats used for behavioral studies (Table II). During the first two weeks their spontaneous motor activity was measured in two actophotometers described earlier (10). During this period they were trained to run in a T-maze for food reinforcement. Fifteen trials were made every day for 5 days a week for 2 weeks. During the next three weeks they were trained for fixed-ratio (FR) food reinforcement as already described (10). The rats were deprived of food for 23 hours before the food-reinforced behavioral sessions. In the FR food reinforcement schedule, FR was gradually raised up to 24 over a 3-week period. When the response rates of these rats were stabilized, they were injected i.p. with amphetamine sulfate (0.1–1 mg/kg) according to our previously described procedure (11).

Results and Discussion

Table I shows the average daily food intake and total weight gain of normal and MSG-pretreated rats during the first 3 months of age. Weight gain appears to be less in the MSG-pretreated rats when compared to the controls within

TABLE I

Food intake and weight gain in normal and MSG-pretreated rats

MSG (g/kg)	Food intake (g)*		Weight gain (g)*	
	Male	Female	Male	Female
–	21 (2)	15 (6)	324	196
1.25	19 (8)		265	
2.50	18 (2)	18 (4)	276	182
5.00	15 (5)	20 (3)	237	159

* Average daily food intake and average total weight gain. — Figures within parentheses indicate number of rats in each dose group for both parameters.

the same sex groups, the gain being least in the 5 g/kg dose group. Similar growth suppression has also been reported in other studies with MSG in larger number of rats (12). Food intake varied in both sex groups and could not be correlated to their weight gain.

Table II summarizes the data on behavioral activities. Measurement of spontaneous motor activity did not show any significant difference between the control group and the groups pretreated with 1.25 and 2.5 g/kg of MSG. However, the rats pretreated with 5 g/kg of MSG showed significantly less activity.

TABLE II

Behavioral activities in normal and MSG-pretreated rats

Behavioral situations	Dose (g/kg) group (1)			
	0(6)	1.25(4)	2.5(6)	5(6)
Spontaneous activity (2)	933 ± 48	901 ± 100	832 ± 69	750 ± 50 (5)
Maze running (3)				
% score	92 ± 2	85 ± 6	83 ± 5	47 ± 12 (6)
Latency (sec)	4.6 ± 0.2	9.1 ± 1.5 (6)	6.0 ± 0.4 (6)	9.3 ± 1.7 (6)
FR (food) reinforcement (4)				
Number of responses	1405 ± 219			1085 ± 230
Effect of amphetamine	−67 ± 17			−54 ± 15

(1) Figures in parentheses indicate number of rats in each group, except for FR reinforcement in which 5 rats were in each group. — (2) Average of 5-day data during the second week of trial. — (3) Data from the 10th session. — (4) Data on responses are from the 19th session. Effect of amphetamine (0.5 mg/kg) on responses from a subsequent session is given as % change from control. — (5) $P < .05$, and (6) $P < .01$ compared to controls.

A similar trend was also observed in maze learning. The number of correct scores made at the 10th session by the rats pretreated with 5 g/kg of MSG was significantly less than those of the control or other MSG dose groups. The average latency for the correct responses (i.e. the amount of time required to run the maze) was significantly longer in all MSG-pretreated rats, but the effect was most marked in the 5 g/kg dose group.

Since the behavioral changes were most marked in the group pretreated with 5 g/kg, only this group along with t[illegible] for training

in FR food reinforcement schedule. Five rats were in each group. On the 19th session FR could be raised up to 24 for 4 rats from each group. The average total response of these rats appears to be higher for the control group, although their difference was not statistically significant.

In absence of any significant difference between the responses of these two groups, it was considered pertinent to challenge these groups with some drugs. Amphetamine was selected for this purpose because of our previous investigation (11) on the effect of this drug on FR reinforcement schedule. It did not show any significant difference in its effects on the rats of the respective groups at the doses used. Table II presents the effects of 0.5 mg/kg dose.

Thus these behavioral studies showed that FR reinforcement was least affected, whereas maze learning was affected most due to MSG treatment. It may be parenthetically added that a similar effect of MSG was indicated in an earlier preliminary experiment on maze learning with 7 rats similarly treated with 5 g/kg dose and 5 rats as controls. Average per cent scores of correct trials for the control and the treated rats were 62 ± 14 and 29 ± 11 on the 9th session and 81 ± 13 and 44 ± 14 on the 16th session respectively.

It appears that discrimination learning as in the maze experiment was mostly affected due to MSG administered at the neonatal age. This study thus indicates the development of a behavioral deficit in the adult following treatment of new born rats with a high dose of MSG. It is difficult to project the implications of the effects of such a high dose of MSG to human infants at this stage.

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dl-GLUTAMIC ACID HYDROCHLORIDE IN TREATMENT OF PETIT MAL AND PSYCHOMOTOR SEIZURES

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In the majority of cases the treatment of epilepsy is moderately satisfactory.[1] Grand mal attacks are controlled completely or reduced in frequency by phenytoin or phenytoin-phenobarbital combinations in the majority of cases. Psychomotor attacks yield less readily to anticonvulsant therapy. Petit mal attacks are infrequently affected by medication but respond favorably to fasting[2] and the ketogenic diet.[3]

The hypothesis advanced by Wilder[3] and supported by Keith's experiments[4] that the mechanism of the ketogenic diet depended on the sedative effect of the ketone bodies was not generally accepted. Fay[5] and McQuarrie[6] attributed the effect to dehydration incidental to ketosis. Bridge and Iob[7] were doubtful that either to acidosis or to ketosis alone could be ascribed anticonvulsant properties.

Maintenance of a dietary regimen that will produce ketonuria is not a simple task. Hospitalization and an intensive educational program for the patient and his parents are necessary. A search for acidosis producing drugs was the outgrowth of these disadvantages of the ketogenic dietary regimen. We have used ammonium chloride (2 cases included in this series) without striking results. Azosulfamide[8] reduced the frequency of seizures moderately and concomitantly lowered the p_H of the blood. Brilliant vital red also seemed to have a moderate anticonvulsant effect.[9]

Putnam and Merritt[10] suggested that the "physiologic" anticonvulsants dextrose, carbon dioxide, pyruvic acid and acetoacetic acid and the synthetic anticonvulsants produce their effect by giving rise to an acid milieu within or about nerve cells. The synthetic anticonvulsants are of a composition which suggests that they are broken down by cellular activity, perhaps chiefly in the brain, to stable acid products such as benzoic acid. Anticonvulsant treatment of epilepsy is suggested as a substitution rather than drug therapy.

The use of *dl*-glutamic acid was suggested by one of us (H. W.) on the basis of the following considerations:

It is known that some racemic amino acids, among them *dl*-glutamic acid, give rise to the excretion of the unnatural isomer in the urine when administered to animals. If *d*-glutamic acid has a similar metabolic inertia[11] in human metabolism, 1 mole of *d*-glutamic acid hydrochloride should furnish 1 mole of hydrochloric acid and two carboxyl groups of which one would be available for acidification. The *l*-glutamic acid hydrochloride also furnishes one equivalent of hydrochloric acid.

Furthermore, *l*-glutamic acid has been stated to be intimately related to brain metabolism. It is the only amino acid known to be metabolized by slices of brain tissue.[12] Brain tissue contains an enzyme which synthesizes glutamine from *l*-glutamic acid and ammonia, decreasing by this mechanism the concentration of free ammonia in the tissue.[13]

Patients with seizures usually associated with slow wave activity in the electroencephalogram were selected for treatment. The amount of the amino acid hydrochloride required to maintain the p_H of the urine at about 5.0 was 4 Gm. three times a day. Larger doses,

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16 and 20 Gm., were administered in 2 cases. Oral administration of the drug in dilute solution or capsules was seldom associated with gastric distress. Anticonvulsant therapy, though ineffective, was continued throughout the trial period.

Nitrazine paper tests of the morning specimen of urine were made by the patient in the majority of instances. This test gives the relative degree of acidity within half a p_H unit approximately. The *dl*-glutamic acid hydrochloride was prepared by one of us (H. W.).

REPORT OF CASES

Case 1.—K. Mac., aged 12 years, had had petit mal seizures which recurred at a frequency of about forty to fifty a day for four years. A six months trial of the ketogenic diet reduced the seizures moderately. The patient refused to continue the rigid regimen.

General physical and neurologic examinations were within normal limits. Negative laboratory tests included a complete blood count, urinalysis, a blood Kline test, psychometric studies and x-ray examination of the skull. Frequent petit mal seizures were accompanied by typical three per second wave and spike formations in all leads of the electroencephalogram.

Maximum tolerated doses of phenytoin sodium and phenobarbital reduced the seizures slightly; that is, the number of days on which fifty seizures occurred were reduced. *dl*-Glutamic acid hydrochloride administration was accompanied by a sharp decrease in seizures. The dose required to maintain a urinary p_H of about 5.0 was 12 Gm. daily. Attacks were reduced to about five to twenty-five a day in contrast to twenty-five to fifty.

In addition to the reduction in seizures, the patient's parents and instructors in school noted a decided improvement in the patient's personality. He accepted disciplinary measures without prolonged arguments. He was more congenial, cooperative and affable. Peculiar eccentric aptitudes were decreased. Concentration and application in his school work were improved.

Ammonium chloride (4.0 Gm. daily) administered for five weeks failed to influence the frequency or severity of seizures.

In this case *dl*-glutamic acid hydrochloride reduced the total number of seizures and produced an improved physical and mental alertness pleasing to patient, parents and other interested parties. Ammonium chloride proved ineffective.

Case 2.—M. B., aged 13 years, had had petit mal seizures (fifteen to thirty daily) for three to four months. Mild depressions were noted occasionally as a result of the recurrent seizures. General physical and neurologic examinations gave negative results. Negative laboratory examinations included a blood count, urinalysis, a blood Kline test, an x-ray examination of the skull, a pneumoencephalogram and psychometric studies. The electroencephalogram showed a pattern commonly associated with convulsive disorders, petit mal type.

Phenytoin sodium and phenobarbital therapy was ineffective. *dl*-Glutamic acid hydrochloride reduced the number of seizures to two to five a day. The p_H of the urine was lowered to 5.0 in forty-eight hours and maintained at this level. The decrease in frequency of the seizures paralleled the lower p_H values of the urine.

The ingestion of the drug was accompanied by an immediate improvement in the clinical condition of the patient. In the past nine months the drug has repeatedly been discontinued with the result that the symptoms have immediately recurred. Improvement has followed readministration of the drug in adequate doses.

The decrease in seizures was accompanied by an improved mental and physical alertness. Exacerbation and remissions of symptoms were closely correlated with glutamic acid therapy.

Case 3.—C. H., aged 7 years, had recurrent petit mal seizures which had resisted phenytoin sodium, phenobarbital and a combination phenytoin-phenobarbital therapy. General physical and neurologic examinations gave negative results. Negative laboratory examinations included x-ray examinations of the skull, psychometric studies, urinalysis, a blood count and a blood Kline test. The electroencephalogram revealed a pattern commonly associated with convulsive disorder, petit mal type.

Following the determination of tolerant doses of phenytoin sodium and phenobarbital, *dl*-glutamic acid hydrochloride was administered. The drug in concentrated solution caused severe gastric distress. He later rejected dilute solution (in water, milk, fruit juices). During this period of inadequate treatment petit mal seizures increased from six to ten to twenty to twenty-five a day.

This is the lone instance of severe gastric distress caused by the drug. Obviously the concentrated solution caused the gastric discomfort. The increased frequency of seizures was related to the emotional response rather than to the effect of the drug. Petit mal seizures are often increased in frequency and severity by excitement, worry, stress and strain.

Case 4.—S. K., a girl aged 8 years, had a recurrent abdominal pain which was first noted at the age of 6 years. Acute pain followed by vomiting recurred weekly for four months. The recurrence of pain ceased, but frequent short periods of staring were noted by the parents. Subsequently this symptom was accompanied by right sided signs (facial asymmetry, clonic movements of the right hand and face and a tendency to fall to the right). Though predominantly on the right, seizures have been noted to occur on the left side for periods of as long as two weeks. The frequency of seizures varied from one hundred to two hundred a day. Personality disturbances (disobedience, destructiveness, cruelty to her playmates and indifference) were not related to the frequency of seizures or medication and responded readily to the use of customary disciplinary measures.

Pneumoencephalograms have been normal on three occasions. Psychometric studies disclose an average intelligence (intelligence quotient 101 Stanford-Binet, 114 Arthur). The electroencephalogram repeatedly demonstrated a focus of slow wave activity in the left temporoparietal area.

Right facial weakness and impairment of motor function, voluntary and involuntary, in the right arm and leg has progressed slowly. She is now unable to write with her right hand.

Various combinations of phenytoin sodium, phenobarbital, thyroid, *dl*-pyrrolidone carboxylic acid and *dl*-glutamic acid hydrochloride, ammonium chloride, ketogenic diet, nicotinic acid and methyl-ethyl phenobarbital were administered. Phenobarbital in increasing dosage to 0.3 Gm. daily was moderately effective over the longest period of time any one drug was administered. *dl*-Glutamic acid hydrochloride in combination with phenobarbital was strikingly effective. Except for an increase in the number of seizures accompanying the excitement of going home, the greatest number of days on which twenty-five seizures or less could be counted was noted within the ensuing two months. Five seizures or less were noted for two consecutive weeks. Seizures recurred at the pretreatment frequency immediately after withdrawal of the drug. Renewal of treatment was not accompanied by a decrease in frequency of the attacks, but this fact is difficult to evaluate because thyroid in toxic doses was administered during the second trial period.

Methyl ethyl phenobarbital in conjunction with nicotinic acid and phenytoin sodium was also accompanied by a striking improvement. Initially the seizures decreased in severity and later in frequency. For two months from one to ten seizures occurred daily, accompanied by a moderate return of function of the right hand. Unavoidably methyl ethyl phenobarbital was discontinued, and the seizures immediately recurred.

On readmission to the hospital, right sided seizures recurred at intervals of five to fifteen minutes.

Evaluation of medication is difficult, but we were impressed with the response accompanying *dl*-glutamic acid hydrochloride therapy, in conjunction with phenobarbital.

CASE 5.—J. R., a youth aged 21, had recurrent psychomotor seizures preceded for an hour of repeated myoclonic twitches and mental confusion, which were decreased by phenytoin sodium, phenobarbital and thyroid.

General physical and neurologic examinations were negative. Negative laboratory examinations included a complete blood count, urinalysis, x-ray examinations of the skull, pneumoencephalogram, psychometric studies and blood Kline and sugar tolerance tests. The basal metabolic rate was minus 18, minus 22 and minus 20 and serum cholesterol was 271 mg. per hundred cubic centimeters. The electroencephalogram showed a pattern commonly associated with convulsive disorders.

Seizures recurred at weekly intervals without treatment. Administration of phenytoin sodium, phenobarbital and thyroid in maximum tolerated doses was accompanied by a reduction in seizures, namely one attack every one to three weeks. Extra phenytoin sodium and phenobarbital administered after the onset of the aura had no effect on the impending seizures.

Ammonium chloride (4 to 6 Gm. daily) reduced the frequency of seizures slightly, but the appearance of severe acne prevented its prolonged administration.

After addition of *dl*-glutamic acid hydrochloride to the foregoing combination, seizures could be prevented by the use of phenytoin sodium (0.1 Gm.) and phenobarbital (0.1 Gm.) during the phase of the aura. Attacks were prevented for a period of three months, the longest interseizure interval in two years.

The dosage required to maintain a urinary p_H of 4.5 to 5.0 was 12 to 20 Gm.

Equally encouraging was the subjective improvement. Parents, friends and business associates volunteered the information that the patient's behavior had changed for the better. He was decidedly more affable. His gymnasium instructor reported an improved physical alertness. His assistant noted an increased mental acuity. For the first time he desired and sought social contacts outside the family circle. Hospital attendants noted a decided improvement in cooperation in respect to both hospital routine and clinical tests.

In this case decided psychologic improvement followed an increased mental and physical alertness, which has aided measurably in social and economic rehabilitation. Seizures recurred at one to four week intervals following withdrawal of the drug irrespective of supplementary medication after the onset of the aura. Though the major portion of the psychologic improvement has been maintained, there has been no progress on other forms of medication.

A regimen designed to determine the effect of *dl*-glutamic acid hydrochloride on the acid-base balance of the blood has recently been instituted. Twelve Gm. doses of the drug have failed to alter the acid-base balance and likewise to affect the frequency of seizures. Failure to respond is probably associated with an intake of excessive doses of thyroid, as in case 4. Symptoms of thyroid intoxication (weakness, sweating, fatigue, restlessness and emotional lability) have been noted recently. After thyroid was discontinued the favorable response to glutamic acid has been reproduced.

Even though seizures may be aborted by supplementary therapy after the onset of the aura, the latter occurs more frequently during the period of *dl*-glutamic acid hydrochloride therapy. The aura is associated with the fast activity and the psychomotor seizures with slow wave activity in the electroencephalogram. This suggests that the drug is effective only in seizures associated with slow wave activity. Improved mental and physical alertness have aided measurably in social and economic rehabilitation.

CASE 6.—E. J., a man aged 25, had a moderately severe head injury at the age of 3 years; this was the only suggestion of an etiologic factor disclosed in the history. Unlike his brother and sister, the patient had always been irritable, restless, emotionally labile (temper tantrums, indifference, depressions) and retarded mentally (school work difficult and far below standards of the family). Anxiety states resulting from recurrent periods of mental dulness prevented employment. He resented his mother's habit of repeatedly recounting his inadequacies to him and others. Though he honestly attempted to maintain employment, recurrent grand mal, petit mal, psychic equivalents and periods of mental dulness prevented contact with the public.

Hemiatrophy of the left arm and leg suggested the presence of diffuse involvement of the right hemisphere of the brain, probably post-traumatic in origin. Confirmation of this opinion was demonstrated in the electroencephalogram, namely a diffuse abnormal pattern was most severe in the right frontal area.

Except for psychic equivalents and periods of mental dulness the patient has been symptom free with phenytoin sodium (0.4 Gm.) and phenobarbital (0.1 Gm.) therapy.

dl-Glutamic acid hydrochloride was administered in 12 Gm. doses daily. Objectively the psychic equivalents and periods of mental dulness were unaffected. Subjectively interseizure periods were accompanied by an increased mental and physical alertness new to the patient. Though not dramatic, the effect was definitely encouraging to him. The drug was discontinued suddenly six weeks later, following which symptoms were intensified and a grand mal seizure occurred, the first in seven months. Readministration of the drug was accompanied by an improvement similar to the previous one. Exacerbation of the symptoms again followed interruption of treatment four weeks later.

In this case subjective improvement accompanied ingestion of *dl*-glutamic acid hydrochloride. Interruption of treatment was followed by an exacerbation of symptoms, and improvement occurred on reinstitution of therapy. Exacerbation of symptoms commonly associated with irregular inadequate treatment of epilepsy is exemplified in this case.

CASE 7.—M. B. S., a woman aged 41, had onset of seizures at the age of 20. Severe social, educational and economic restrictions have been a serious handicap. Seizures were strikingly reduced by phenytoin sodium. Personality disturbances combined with social maladjustment prevented a satisfactory rehabilitation following control of the seizures. Severe emotional instability is far more incapacitating than infrequent psychic equivalents, grand mal, petit mal and psychomotor attacks.

General physical and neurologic examinations have been repeatedly negative. Negative laboratory studies include x-ray examinations of the skull, psychometrics, urinalysis, complete blood count and blood sugar determination. The electroencephalogram disclosed a pattern consistent with convulsive disorder.

dl-Glutamic acid hydrochloride in conjunction with phenytoin therapy reduced petit mal seizures and psychic equivalents. A daily dose of 12 Gm. maintained a urinary p_H of about 5.0. Psychomotor and grand mal attacks were also reduced in frequency.

Improved mental and physical alertness was noted objectively and subjectively. Tolerance of the opinions of others was noticeably improved. Insight was acquired into her pronounced introversion, egocentric responses to minor stimuli, recurrent anxiety states and mild depressions. The efficiency with which she performed the details of her position as a librarian was augmented by the increased mental alertness. Gymnasium exercises became less laborious.

In this case petit mal attacks and psychic equivalents were decreased during the administration of *dl*-glutamic acid hydrochloride. A decided regression of mental and physical alertness followed withdrawal of the drug. Periodic rest in bed and a winter vacation were required to overcome a serious emotional instability.

CASE 8.—J. K., aged 20, had had recurrent grand mal seizures for three years. General physical and neurologic examinations were negative. Negative laboratory examinations included a complete blood count, blood Kline test, urinalysis, x-ray examinations of the skull and psychometric studies. The electroencephalogram demonstrated a pattern commonly associated with convulsive disorders.

dl-Glutamic acid hydrochloride in 12 Gm. daily doses reduced the urinary p_H to about 5.0. Increased mental and physical alertness was noted, but only to a slight degree.

Seizures occurred at the pretreatment rate of one every one to two weeks. Phenytoin sodium in 0.4 Gm. daily doses relieved the patient of seizures but had no effect on mental or physical alertness.

In this case, as noted previously, grand mal seizures were not affected by *dl*-glutamic acid hydrochloride.

COMMENT

Tolerance to *dl*-glutamic acid hydrochloride has proved surprisingly high. Gastrointestinal symptoms have been minimal. Objections to the taste have been alleviated by administration of the drug in capsules. Furunculosis has not occurred. Renal irritation has not been noted.

Improvement was noted in those cases in which the seizures are related to slow wave activity in the electroencephalogram, namely petit mal and psychomotor types. Seizures are decreased in frequency on medication which previously had been proved either ineffective or partially satisfactory. Petit mal seizures have been decreased from thirty to fifty to five to twenty-five daily in 1 case and from fifteen to twenty to three to five in another.

Universally, mental and physical alertness have been increased. The degree of improvement in mental efficiency cannot be correlated with the incidence of seizures. Without solicitation the patient, family and other interested parties have volunteered a favorable response to *dl*-glutamic acid therapy. Usually the patient is noted to be more energetic and happier, mood swings are less pronounced, behavior mannerisms are ameliorated and he is more congenial with associates.

Readministration of *dl*-glutamic acid hydrochloride was ineffective in 2 cases in both of which there was evidence of thyroid intoxication. Withdrawal of thyroid therapy was followed by a response similar to that of the initial treatment. In 2 other cases responses to readministration of the drug were quite similar to that of the initial treatment.

Ammonium chloride was administered in 2 cases (1 and 5). It proved ineffective in case 1, and severe acne resulted from its use in case 5.

Grand mal seizures were unaffected by the drug in 1 case (case 8). Focal motor cortical seizures and myoclonus likewise responded less favorably, as noted in cases 4 and 5 respectively.

It seems hardly necessary to emphasize that the beneficial effects of an additional aid in the treatment of epilepsy can be appraised only after a period of years. The preliminary results reported in this note may serve as a lead in further investigations. A comparison of the effect of *l*-glutamic and *d*-glutamic acid and their hydrochlorides as well as pyrrolidone carboxylic acid will show whether glutamic acid exerts a specific effect on the brain metabolism of epileptic patients.

SUMMARY

dl-Glutamic acid hydrochloride has been used in cases in conjunction with known anticonvulsant therapy. Seizures associated with slow wave activity in the electroencephalogram, namely petit mal and psychomotor types, were decreased in frequency. Increased mental and physical alertness have been most gratifying to patient, family and physician. Grand mal seizures were unaffected by the drug.

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APPLICATIONS OF AMINO ACIDS AND PROTEIN HYDROLYSATES IN THE FOOD INDUSTRIES

By H. G. Rees

(Delivered at the Food Group Symposium on Amino Acids and Protein Hydrolysates on September 30, 1949)

The development of protein hydrolysates in one form or another for incorporation into foodstuffs was initiated in the Far East in the early years of the present century. The first commercial use of hydrolysates appeared to be in China and Japan about 1908 and several patents were granted to Ikeda in 1912. In the early stages they were used chiefly as condiments but during the past 10—15 years rapid progress in their manufacture has been made in both this country and the United States.

The growth of interest in these products is shown by the numerous patent specifications covering the manufacture of protein hydrolysates and flavouring materials from hydrolysed proteins which are evidence of the amount of industrial research that has been carried out to develop the use of protein hydrolysates in the food industry.

The incorporation of protein hydrolysates into food products has the dual purpose of increasing the nutritional value by the addition of a concentrated predigested protein and improving the flavour of the products in which they are used. Flavour is of primary importance and is influenced very largely by the conditions of hydrolysis and by the raw materials hydrolysed. The broad principles of manufacture are well known but the finer points necessary to ensure that smoothness and roundness of flavour which is compatible with more delicate types of flavour is the result of long experience in the manufacture of this type of product.

For the manufacture of hydrolysed protein there is a choice of three methods of hydrolysis (*a*) enzymic, (*b*) alkaline, and (*c*) acid hydrolysis. The former two find little application on the manufacturing scale when the flavour of the product is of primary importance. Obviously, from the nutritional standpoint alone, an enzymic process has many advantages, particularly in that racemization and destruction of the amino acids do not occur. The process of hydrolysis is however slow, seldom goes to completion resulting in a product with a high content of polypeptides and peptones, and requires specialized equipment for large-scale production to ensure freedom from bacterial contamination with resultant development of off-flavour. However, there is one important industrial application of this type of hydrolysis which will be described briefly later. Finally, and of primary importance, the product has a pronounced bitter flavour which renders it frequently unsuitable for use as an adjuvant to a food product. Similarly alkaline hydrolysis involves considerable breakdown of nitrogenous products during the process and the resulting flavour is harsh and unpleasant, though this process has the advantage that little destruction of tryptophan occurs and the amount of humin formed is smaller. From the point of view of the chemical engineer it also entails fewer problems in protection of the plant from corrosion.

The general manufacturing process therefore consists of hydrolysis with acid, generally hydrochloric, though sulphuric is sometimes used when a salt-free hydrolysate is required, either by boiling or under moderate pressure. The use of sulphuric acid is not to be recommended as the flavour of the final product is inferior to that from a hydrochloric acid hydrolysis. The non-volatile acid does assist corrosion problems but the neutralization process with milk of lime can be very tedious. Excess acid is neutralized with sodium hydroxide or carbonate to a p_H of 5.0 to 6.0, the insoluble residue filtered out and the resulting filtrate concentrated. Care must be taken that the neutralization process is not carried too far as alkaline conditions produce a rapid deterioration of flavour with liberation of ammonia.

Hydrolysed protein is usually produced in the form of a thick paste containing 12—15% of water, 40—45% of salt, 4.75 to 5% of nitrogen and 3.7—4.0% of amino nitrogen. It is also supplied, particularly in America, in the form of a spray-dried powder containing about 50% of salt and 3% of water, but the extreme hygroscopicity of the product does not favour this method of presentation. Elimination of this property has received some attention in the United States and one of the few satisfactory methods appears to be the addition of 15—25% of starch prior to spray-drying. The technical difficulties encountered in the design and construction of plant are considerable and are usually developed only after many years experience. Major difficulties encountered include selection of suitable acid-resisting linings of either rubber, ceramic or glass to the reaction vessels which will not impart flavour to the finished product and the provision of suitable glands to house stirrers, etc.

Raw materials for hydrolysed protein

Any reasonably concentrated source of protein can be used as a starting material, but in order to minimise practical difficulties it is inadvisable that materials containing less than 50% of protein should be used.

In general the chief sources are the glutens, casein, soya bean meal, meat meal, and various vegetable meals, blends being more frequently employed than single protein sources. The reason is two-fold, firstly it enables the desired flavour to be more readily obtained and secondly, from our knowledge of the amino-acid composition of the proteins used, it enables us to devise a blend which will be nutritionally satisfactory.

Upon the conditions employed depends very largely the flavour of the product, with a given raw material quite a wide range of flavour can be obtained which can be very useful when more than one application of the product is desired. It may be mentioned here that very frequently small quantities of special materials may be used to impart a particular flavour. For example, coconut meal containing approximately 20% of protein may be used in small quantities to impart a sharp piquant flavour to the hydrolysate. The original flavours of the meals are largely destroyed by the process and it appears that these special flavours are due probably to the non-protein fraction of the meal, as is also, largely, the amount of colour produced on hydrolysis. The carbohydrate fraction of the meal is also important as regards the production of humin formed largely by condensation of tryptophan with aldehydic material. The presence of appreciable quantities of colloidal humin in the finished product has a detrimental effect on flavour and selection of the proper process involves removal of humin as far as possible from the amino-acid mixture.

Some processes involving the use of reducing substances, such as stannous chloride, to minimize production of humin, have been patented. It is not possible to tabulate flavour against raw material but only to make generalisations. The finest and most bland flavours are obtained from the most concentrated protein sources such as casein and the glutens, possibly on account of a lack of crude flavour derived from the carbohydrate fraction. On the other hand animal protein material such as meat meal, "cracklings," gelatin, and epidermal proteins are not entirely satisfactory as the basic flavour has a sweetish character, not always compatible with the food flavour with which hydrolysed protein is generally associated. The Americans believe that the most acceptable flavour is largely bound up with the glutamate content of the product

but a protein hydrolysate containing all or most of the amino-acids has the constituents present necessary to produce a full satisfying flavour.

The chief types of food products of which hydrolysed protein form an important ingredient are meat products of one type or another—soups, broths, bouillon cubes, sauces, etc., where a distinctive meat broth flavour is the dominant flavour of the finished product. In many cases it is used chiefly to give added strength to the product.

One factor of the importance of hydrolysed protein to the food industry lies in the fact that the process enables a manufacturer not only to fortify a product with what might almost be termed a pre-digested protein concentrate but also to utilize sources of protein otherwise unsuitable for human consumption. Protein material in an insoluble form or accompanied by unwanted material, possibly cellulosic in composition, may be converted to such a form as to render it compatible with a large number of food products and by careful selection of the raw materials it is possible to devise a nutritionally useful hydrolysate which enables the manufacturer to fortify a foodstuff with a highly-concentrated source of pre-digested protein.

The other factor of importance is the value of flavour of these hydrolysates. In general the flavour of hydrolysed protein is described as meaty and by one manufacturer as "akin to those of the broth from well-browned beef." Admittedly this may seem rather an exaggeration in the case of a large number of protein hydrolysates but the basic flavour is, if not similar to, readily compatible with, that of a meat and in some cases a vegetable stock. Some American manufacturers recently have been using the glutamate content of their hydrolysed protein as an index of flavour and quality. This is an aspect which has so far received no attention in this country but it is of interest to note that the recent B.I.O.S (British Intelligence Objectives Sub-Committee) "Overall Report on the Food Manufacturing Industry in Germany 1939-45" states that one authority claims that proteins capable of producing a hydrolysate with a high glutamic acid content give the best flavoured products and that he has developed a simple method of examining raw materials for this purpose.

Proteins, in general, have little attractive flavour as also have the amino-acids, other than glutamic acid, though it has been stated that the non essential are superior to the essential amino-acids as regards flavour. When heated together or prepared in a crude conditions the mixtures develop characteristic odours and flavours which are best described as "hydrolysate flavour." Possibly this is largely owing to interaction with breakdown products of other fractions of the raw materials.

Amino-acids

Individual amino-acids, with one exception, have little application in the food industry. That exception is glutamic acid which, as the monosodium salt, is now finding extensive use, particularly in the United States.

In the early years of the present century the use of dried seaweed in Japanese cookery was investigated by Ikeda who detected considerable quantities of glutamic acid among the hydrolysis products. He found that the crude monosodium salt exhibited a desirable meat-like flavour and was the originator of the first British Patent, filed in 1900, covering the manufacture of this substance as a flavouring material. This work aroused the interest of the Suzuki Company in Tokyo who obtained a number of patents covering the manufacture of glutamate under their trade mark "Aji-No-Moto." The basic Japanese patents expired in 1929, since which time manufacture has received considerable attention in the U.S.A.

Oriental diets are, on the whole, monotonous in flavour and this probably accounts for the remarkable development of the soya sauce and glutamate industries—it is stated that the annual output of Japanese manufacturers of glutamate reached £10,000,000 in 1933 and their exports of "seasoning materials," principally glutamate and chiefly to Asiatic countries, was more than £2,250,000 in 1934. The chief raw material was initially wheat gluten but this was generally superseded by extracted soya bean meal in Japan, though gluten is still largely used in the United States. Large scale production by the Huron Milling Company whose manufacture of a variety of special starches renders wheat gluten available as a valuable by-product and logical starting point for glutamate manufacture. This company has now become one of the largest American manufacturers of glutamate and hydrolysed protein.

As interest in glutamate developed, the search for fresh sources of raw materials other than protein hydrolysates increased. Processes have been evolved for utilization of the nitrogenous compounds of Steffen's waste liquor from the beet sugar industry and it has been reported that one plant in the U.S.A. now produces 9,000 lb. of sodium glutamate daily by this process.

When glutamate was first produced in Japan it was claimed to have a "meaty flavour" and was capable of producing a meat-like flavour in non-meat dishes but this has been questioned of late. It is true that vegetable protein hydrolysates do have a broth-like flavour and it is probable that the early crude commercial forms of glutamate were higly contaminated with hydrolysate flavour.

The literature on the flavour and uses of glutamate is largely American in origin, in which country its potentialities as an ingredient in foodstuffs is having considerable attention. In solutions of 0.1—0.5% it has a sweet, saline taste with some astringency, and a mild but very lasting after-taste. When added in small amounts to foodstuffs (0.05—0.5%) it is said to increase the flavour level without overshadowing the original taste and without itself being noticeable. This is due particularly to a rounding-off or balancing of the flavour, though in some cases rawness and other undesirable aspects of flavour appear to be suppressed. The effect is slightly affected by the p_H of the medium but is most marked in the range of 5.5—6.5% and it is also desirable to have a proportion of salt present.

Glutamate flavour does not blend well with cereals, fruits, vegetables, or dairy products. It is usually used in conjunction with meat and fish products, soups, and gravies and has been particularly recommended in the United States for intensification of chicken and general meaty flavours.

The flavours we have been discussing are in general of a meaty type and it is of interest to summarize briefly some work that has been reported in the last few years from the United States on the nature of the flavour of meat, particularly that which develops on cooking, and to gain some knowledge of the nature of the flavouring substances produced.

It was found that the odour and flavour of raw meat reside mostly in the juices and is due largely to the sweetness and saltiness of actual blood and only incidentally to the presence of the bases creatine and creatinine which have but weak flavours and that on cooking very little flavour is developed in these juices. The distinctive flavour of cooked meat is largely developed in the fibre and is presumably due to cracking of amino-acid units of the protein. In an attempt to characterize the volatile fraction which is very largely responsible for the flavour of cooked meat, hydrogen sulphide was found to be an important constituent together with unidentified acidic substances, and, in the basic fraction, a simple low amine such as methylamine, a piperidine-like amine and probably indole.

The wide variety of amines, acids, sulphur compounds and possibly phenols present in small proportion implies that amino-acid units of several types are affected in the cooking. The flavours developed on cooking are similar for various meats but modified by species. The meaty flavour is due chiefly to volatile substances detected by the sense of smell though chewing is sometimes needed to release it. In this respect glutamate can, therefore, not be regarded as a true substitute for meat, in that though it has flavour it has no odour, and its association with meatiness in the early days was largely due to the odour of the crude glutamate then available.

Examination of other individual amino-acids in a state of purity have shown that they, their sodium salts and hydrochlorides are generally without odour. The characteristic flavour is chiefly sweetness, which is most marked in glycine, alanine and hydroxy-proline. The presence of appreciable amounts of these acids in epidermal proteins probably largely accounts for the very marked sweetness of hydrolysed animal proteins manufactured from such protein sources as hoof, horns, fish scales, etc.

Enzymic processes

One further aspect of this subject which involves the production of a protein hydrolysate by an enzymic process, namely that of yeast extract by autolysis, may briefly be described. Yeast extracts may be obtained by direct aqueous extraction of fresh yeast but the yield is low and flavour yeasty. The yield can be considerably increased and the flavour markedly improved with the production of a broth-like quality if the yeast is subjected to autolysis prior to extraction.

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Briefly the process of manufacture is as follows: Fresh, pressed yeast is plasmolysed, generally by blending with 2–5% of salt, and then maintained for periods ranging from 12 to 30 hours at a temperature between 40° and 50°. The autolysate is then brought to the boil to precipitate unchanged protein and to inactivate the enzymes and the insoluble portion removed by filtration. The extract is then concentrated to a pasty consistency and the residue frequently dried for cattle feeding. As a rule pressed Brewers' yeast is used but this is inclined to give a bitter-flavoured product due mainly to the hop resins associated with the yeast. To obviate this, processes have been devised to remove these bitter principles, based on washing the yeast with a dilute bicarbonate solution.

Hydrolysis of the protein of the yeast cells is in this case effected by the proteolytic enzymes of the yeast and results only in a partial hydrolysis of the protein. The yeast protein is solubilized to give a mixture of peptides together with some degradation to the amino-acid stage as may be seen from the following typical analysis of a commercial yeast extract:—

Water	40%
Salt	20%
Total nitrogen	4·5%
Amino nitrogen	2·5%

Though the hydrolysis is incomplete the flavour of the extract does not contain any of the bitterness usually associated with an enzymatically-digested protein and, provided that the yeast enzymes are active, only a trace of yeastiness. The autolysis process is subject to more variation than an acid hydrolysis and is probably due to the somewhat variable nature of the enzymes present in the yeast cells. The resulting extract is rich in vitamins of the B group providing that a potent yeast is used but the vitamin B_1 content is unstable on storage.

These extracts have an excellent flavour and are frequently used in conjunction with meat extracts in the manufacture of canned soups, composite meat extract cubes and the like.

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REVUE ROUMAINE DE BIOCHIMIE

MICRODETERMINATION OF ALANINE, GLUTAMATE, ASPARTATE, γ-AMINOBUTYRATE, GLYCINE AND TOTAL FREE AMINO ACIDS IN THE SAME SAMPLE OF NERVE TISSUE: SOME MODIFICATIONS

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The modifications described in this communication considerably shorten the extraction procedure of Graham and Aprison (1966) both in time and number of manipulations.
The alanine determination of Young and Lowry (1966) was also simplified.
The problem of enzymatic inhibition by TCA used in analytical determinations was avoided by prior volatilization of the TCA.
On the same TCA extract it is possible to measure microamounts of alanine, glutamate, aspartate, GABA, glycine and total free amino acids.

Neurobiologists have found it necessary to measure microlevels of specific amino acids in presence of other tissue constituents extracted from small discrete areas of the brain and spinal cord (2–30 mg). The analyses of large numbers of such small samples by the technique of column chromatography or paper chromatography are time consuming and not practical. Recently Graham and Aprison (1966) and Young and Lowry (1966) have published elegant enzymic techniques for measuring substrate levels of amino acids in the range required.

Since these procedures can be further improved it appeared worthwhile to report some modifications to the above techniques.

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EXPERIMENTAL

The tissue was homogenized in cold 5% TCA at a concentration of 5 mg/100 ml [illegible] with [illegible] 2 [illegible] Homogenization was performed in small [illegible] The homogenate was poured into 7 [illegible] ml [illegible] and centrifuged for 20 min at 23,000 g. The supernatant [illegible] These tubes were capped and kept in ice to prevent evaporation [illegible]

[illegible] were [illegible] used for the determination of glutamate. To each tube [illegible] 1.5 ml of 0.1 M glycine-hydrazine buffer (pH 8.6) was added, either 100 µl supernatant [illegible] of standard [illegible] TCA or 10 µl of 5% TCA. No inhibition of the reaction was observed due to the small amount of TCA added. If the quantity of TCA was increased, it was served some decrease in the formation of NADH. We found it advantageous to substitute the hydrazine hydrate with hydrazine sulphate, since the last is more stable and does change during storage.

A 5.2 g of hydrazine sulphate was added to 5.75 g of glycine; the pH of the [illegible] was subsequently adjusted to 8.6 with 10 N NaOH and then the volume was brought to 100 [illegible]

A convenient amount of the remaining samples was transferred to 1 mm o.d. microtubes; a dilution might be necessary because of the smaller quantity of aspartate and GABA detectable with their more sensitive assays. The content of the tubes was then dried for at least 30 min in the vacuum centrifuge evaporator. An infrared lamp was applied to the vacuum centrifuge for 20 min to ensure a complete evaporation of TCA, since TCA had an inhibitory effect upon the enzymes used in the assays. Alanine was determined by a method similar to that of Young and Lowry (1959). However the two reactions

Alanine + α-oxoglutarate → Pyruvate + glutamate

Pyruvate + NADH → Lactate + NAD⁺

were run simultaneously as in the aspartic acid determination.

The incubation mixture contained:

- 2.00 ml Na pyrophosphate buffer (0.067 M; pH 7.2)
- 10 µl NADH (0.01 M)
- 2 ml α-oxoglutarate (0.1 M) (freshly prepared)
- [illegible] µl Glutamic Pyruvate Transaminase 2 mg/ml (Boehringer)
- 5 µl L-Lactate Dehydrogenase 5 mg/ml (Boehringer)

Twenty ml of this mixture were added to the dried 1 mm o.d. tubes containing sample, standard, or blank. During addition the tubes were kept in ice water. Subsequently they were capped, buzzed, and incubated for 15 min at 38°. The blanks were run simultaneously in which 5 µl of Lactate Dehydrogenase or 10 µl of Glutamic Pyruvate Transaminase were left out of the incubation mixtures. Two different non-tissue blanks were also used. Fifty µl of 0.5 N HCl were added to each tube at the end of the incubation period. During addition the tubes were kept in ice water. The tubes were buzzed vigorously and a 50 µl portion was removed and added to a fluorometer tube containing 100 µl 2 N NaOH. The fluorometer tubes were subsequently heated at 60° for 10 min. One ml of distilled water was added to each tube, buzzed, and the fluorescence was read.

A standard curve for alanine is shown in figure 1.

A sufficient volume of sample is also available to measure glycine and total amino acids concentration according to Graham *et al.* (1967).

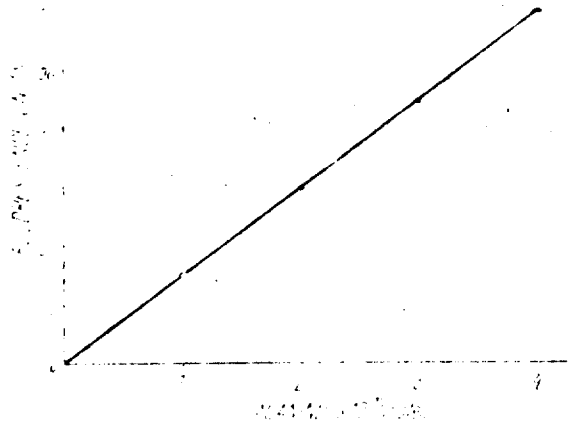

Fig. 1. — Relationship between net fluorescent product of NAD[illegible] in strong alkali and [illegible] levels. Each point is the mean of five determinations. Fluorescence units represent arbitrary galvanometer readings.

RESULTS

Standards made up in 5% TCA as well as in water can be used. The results are the same since there is volatilization of the TCA during evaporation (table 1).

Table 1

Determination of GABA standards dissolved in TCA and in water

		Fluorescence Units		
Blank		Water	TCA 5%	TCA 2.5%
		13	13	13
Moles 10^{-11}	4	21	20	21
	12	38	38	39
	20	57	58	59

We have compared the extraction of three amino acids both in 75% ethanol and 5% TCA. Table 2 reports a recovery experiment. Glutamine,

when added to tubes containing glutamate, did not undergo any hydrolysis after TCA or ethanol extraction, as measured by an increase in glutamate.

Table 2

Recovery of some amino acids extracted with TCA and ethanol

	Extraction	Calculated	Found	Recovery
Glutamic acid Moles × 10^{-10}	5% TCA	9.48	9.61	101.6%
	75% EtOH	10.15	10.03	98.6%
Glutamic acid + Glutamine Moles × 10^{-10}	5% TCA	18.13	18.22	98.8%
	75% EtOH	16.95	17.15	101.1%
Aspartic acid Moles × 10^{-11}	5% TCA	23.69	22.62	95.8%
	75% EtOH	23.28	22.85	96.2%
GABA Moles × 10^{-11}	5% TCA	17.96	17.51	97.4%
	75% EtOH	15.20	15.26	100.3%

Alanine values obtained in cat spinal cord (L_6—L_7 segments) were somewhat lower than those reported by Shaw and Heine (1965) in different areas of the rat brain (table 3).

Table 3

Alanine distribution in six areas of the cat spinal cord (L_6—L_7)

Alanine * (μmoles/g)	Dorsal funiculus 0.160 ± 0.020	Dorsolateral funiculus 0.126 ± 0.007
	Dorsal gray 0.400 ± 0.050	Ventral gray 0.380 ± 0.042
	Ventral funiculus 0.110 ± 0.018	Ventrolateral funiculus 0.127 ± 0.010

* Media of 9 animal ± SEM.

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Effects of Casein Hydrolysate on the Induction and Regulation of Tyrosine-α-Ketoglutarate Transaminase in Rat Liver*

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SUMMARY

A several fold increase in the activity of tyrosine-α-ketoglutarate transaminase occurs in intact or adrenalectomized rats following treatment with an enzymatic hydrolysate of casein, whereas tryptophan pyrrolase is unresponsive to this treatment. Doses of tyrosine, tryptophan, or the combination of these amino acids equivalent in amount to those contained in an effective dose of casein hydrolysate did not produce a rise in tyrosine transaminase activity. The effect of casein hydrolysate is inhibited by actinomycin D or cycloheximide, indicating that the synthesis of new RNA and protein is associated with the response of tyrosine transaminase to casein hydrolysate.

Additive and, in some cases, synergistic effects on tyrosine transaminase are observed when casein hydrolysate and hydrocortisone are given together or sequentially. In contrast to the response of this enzyme, no additive effect on tryptophan pyrrolase or serine dehydrase occurs when 1 g of casein hydrolysate is given at the same time as a large dose of hydrocortisone. Treatment of adrenalectomized rats with casein hydrolysate, 4 or 8 hours after they were treated with hydrocortisone, stimulates a marked further increase in the activity of tyrosine transaminase. In intact rats, tyrosine transaminase shows a synergistic response to casein hydrolysate when it is given concomitantly with a small dose of hydrocortisone; under these conditions tryptophan pyrrolase is also responsive, but this effect appears to be due to tryptophan stabilization of the enzyme against degradation.

When a low dosage of hydrocortisone is administered to intact rats, tryptophan pyrrolase, but not tyrosine transaminase, shows an increase in activity. On the other hand, of these two enzymes, only the transaminase responds to casein hydrolysate. Each of these treatments stimulates the incorporation of orotic acid-6-^{14}C into liver RNA to about the same extent, indicating either that this is a selective effect or that factors in addition to new RNA synthesis are involved in the induction of these enzymes.

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The induction of specific enzymes in mammalian liver by treatment with glucocorticoids has now been observed in several species (2), including man (3). In rats, two rapidly responding enzymes, tyrosine-α-ketoglutarate transaminase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) and tryptophan pyrrolase (L-tryptophan:H_2O_2 oxidoreductase, EC 1.11.1.4), show maximum increase in activity in liver within 4 to 6 hours after the intraperitoneal administration of hydrocortisone, and by 12 hours the activity of these enzymes returns to normal. Also, in intact rats subjected to experimental conditions known to increase the rate of gluconeogenesis (high protein diet, alloxan diabetes, or starvation) both of these enzymes show a significant increase in activity (2, 4, 5). In contrast to this similarity in response, other treatments have been found to act specifically with respect to the regulation of activity of these enzymes in rat liver. Thus, tyrosine transaminase shows a significant rise in activity in intact or adrenalectomized rats treated with glucagon (6-8), insulin (8), or the inhibitor of protein synthesis, cycloheximide (9), whereas tryptophan pyrrolase is refractory to induction by these agents.

Some evidence has been obtained indicating that the increase in tyrosine transaminase following cycloheximide treatment might be due to an increase in the levels of amino acids in liver (10). This possibility has also been suggested to explain the elevated levels of certain adaptive enzymes observed in the liver of rats treated with small doses of actinomycin D (11). The present study describes a specific effect of an enzymatic hydrolysate of casein on the induction and regulation of tyrosine transaminase activity. This effect of casein hydrolysate is inhibited by actinomycin D, and in adrenalectomized rats cannot be duplicated by tyrosine or tryptophan administered in amounts equivalent to those present in an effective dose of the casein digest.

EXPERIMENTAL PROCEDURE

Materials

The substrates for the enzyme assays were obtained from Nutritional Biochemicals or Sigma. Casein hydrolysate (Lot 54314) was an enzymatic digest purchased from General Biochemicals. Orotic acid-6-^{14}C (44.5 mC per mmole) was obtained from Nuclear-Chicago. Hydrocortisone acetate (cortisol)

was obtained as a commercial preparation (Hydrocortone) from Merck Sharp and Dohme. Hydrocortisone hemisuccinate was purchased from Mann. Cycloheximide was purchased from Nutritional Biochemicals. Actinomycin D was a gift from Merck Sharp and Dohme.

Animals and Diets

Sprague-Dawley rats were used throughout; the specific range of body weights is given for each experiment. Teklad laboratory chow and water were supplied *ad libitum*. The composition of the purified diets was previously reported (12). After bilateral adrenalectomy (performed under anesthesia with Nembutal) the animals were maintained on 1% NaCl as drinking water; they were used within 5 to 7 days following the operation. The adrenalectomized animals were examined for adrenal remnants at the time they were killed, and were not included in the experiment if such tissue was found.

Recent reports indicate that tyrosine transaminase (13, 14) and tryptophan pyrrolase (15) show diurnal variation when rats are maintained in animal rooms in which the periods of feeding and lighting are rigorously controlled. The variation seen in tryptophan pyrrolase activity was found to be adrenal mediated, while the variation in transaminase activity persisted even after adrenalectomy.

Most of our experiments were started between 9 and 10 a.m. The animals were killed during the midafternoon, with appropriate controls included for each time. The variation in transaminase activity during this period was less than 20% in animals fed *ad libitum* and housed in rooms lighted from 7 a.m. to 5 p.m. We have not seen any significant increase in the control values in experiments extending into the early evening, possibly because the daytime levels of lighting were maintained for experiments in which the animals were killed during this period.

Analytical Procedures

Enzyme Assays—Tyrosine-α-ketoglutarate transaminase activity was measured by a colorimetric procedure described previously (16). Tryptophan pyrrolase was determined by the method of Knox (17) with the addition of hematin as reported by Feigelson and Greengard (18). The procedure for determination of serine dehydrase (L-serine hydrolyase (deaminating), EC 4.2.1.13) was similar to that described by Freedland and Avery (19), except that the NAD formed was measured fluorometrically (12).

The animals were stunned by a blow on the head and killed by exsanguination. The livers were rinsed in cold water, blotted dry, frozen in Dry Ice and acetone, and stored on Dry Ice until assay. For the tryptophan pyrrolase assay, a 10% homogenate of liver in ice-cold 0.14 M KCl was prepared; appropriate dilutions of this homogenate were made for the tyrosine transaminase determination. A 5% homogenate of liver in ice-cold water was used for the serine dehydrase assay.

Protein—Protein was determined by the method of Lowry *et al.* (20), with bovine serum albumin as a standard.

RNA Isolation and Measurement—RNA was isolated as a crude fraction by the procedure of Ralph and Bellamy (21); since orotic acid is not incorporated into glycogen, which was the major contaminant of this fraction, no further purification was attempted. The concentration of RNA was determined spectrophotometrically by its absorption at 260 mμ on the basis that 1 unit of optical density was equivalent to 45 μg of RNA (22). The isolated RNA fraction was dried and dissolved in water (1 mg per ml), and 1-ml aliquots were dried on stainless steel planchets. Radioactivity was counted to 3% precision in a Nuclear-Chicago thin window gas flow counter with a background of 13 to 15 cpm.

RESULTS

Response of Tyrosine Transaminase and Tryptophan Pyrrolase to Delayed Administration of Casein Hydrolysate—Many of the enzymes in liver which are responsive to the glucocorticoids also show an adaptive response to the level of protein in the diet (2, 23). Thus, feeding a diet which is lacking or low in protein results in a depression in the activity of these enzymes, whereas when rats are fed a high protein diet for several days a significant elevation in enzyme activity occurs. Both the level of dietary protein and treatment with hydrocortisone were found to be of importance in obtaining a maximal alanine-α-ketoglutarate transaminase response in the livers of adrenalectomized rats (12). It has also been shown that oral administration of casein hydrolysate can stimulate increases in the activities of threonine (or serine) dehydrase and ornithine δ-transaminase in rats fed a 0% protein diet for 5 days (24). These observations suggested that the dietary level of protein and the intracellular pool of amino acids could play an important role in mediating the response of an enzyme to an adrenal corticoid. With this possibility in mind, the provocative observation by Garren *et al.* (25) of a paradoxical effect of actinomycin D on the induction of tyrosine transaminase and tryptophan pyrrolase suggested to us that an alternative explanation for this phenomenon might well be the limitation of amino acids required for continued enzyme synthesis. As indicated in Fig. 1*A*, the fall in activity of tyrosine transaminase which begins about 4 hours after an intraperitoneal injection of hydrocortisone hemisuccinate was prevented and an increase in enzyme activity occurred after oral intubation of 1 g of casein hydrolysate. With the same treatment, tryptophan pyrrolase activity remained constant for 2 hours but thereafter decreased in activity, so that by 4 hours the values for this enzyme were in the range of hydrocortisone controls (Fig. 1*B*).

Selectivity, Enhancement, and Basis of Tyrosine Transaminase Response to Casein Hydrolysate—The data in Table I show the response of three enzymes to a large dose of hydrocortisone hemisuccinate only and to this hormone plus casein hydrolysate given at the start of the experiment. It can be seen that the combination is considerably more effective than the corticoid alone in stimulating the response of tyrosine transaminase. Serine dehydrase, which requires about 8 hours following an intraperitoneal dose of hydrocortisone to show a doubling in activity, did not show any further increase with the combined treatment. Also, no additive effect was observed with this combination on tryptophan pyrrolase measured at 4 and at 8 hours ($p > 0.10$).

The stimulatory action of 0.5 to 3.0 g of casein hydrolysate on hepatic tyrosine transaminase activity in adrenalectomized rats is shown in Table II. In contrast, the level of tryptophan pyrrolase in the livers of these rats is unresponsive to treatment with casein hydrolysate; this is also the case in intact rats. Although the data are not shown, the response of tyrosine transaminase to 1 g of casein hydrolysate given intraperitoneally was at least equal to that obtained by the oral route.

A comparison of the time course of response of tyrosine transaminase to hydrocortisone, casein hydrolysate, and the combination of these inducing agents is presented in Fig. 2. Previous studies had shown that small doses of hydrocortisone were effective in inducing tyrosine transaminase in adrenalectomized rats (16) and that this response was significantly enhanced when the hydrocortisone was combined with tyrosine (26) or tryptophan (27). In the present study, the effects of a large dose of hydrocortisone on transaminase activity measured at 4 hours were further stimulated by the concomitant administration of casein hydrolysate. The high level of enzyme activity observed at 8 hours with the combined treatment might be the result of impaired degradation of the enzyme due either to stabilization by interaction with specific amino acids or to prolongation of the period during which the increased rate of synthesis of this enzyme is maintained by continued or additional RNA synthesis.

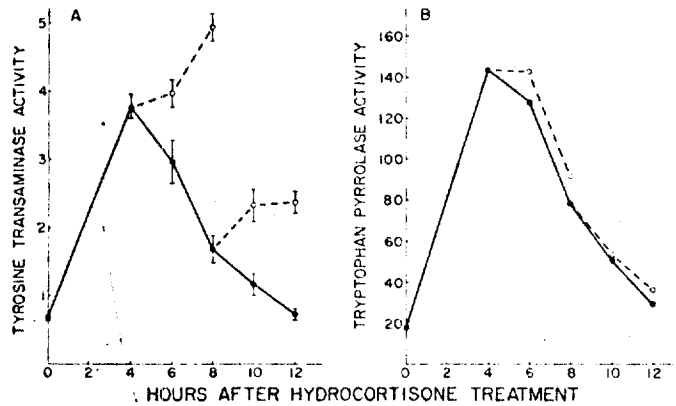

FIG. 1. Comparative response of tyrosine transaminase and tryptophan pyrrolase to the delayed administration of casein hydrolysate. Adrenalectomized rats weighing from 98 to 125 g were given intraperitoneal injections of hydrocortisone hemisuccinate (30 mg per kg) at the start of the experiment. Casein hydrolysate (1 g) in 3 ml of 0.9% NaCl solution was given by stomach tube either at 4 or at 6 hours. Tyrosine transaminase (*A*) or tryptophan pyrrolase (*B*) activity following treatment with hydrocortisone, alone (●——●) or combined with casein hydrolysate (○---○), was measured in the livers of rats killed at the times indicated. Each *point* represents the mean value from four rats. The vertical *bars* indicate standard error of the mean and, where used, indicate *p* values less than 0.05. Tyrosine transaminase activity is expressed as micromoles of *p*-hydroxyphenylpyruvate formed per mg of protein per hour at 37°; tryptophan pyrrolase activity as micromoles of kynurenine formed per g of protein per hour at 37°.

TABLE II

Effects of different doses of casein hydrolysate on tyrosine transaminase and tryptophan pyrrolase activities in livers of adrenalectomized rats

An enzymatic digest of casein was given orally as a suspension in 3 ml of isotonic NaCl solution. Control animals received isotonic NaCl only. All animals were killed 4 hours after treatment. Results are expressed as the mean value ± standard error for five rats weighing from 172 to 219 g.

Treatment and dosage per rat	Tyrosine transaminase	Tryptophan pyrrolase
	μmoles/mg protein/hr	*μmoles/g protein/hr*
Control	0.41 ± 0.04	11.9 ± 0.54
Casein hydrolysate, 0.5 g	1.03 ± 0.20	13.4 ± 1.4
Casein hydrolysate, 1.0 g	1.46 ± 0.18	12.5 ± 0.85
Casein hydrolysate, 2.0 g	2.05 ± 0.24	11.3 ± 1.0
Casein hydrolysate, 3.0 g	2.24 ± 0.15	13.1 ± 0.63

It is well established that the induction of tyrosine transaminase and tryptophan pyrrolase by glucocorticoids involves new RNA synthesis and that actinomycin D can block this effect, whereas the substrate-mediated increase in the activity of the latter enzyme is due to inhibition of its degradation, an effect which is insensitive to actinomycin D (28). The results shown in Table III indicate that when actinomycin D was given at the same time as casein hydrolysate and the animals were killed 4 hours later no increase in enzyme activity was observed. Furthermore, when two doses of casein hydrolysate were given 4 hours apart the response due to the second dose was also blocked by actinomycin D. It appears then that the induction of tyrosine transaminase by casein hydrolysate, as well as the additional response to this treatment seen following the earlier administration of casein hydrolysate, is dependent on new RNA synthesis.

These results are to be contrasted with the influence of actinomycin D on the responses of threonine dehydrase and ornithine δ-transaminase to casein hydrolysate. Treatment with the antibiotic and the protein hydrolysate at the start of the experiment completely blocked the rise in activity of these enzymes, but when actinomycin D was given 4 hours later, along with a

TABLE I

Comparison of induced responses of several enzymes in livers of adrenalectomized rats treated with hydrocortisone hemisuccinate and casein hydrolysate

The hydrocortisone hemisuccinate was given intraperitoneally and the casein hydrolysate orally as a suspension in 3 ml of NaCl solution. Results are expressed as the mean value ± standard error for five rats weighing from 118 to 154 g.

Compound and dosage per rat	Period between treatment and killing	Tyrosine transaminase	Serine dehydrase	Tryptophan pyrrolase
	hrs	*μmoles/mg protein/hr*		*μmoles/g protein/hr*
None		0.68 ± 0.09	2.66 ± 0.32	10.6 ± 0.67
Hydrocortisone hemisuccinate, 5 mg	4	3.88 ± 0.22	3.35 ± 0.54	80.9 ± 2.9
Hydrocortisone hemisuccinate, 5 mg, + casein hydrolysate, 1 g	4	6.54 ± 0.24	4.65 ± 0.49	88.8 ± 8.2
Hydrocortisone hemisuccinate, 5 mg	8	2.04 ± 0.12	5.27 ± 0.34	56.5 ± 3.94
Hydrocortisone hemisuccinate, 5 mg, + casein hydrolysate, 1 g	8	3.59 ± 0.12	6.01 ± 0.28	69.1 ± 6.9

second dose of casein hydrolysate, enzyme activity continued to increase (29).

When protein synthesis was inhibited by injecting cycloheximide concomitantly with casein hydrolysate at 4 hours, the fall in enzyme activity was comparable to that seen in the control animals (Fig. 3). Thus, the second dose of casein hydrolysate is acting to stimulate the synthesis of new tyrosine transaminase rather than to prolong the rate of turnover or degradation of this enzyme with the synthesis of this protein continuing at a normal rate.

Enzyme Responses to Hydrocortisone and Casein Hydrolysate as Influenced by Intake of Dietary Protein—The observations presented in Fig. 2 suggested that the protein intake might modify the response of tyrosine transaminase to casein hydrolysate and hydrocortisone. The results of this study, in which the transaminase is compared with tryptophan pyrrolase, are presented in Table IV. Casein hydrolysate was more effective than a small dose of hydrocortisone in producing an increase in tyrosine transaminase in animals fed the 0% or 18% protein diets. However, on the 95% protein diet further treatment with casein did not elicit a rise in transaminase activity and hydrocortisone treatment effected only a 50% increase in this enzyme. In the rats fed either the 0% or the 18% casein diet, the induction of tyrosine transaminase following the combined treatment with hydrocortisone and casein hydrolysate was synergistic when compared to either treatment alone.

Of interest was the finding that in rats fed the 95% casein diet for 1 week the level of tyrosine transaminase was 5 times higher than it was in animals fed the 0% or 18% casein diets, whereas for tryptophan pyrrolase the value only doubled. The slight stimulation in tryptophan pyrrolase activity observed in intact rats that had received casein hydrolysate can be attributed to the stress of this treatment and the subsequent release of corticosterone or to the small amount of tryptophan supplied with the protein digest or to both causes. The combined treatment

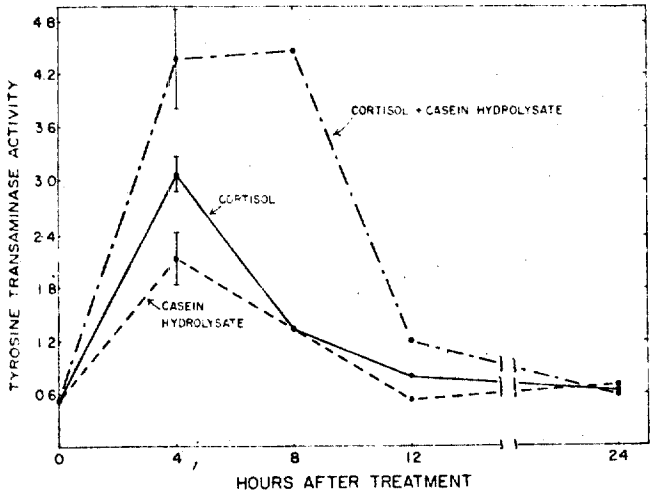

FIG. 2. Effect of different treatments on the time course response of tyrosine transaminase. Adrenalectomized rats weighing from 105 to 139 g were treated at zero time with ●——●, cortisol, 30 mg per kg, intraperitoneal; ●- - -●, casein hydrolysate, 1 g/3 ml of isotonic NaCl by stomach tube; or ·—·, the combination of cortisol and casein hydrolysate, and were killed at the times indicated. Enzyme activity is expressed as in Fig. 1 and each point represents the mean value for four rats. The standard error of the mean is shown for the 4-hour values; these values are significant ($p < 0.05$).

TABLE III

Effects of actinomycin D on induction of tyrosine transaminase by casein hydrolysate in livers of adrenalectomized rats

The casein hydrolysate was given orally as a suspension in 3 ml of NaCl solution; actinomycin D (1 mg) was injected intraperitoneally. Control animals were treated orally and intraperitoneally with equivalent volumes of NaCl solution. Results are expressed as the mean value ± standard error for four to six rats weighing from 120 to 150 g.

Treatment and dosage	Period between start of treatment and killing	Tyrosine transaminase
	hrs	*μmoles/mg protein/hr*
Control	4	0.49 ± 0.06
Casein hydrolysate, 1 g per rat	4	1.62 ± 0.12
Actinomycin D, 1 mg per kg	4	0.29 ± 0.08
Casein hydrolysate, 1 g per rat, + actinomycin D, 1 mg per kg	4	0.52 ± 0.04
Casein hydrolysate, 1 g per rat, at 0 and at 4 hours	8	2.81 ± 0.21
Casein hydrolysate, 1 g per rat, at 0 and at 4 hours + actinomycin D, 1 mg per kg, at 4 hours	8	0.88 ± 0.12

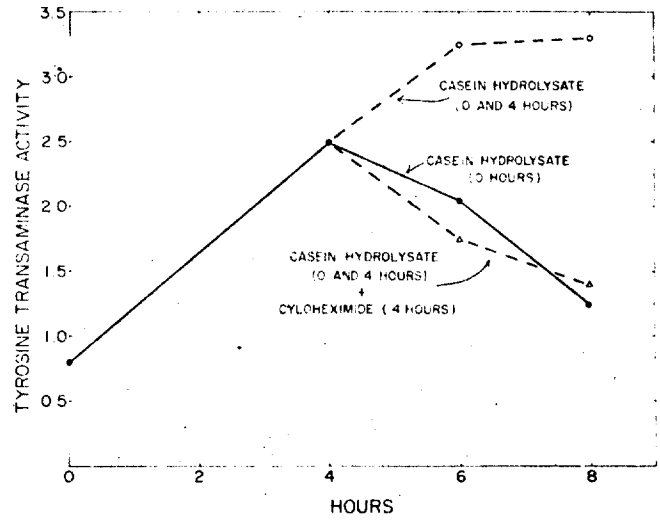

FIG. 3. Inhibitory effect of cycloheximide on the tyrosine transaminase response to casein hydrolysate in intact rats weighing from 99 to 119 g. All rats were intubated orally with 1 g of casein hydrolysate in 3 ml of isotonic NaCl at zero time. At 4 hours the rats were divided into three groups: ●——●, no additional treatment; ○- - -○, a second intubation of 1 g of casein hydrolysate; △- - -△, intraperitoneal injection of cycloheximide, 10 mg per kg, as well as a second intubation of 1 g of casein hydrolysate. Five rats from the control group (●——●) were killed at 4 hours and five rats from each of the three groups were killed at 6 and at 8 hours. Enzyme activity is expressed as in Fig. 1.

led to an additive effect on the induction of tryptophan pyrrolase only in the animals fed the diet lacking protein.

Effects of Tyrosine and Tryptophan in Comparison to Casein Hydrolysate—It was important to rule out the possibility that the effects of casein hydrolysate on tyrosine transaminase could be attributed to the quantities of tryptophan and tyrosine contained in this hydrolysate. Previously it had been found that the intraperitoneal administration of a large dose of L-

TABLE IV

Influence of protein intake on induction of tyrosine transaminase and tryptophan pyrrolase by cortisol and casein hydrolysate

Intact rats, maintained on the diets for 7 days, were used. On Day 7 the weights of the animals on the 0%, 18%, and 95% protein diets ranged from 87 to 113 g, 98 to 131 g, and 165 to 200 g, respectively. The treatments were given on the 7th day only. Cortisol was injected intraperitoneally and casein hydrolysate was given orally; the animals were killed 4 hours after these treatments. Control animals were treated orally and intraperitoneally with equivalent volumes NaCl solution. Results are expressed as mean value ± standard error for four or five animals per group.

Treatment	0% protein diet		18% protein diet		95% protein diet	
	Tyrosine transaminase[a]	Tryptophan pyrrolase[b]	Tyrosine transaminase[a]	Tryptophan pyrrolase[b]	Tyrosine transaminase[a]	Tryptophan pyrrolase[b]
Control	0.46 ± 0.10	25 ± 1.5	0.37 ± 0.04	24 ± 1.6	2.4 ± 0.34	50 ± 2.7
Hydrocortisone, 2 mg per kg	0.74 ± 0.04	46 ± 5.2	0.87 ± 0.05	78 ± 4.1	3.7 ± 0.26	104 ± 6.1
Casein hydrolysate, 1 g per rat	1.6 ± 0.13	40 ± 1.3	1.6 ± 0.21	38 ± 1.8	2.8 ± 0.27	58 ± 4.4
Hydrocortisone, 2 mg per kg, + casein hydrolysate, 1 g per rat	6.2 ± 0.71	57 ± 1.5	3.8 ± 0.23	75 ± 4.1	3.9 ± 0.16	105 ± 12

[a] Values are expressed as micromoles of *p*-hydroxyphenylpyruvate per mg of protein per hour.
[b] Values are expressed as micromoles of kynurenine per g of protein per hour.

TABLE V

Comparative effects of casein hydrolysate, tryptophan, tyrosine, or tryptophan and tyrosine on tyrosine transaminase and tryptophan pyrrolase activities in livers of adrenalectomized rats

Animals weighed 110 to 125 g. All compounds were given orally in 3 ml of isotonic NaCl solution. The animals were killed 4 hours after treatment. Results are expressed as the mean value ± standard error for five to seven rats in each group.

Treatment and dosage per rat	Tyrosine transaminase	Tryptophan pyrrolase
	μmoles/mg protein/hr	*μmoles/g protein/hr*
Control, 3 ml of NaCl solution	0.61 ± 0.04	24.4 ± 2.7
Casein hydrolysate, 1 g	1.95 ± 0.17[a]	21.0 ± 1.6
L-Tryptophan, 14 mg	0.71 ± 0.02	22.9 ± 2.0
L-Tyrosine, 61 mg	0.70 ± 0.04	22.1 ± 2.8
L-Tryptophan, 14 mg, + L-tyrosine, 61 mg	0.80 ± 0.08[b]	24.0 ± 1.5

[a] $p < 0.01$.
[b] $p > 0.05$.

TABLE VI

Comparison of tryptophan and tyrosine with casein hydrolysate on response of transaminase and tryptophan pyrrolase in intact rats

Animals weighed 110 to 125 g. Hydrocortisone was given intraperitoneally and the casein hydrolysate and amino acids orally in 3 ml of NaCl solution. The animals were killed 4 hours after treatment. Results are expressed as the mean value ± standard error for five animals in each group.

Treatment and dosage per rat	Tyrosine transaminase	Tryptophan pyrrolase
	μmoles/mg protein/hr	*μmoles/g protein/hr*
Control, 3 ml of NaCl solution	1.06 ± 0.04	29.1 ± 2.0
Hydrocortisone, 0.1 mg	1.24 ± 0.10[a]	52.3 ± 3.5
Casein hydrolysate, 1 g	2.74 ± 0.05	38.9 ± 3.7
Hydrocortisone, 0.1 mg, + casein hydrolysate, 1.0 g	3.53 ± 0.23	68.8 ± 2.9
Tryptophan, 14 mg, + tyrosine, 61 mg	1.72 ± 0.18[b]	43.5 ± 5.7
Hydrocortisone, 0.1 mg, + tryptophan, 14 mg, + tyrosine, 61 mg	2.18 ± 0.31	92.6 ± 3.4

[a] $p > 0.05$.
[b] $p < 0.03$.

tryptophan (3 mmoles per kg) produced significant increases in the activities of both tryptophan pyrrolase and tyrosine transaminase in the livers of adrenalectomized rats (30). L-Tyrosine administration produces a significant rise in hepatic tyrosine transaminase in intact rats, but is ineffective in adrenalectomized animals unless it is given with small doses of hydrocortisone (26); the response to relatively large doses of tyrosine in normal animals is most likely due to a stress-induced release of corticosterone from the adrenals.

As assayed by the colorimetric procedure of Bates (31) there were 14 mg of tryptophan per g of casein hydrolysate. The average value for tyrosine in casein, taken from the literature, was 61 mg per g. These doses of tyrosine and tryptophan were used singly and in combination in the following studies. The oral administration of tryosine and tryptophan to adrenalectomized rats failed to elicit an increase in the activity of either enzyme (Table V). An experiment in adrenalectomized animals to test the delayed response of tyrosine transaminase to these amino acids revealed that, in comparison to casein hydrolysate, there was only a modest change (less than 15%) in the activity of this enzyme when tyrosine, tryptophan, or a combination of the two was given at 4 hours, and the rats were killed at 6 and at 8 hours after the administration of hydrocortisone.

As might have been anticipated, in intact rats both of these adaptive enzymes responded to tryptophan and tyrosine given together in doses equal to those contained in 1 g of casein hydrolysate (Table VI). It is evident, however, that the combination of hydrocortisone and casein hydrolysate was more effective than the steroid plus amino acids in stimulating a rise in the transaminase, whereas the opposite relationship existed for tryptophan pyrrolase. The results of this experiment suggest that, in intact rats, substrate stabilization could readily account for the stimulatory action of casein hydrolysate and hydrocortisone on tryptophan pyrrolase but that this mechanism could play only a minor role, if any, in mediating the response

TABLE VII

Selective effects of hydrocortisone and casein hydrolysate on enzyme induction

Rats weighing from 125 to 175 g were used in these experiments. The control rats received 3 ml of NaCl solution orally. The casein hydrolysate was given by stomach tube as a suspension in 3 ml of NaCl solution, and the hydrocortisone intraperitoneally, at zero time. Orotic acid-6-^{14}C was prepared in sterile NaCl solution and given intraperitoneally 2 hours after the administration of the steroid and casein hydrolysate. The rats were killed 4 hours after the first treatment. In Experiment 2, the rats were treated with 5μC of the ^{14}C-labeled orotic acid and the RNA was isolated from a pooled sample of liver; in Experiment 3 the dose of radioactive orotic acid was 3 μC and the RNA was isolated from individual livers. The values for both enzymes with the combination treatment were statistically significant ($p < 0.01$). For tyrosine transaminase, the differences obtained with cortisol were not significant ($p > 0.2$), but they were with casein hydrolysate ($p < 0.01$). The effect of cortisol on the activity of tryptophan pyrrolase, while of limited statistical significance ($p = 0.05$), is consistently observed; however, the differences seen following the administration of casein hydrolysate are quite variable and of a low order of significance ($p > 0.10$). On the basis of the incorporation of orotic acid into RNA (Experiments 2 and 3), all treatments effected a significant increase ($p < 0.05$). Mean values ± standard error for four to five rats in each group are given.

Treatment and dosage	Tyrosine transaminase			Tryptophan pyrrolase			Radioactivity	
	Experiment 1	Experiment 2	Experiment 3	Experiment 1	Experiment 2	Experiment 3	Experiment 2	Experiment 3
	μmoles/mg protein/hr			μmoles/g protein/hr			cpm/mg RNA	
Control, 3 ml of NaCl solution	0.60 ± 0.03	0.69 ± 0.11	0.51 ± 0.04	17.9 ± 1.3	26.2 ± 4.7	19.0 ± 2.7	4754	2696 ± 149
Hydrocortisone acetate, 1 mg per kg	0.62 ± 0.09	0.93 ± 0.21	0.61 ± 0.05	34.0 ± 1.7	41.8 ± 3.5	30.1 ± 3.2	8807	3643 ± 135
Casein hydrolysate, 1 g per rat	1.59 ± 0.13	2.71 ± 0.33	1.16 ± 0.12	24.5 ± 1.6	33.7 ± 5.8	24.1 ± 0.85	5988	3703 ± 322
Casein hydrolysate, 1 g per rat, + hydrocortisone acetate, 1 mg per kg	2.57 ± 0.10	4.48 ± 0.25	2.62 ± 0.10	62.3 ± 5.5	66.6 ± 5.2	42.5 ± 4.2	7812	3869 ± 200

of tyrosine transaminase to the enzymatic hydrolysate of casein.

Effects of Casein Hydrolysate and Small Doses of Hydrocortisone on Enzyme Induction and RNA Synthesis—In a previous report (16), Rosen *et al.* had noted that although the injection of small doses of hydrocortisone acetate into intact rats stimulated a rise in tryptophan pyrrolase activity, the levels of tyrosine transaminase were not significantly changed. The results reported here (Table II) indicate that tyrosine transaminase, but not tryptophan pyrrolase, can be induced by treatment with casein hydrolysate. It was of interest, therefore, to determine if the concomitant administration of 1.0 g of casein hydrolysate with 1 mg of hydrocortisone acetate per kg would effect an increase in the activity of these enzymes greater than that produced by either treatment alone. The results of these experiments are summarized in Table VII. The specificity of these treatments is shown by the effect of the small dose of the corticoid on tryptophan pyrrolase and by the fact that tyrosine transaminase was induced only following the administration of casein hydrolysate. When these treatments were combined, the extent of increase in activity of each of these enzymes indicates a synergistic rather than an additive response.

Studies in several laboratories have indicated that stimulation of liver RNA synthesis occurs in rats treated with doses of glucocorticoids sufficient to induce enzyme synthesis (28, 32-34). Table VII shows that, in rats given a low dose of hydrocortisone acetate or casein hydrolysate, the incorporation of orotic acid-6-^{14}C was increased from 25 to 85%. About the same magnitude of stimulation of the incorporation of this precursor into RNA was seen when both the steroid and the amino acid digest were given together. In another experiment (data not shown), the possibility that casein hydrolysate given to intact rats was affecting RNA synthesis by elevating the endogenous level of corticosterone was ruled out, since the incorporation of orotic acid-6-^{14}C into RNA in adrenalectomized animals also showed a significant increase (80%) within 4 hours after the administration of the hydrolyzed protein.

DISCUSSION

The present experiments show the selective action of casein hydrolysate on the induction of tyrosine transaminase in adrenalectomized rats maintained on a stock diet. Previously, tyrosine transaminase was shown to be responsive to casein hydrolysate in intact animals fed a protein-free diet (29). It is likely that part of this effect was due to increased levels of corticosterone—produced by the stress of maintaining the animals on a diet lacking protein—acting together with the casein hydrolysate. Threonine dehydrase and ornithine δ-transaminase have previously been shown to be responsive to casein hydrolysate administration, but to obtain significant responses of these enzymes the animals have to be fed a protein-free diet for 5 days; under these conditions their induction in adrenalectomized rats is accompanied by high toxicity and is technically difficult to achieve (24). That the induction of tyrosine transaminase by a mixture of amino acids is dependent on newly synthesized RNA is suggested by two observations: (*a*) the incorporation of orotic acid-6-^{14}C into liver RNA was increased following the administration of 1 g of casein hydrolysate, and (*b*) the administration of actinomycin D together with casein hydrolysate abolished the increase in enzyme activity normally observed in 4 hours. Whether the action of the amino acids can be attributed to another mechanism in addition to their effect on RNA synthesis is not known. However, another site of action seems likely in view of the failure of the combined treatment

with casein hydrolysate and hydrcortisone to produce an additive effect on RNA synthesis.

On the basis of studies with actinomycin D given at delayed times after the injection of hydrocortisone hemisuccinate, Garren *et al.* (25) postulated that the plateau and fall in the activities of tyrosine transaminase and tryptophan pyrrolase following treatment with the hormone could be due to the synthesis of a repressor (or repressors) of these enzymes. Numerous experiments were carried out in our laboratory in an attempt to confirm these studies, and although in a few experiments the antibiotic did seem to effect a slight increase in the activity of these enzymes, in no instance were these values significantly different from the controls (1). In general, our over-all experience in these studies is in agreement with the recent reports of Mishkin and Shore (35) and Csányi, Greengard, and Knox (7), who have also been unable to repeat the experiment reported by Garren *et al.* (25).

Tomkins *et al.* (36) reported that the amino acid antagonist canavanine, when given together with hydrocortisone hemisuccinate, further stimulated the steroid-induced levels of tyrosine transaminase and tryptophan pyrrolase in rat liver; this effect could be reversed with arginine. These results were interpreted to mean that "repression of tryptophan pyrrolase and tyrosine transaminase synthesis after hydrocortisone administration requires protein synthesis and that canavanine interferes with the formation of the repression system by virtue of its activity as an arginine analogue." It seems unlikely that canavanine would act selectively to inhibit the synthesis of the "repressor protein" and yet not interfere with the continued synthesis of the two adaptive enzymes. These investigators also raised the possibility that an amino acid antagonist, present in small amounts in casein hydrolysate, could explain the results we obtained with this mixture of amino acids (1, 36). This explanation appears to be ruled out on the basis of the results, which indicate that when given alone casein hydrolysate stimulates the induction of tyrosine transaminase only (Table II) and that when given with small amounts of hydrocortisone it acts synergistically to enhance the responses of both tyrosine transaminase and tryptophan pyrrolase (Table VII).

Treatment with large doses of L-tryptophan or other indoles can produce an increase in the activity of tyrosine transaminase in adrenalectomized rats (30). However, tryptophan and tyrosine, when given alone or in combination to adrenalectomized rats in amounts equal to those present in 1 g of casein hydrolysate, did not stimulate a rise in the activity of the transaminase or of tryptophan pyrrolase.

We have observed a significant increase (2-fold) in tyrosine transaminase activity in the livers of adrenalectomized rats which received orally a synthetic mixture of amino acids that was equivalent in composition to 1 g of casein hydrolysate.[1] Further studies with mixtures of pure amino acids are required to determine whether all of the essential amino acids are needed for the induction of tyrosine transaminase, or whether only certain amino acids present in casein hydrolysate are required to obtain this effect. Specific mixtures of amino acids have been found to have selective effects on the induction of threonine dehydrase and ornithine δ-transaminase (24).

It is evident from this study that hydrocortisone and casein hydrolysate can act independently, as well as additively and, in some instances, synergistically, to induce an increase in tyrosine transaminase, whereas tryptophan pyrrolase is responsive to the hormone but not to casein hydrolysate. That this diversity is not unique is indicated by the recent report of Peraino (23), who showed that several adaptive enzymes differed widely with respect to their responses to protein, carbohydrate, and cortisone; protein, but not cortisone, was found to be necessary for the induction of serine dehydrase and ornithine transaminase, while glucose 6-phosphate dehydrogenase was responsive to both of these agents and to carbohydrate. Thus, evidence continues to accumulate that the intracellular concentration of amino acids in liver can selectively direct protein synthesis.

There are certain features common to the induction of tyrosine transaminase by either glucagon (6-8) or casein hydrolysate. Unlike hydrocortisone, neither of these agents elicits an increase in tryptophan pyrrolase. However, in common with the hormone, the induction of tyrosine transaminase by each of these treatments is inhibited by concomitant administration of actinomycin D (7, 8). Like casein hydrolysate, glucagon acts alone and, when given with hydrocortisone, acts additively and, in some cases, synergistically to stimulate tyrosine transaminase activity. These results suggest that the effects of glucagon and casein hydrolysate on RNA synthesis are different from those of the glucocorticoid, but do not rule out the possibility that the former two agents have a common basis for their action. In this regard, Csányi *et al.* (7) recently drew attention to the possibility that any treatment which produced a rise in tyrosine transaminase activity could be acting indirectly by releasing glucagon from the pancreas. We have carried out the following two experiments[2] which indicate that such a mechanism is probably not involved in the casein hydrolysate-mediated response of tyrosine transaminase: (*a*) in the isolated, perfused rat liver, the perfusion of casein hydrolysate for a period of a few hours acts synergistically with hydrocortisone to stimulate increases of both tyrosine transaminase and tryptophan pyrrolase, and (*b*) in intact or adrenalectomized rats, combined treatment with hydrocortisone (1 mg per kg) and glucagon (3.0 mg per kg) stimulates tyrosine transaminase markedly, but depresses the response of tryptophan pyrrolase to values significantly lower than those obtained by steroid treatment alone. The latter results are to be contrasted with those obtained when casein hydrolysate is given with the same dosage of hydrocortisone (Table VII).

There are several reports indicating that RNA synthesis is stimulated in the livers of rats treated with specific amino acids (37) or protein hydrolysate (29). Although each of the treatments described in Table VII produced a significant increase in the incorporation of orotic acid-6-^{14}C into liver RNA, they differed qualitatively with regard to their effects on the induction of tyrosine transaminase and tryptophan pyrrolase. The lack of response of tryptophan pyrrolase to casein hydrolysate is difficult to understand, especially since such treatment stimulates RNA synthesis to about the same extent as that observed following the injection of hydrocortisone and provides a supply of amino acids that could be used for the synthesis of this enzyme. It may be that while the total incorporation of orotic acid-6-^{14}C into RNA by hydrocortisone and casein hydrolysate is about the same, differences occur in the particular RNA

[1] F. Rosen and R. J. Milholland, unpublished results.

[2] P. N. Raina, R. J. Milholland, and F. Rosen, unpublished results.

species that are labeled. Thus the lack of an enhanced synthesis of RNA specific for tryptophan pyrrolase might offer an explanation for the failure of this enzyme to respond to casein hydrolysate.

Treatment with glucocorticoids is known to produce a marked catabolic effect on protein metabolism in peripheral tissues (38, 39) and to decrease the rate of incorporation of amino acids into the proteins of such tissues as skeletal muscle, thymus, and cartilage (40–43). Such effects in extrahepatic tissues would be expected to elevate the level of amino acids in plasma (44) and to favor the accumulation of amino acids in liver. There are also reports indicating that hydrocortisone stimulates the uptake of amino acids into liver (45, 46) and enhances the rate of incorporation of amino acids into liver proteins (47). Furthermore, glucagon and insulin, as well as hydrocortisone, stimulated the uptake of α-aminoisobutyric acid-1-^{14}C into the isolated, perfused rat liver (48). These findings and the results of the present study reinforce the hypothesis that the response of tyrosine transaminase to certain hormones and other treatments may be due to increased levels of amino acids in liver.

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TOXICOLOGY AND APPLIED PHARMACOLOGY 18, 367–373 (1971)

Single and Double Blind Studies with Oral Monosodium Glutamate in Man

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Single and Double Blind Studies with Oral Monosodium Glutamate in Man. ROSENBLUM, IRA, BRADLEY, JAMES D., and COULSTON, FREDERICK (1971). *Toxicol. Appl. Pharmacol.* **18**, 367–373. Single and double blind studies were done with single oral doses of monosodium glutamate (MSG) in human volunteers. A total of 169 doses were given, including controls. Ninety-eight received 5 g of MSG in single blind studies, 6 received 8 g, and 5 received 12 g in double blind studies. Physical examinations were done on all subjects. Those taking 5 g of MSG filled out a questionnaire 1 hr after ingestion. Based on the questionnaire, as few as 23% or as many as 80% of the subjects in any group registered complaints. There was a low incidence of most complaints except for lightheadedness and tightness in the face. In the double blind studies in which clinical chemistry, blood pressure, and pulse were measured in addition to clinical examination, no significant differences between MSG and sodium chloride were detected. No subject reported or was observed to have experienced the triad of symptoms described as the Chinese Restaurant Syndrome.

Glutamic acid (GA) is a natural component of food and is ingested at an estimated daily level of 38 g in protein. GA is absorbed by the body in the normal digestive processes. Both GA and its sodium salt, monosodium glutamate (MSG), have been used extensively in medicine. From a clinical view point, GA and MSG have been used in psychiatric disorders (Himwich *et al.*, 1955; Albert *et al.*, 1946), geriatrics (Gasster, 1961) and to a lesser extent in hepatic coma (Alexander *et al.*, 1955). A further extensive but nonmedical use of MSG is as a food flavor, and it is this application rather than its medical use for which its safety has been recently questioned (Schaumburg *et al.*, 1969).

A series of letters appeared in the *New England Journal of Medicine* in 1968 (Kwok, 1968; Schaumburg and Byck, 1968; Ambos *et al.*, 1968) which described a reaction brought on in susceptible subjects by MSG (Schaumburg and Byck, 1968) which reproduced symptoms reportedly experienced by persons after eating in a Chinese restaurant (Ambos *et al.*, 1968). These reactions have been called the Chinese Restaurant Syndrome (Kwok, 1968).

Animal experimentation with MSG, as a basis for explaining the Chinese Restaurant Syndrome, has been going on at this Institute for approximately 4 years. Because of this long-standing work with MSG, and in the public interest, it was decided to investigate also the possible adverse reaction to MSG in man.

MATERIALS AND METHODS

Ninety-nine male volunteers between the ages of 21 and 59 were used in this study. Each subject was given a physical examination prior to every experiment. At that time they were told the nature of the experiment and their voluntary consent was obtained according to established standards (Coulston, 1967). Between July 10, 1969 and September 23, 1969, 4 groups of approximately 25 subjects each (total 98) were asked to ingest 5 g of MSG (purity, 99.5%, chemical analysis available on request). For 2 groups, the MSG was dissolved in 100 ml of tap water (5% solution) while the other 2 groups received their MSG dissolved in an equal volume of chicken stock (to disguise the taste) diluted 1 : 1 with tap water. Analysis of the chicken stock showed that it contained less than 0.1% MSG, naturally. Ingestion by drinking by all groups was approximately 15 hr after they had last eaten.

The subjects in each group were observed for 1 hr after drinking a mixture of MSG. This period was interrupted once for a 5-min interval of mild exercise, but subjects were otherwise allowed to behave in their usual manner. During the hour, the subjects were observed closely for frank evidence of adverse reaction, i.e., vomiting, ataxia, flushing; they were also free to communicate any untoward response to the observers. At the end of 1 hr, each subject was requested to fill in a questionnaire which consisted of 14 common complaints plus a space for a description of any response that did not fit the list.

Between October 28 and November 4, 1969, 2 groups of 25 and 24 subjects were organized from the original 98 subjects and 1 new member. These were used to determine the number of placebo reactors to MSG. One group received NaCl in an amount osmotically equivalent to 5 g of MSG (1.7 g, NaCl) in 100 ml of diluted chicken stock, while another group received only 100 ml of diluted chicken stock. The observation period and questionnaire employed were the same as with the groups that had received MSG.

After a careful review of the results of the questionnaire, further experiments were designed using fewer subjects and larger doses of MSG. Eleven subjects were randomly divided into those who were to receive 8 g of MSG (6) or 2.8 g of NaCl (5) in chicken stock. The distribution of the 2 treatments was known to neither the subjects nor the observers during the test period. The reason for choosing these subjects from among the original 99 volunteers was that about one-half reported multiple complaints on the questionnaire while the other one-half reported no complaints.

Each of the 11 volunteers was given a clinical examination the day before the experiment was done on November 18, 1969. On the day of the experiment, each subject was again examined, and 3 readings of blood pressure and radial pulse were taken followed by drawing of a 10-ml blood sample from the antecubital vein. The serum from these blood samples was used for clinical chemistry tests for calcium, phosphorus, glucose, urea nitrogen, uric acid, total cholesterol, total protein, albumin, total bilirubin, alkaline phosphatase, lactic dehydrogenase, and glutamic–oxaloacetate transaminase. Each was then asked to drink either 8 g of MSG or 2.8 g of NaCl dissolved in 100 ml of diluted chicken stock. Approximately 15 min after ingestion, another blood sample was drawn, and the blood pressure and pulse were again read. Thereafter, and for the remainder of the hour, the subjects were questioned, examined clinically and final readings of blood pressure and pulse were taken.

On November 24, 1969, another study was performed with this group of subjects with the exception of 1 volunteer who had withdrawn from the study. The procedure was again the double-blind type as before except that the dose of MSG was increased to 12 g in 200 ml of diluted chicken stock (6% solution) and the NaCl was increased to 4.2 g in the same amount of chicken stock.

The experimental design for all the experimental groups is shown in Table 1.

TABLE 1

EXPERIMENTAL DESIGN

Group I, 24 subjects received 5 g MSG in tap water	Group II, 25 subjects, received 5 g MSG in tap water	Group III, 25 subjects, received 5 g MSG in chicken stock	Group IV, 24 subjects, received 5 g MSG in chicken stock
Group V, 24 subjects, received 1.7 g NaCl in chicken stock		Group VI, 25 subjects, received chicken stock	
Group VII, 11 subjects, 6 received 8 g MSG in chicken stock, 5 received 2.8 g NaCl in chicken stock			
Group VIII, 10 subjects, 5 received 12 g MSG in chicken stock, 5 received 4.2 g NaCl in chicken stock			

RESULTS

Questionnaire Results (Table 2)

The incidence of complaints reported varied between groups. Sixty-seven percent of the subjects in group III (5 g MSG) reported no complaints. These results were similar to those in the control group V (71%) and VI (80%). However, in group II (5 g MSG), only 20% of the subjects reported no complaints. A chi-square test was performed comparing the total complaints in subjects receiving MSG (groups I, II, III, and IV) with the totals for control groups V and VI. The test showed a significantly greater, $P < 0.01$, incidence of complaints in the subjects receiving MSG than in the control subjects. Some complaints were reported in each of the 4 groups, these being headache, itching, lightheadedness, grogginess, dry mouth, and tightness in the face. These complaints were also registered by the control groups, and when the results with MSG were compared to those reporting in the control groups using the chi-square test, only lightheadedness (18%) and tightness in the face (12%) appear with a significantly greater ($P < 0.05$) frequency in the subjects receiving MSG than in the control subjects. Other complaints appeared with proportionally low incidence. Where a statistical comparison was possible, the incidence of other complaints did not differ significantly ($P > 0.05$) between subjects receiving MSG and control subjects.

TABLE 2

COMPLAINTS REPORTED AFTER INGESTION OF 5 GRAMS MSG OR CONTROL SUBSTANCES[a]

Complaint	GP I (25) MSG 5g		GP II (25) MSG 5g		GP III (24) MSG 5g		GP IV (24) MSG 5g		Total (98) MSG		GP V (24) NaCl 1.7g		GP VI (25) Stock		Total (49) Control	
	Yes	%	Yes	%	Yes	%	Yes	%	Yes	%	Yes	%	Yes	%	Yes	%
Headache	3	12	5	20	3	13	6	25	17	17	3	13	1	4	4	8
Nausea	—	—	1	4	—	—	6	25	7	17	—	—	—	—	—	—
Nervousness	1	4	1	4	1	4	—	—	3	3	—	—	2	8	2	4
Itchy	2	8	1	4	1	4	1	4	5	5	1	4	—	—	1	2
Lightheadedness	4	16	10	40	2	8	9	38	25	26	2	8	2	8	4	8
Flushed	—	—	4	16	—	—	5	21	9	9	1	4	1	4	2	4
Heart palpitations	1	4	2	8	—	—	—	—	3	3	—	—	—	—	—	—
Heartburn	—	—	2	8	—	—	1	4	3	3	—	—	—	—	—	—
Grogginess	1	4	4	16	1	4	5	21	11	11	1	4	—	—	1	2
Cold and clammy	—	—	—	—	3	13	—	—	3	3	—	—	—	—	—	—
Sweating	—	—	7	28	—	—	2	8	9	9	—	—	—	—	—	—
Dry mouth	2	8	6	24	2	8	1	4	11	11	2	8	1	4	3	6
Tightness in face	2	8	5	20	3	13	8	33	18	18	2	8	1	4	3	6
Sour stomach	—	—	2	8	—	—	1	4	3	3	—	—	—	—	—	—
No symptoms	15	60	5	20	16	67	7	29	43	44	17	71	20	80	37	76

[a] Groups I and II, the MSG was dissolved in 100 ml of tap water, groups III and IV in 100 ml of soup stock diluted 1:1 with water. Group V received NaCl in 100 ml of soup stock and group VI received 100 ml of diluted soup stock. Dashes indicate that no complaints were reported.

Clinical Evaluation with Higher Doses of MSG (Table 3)

A total of 12 clinical chemistry tests were performed before and after ingestion of MSG or NaCl. Results of these tests in both group VII (MSG, 8 g) or group VIII (MSG, 12 g) showed no significant difference ($P > 0.05$) between those ingesting MSG and NaCl. Furthermore, in the same groups, changes in blood pressure and pulse in the subjects taking MSG or NaCl did not differ significantly ($P > 0.05$). Other information solicited in this phase of the study concerned the presence of symptoms previously reported as being associated with ingestion of MSG or GA (Table 3). At 8 and 12 g of MSG, subjects reporting no symptoms did not differ significantly ($P > 0.05$) from the subjects on NaCl. Whenever subjects did report symptoms in both MSG and NaCl categories, the incidence did not differ significantly ($P > 0.05$).

TABLE 3

SYMPTOMS[a] REPORTED AFTER INGESTION OF 8 OR 12 GRAMS OF MSG

Symptoms	MSG 8 g GP VII (6) Yes	NaCl 2.8 g GP VII (5) Yes	MSG 12 g GP VIII (5) Yes	NaCl 4.2 g GP VIII (5) Yes
1. Burning sensation radiating out to forearms and thorax	—	—	—	—
2. Infraorbital tightness	1	—	3	1
3. Substernal pain	2	—	—	—
4. Headache	3	2	2	—
5. Fullness in stomach	1	—	2	2
6. Vomiting	—	—	—	—
7. Nausea	1	—	2	—
8. Diarrhea	—	—	—	—
9. Flushing	1	—	—	—
10. Dizziness	1	1	1	1
11. No symptoms	1	4	2	3

[a] Symptoms 1–4 reported by Schaumburg *et al.* (1969) and Schaumburg and Byck (1968), symptoms 5, 6, and 10 reported by Albert *et al.* (1946), and symptom 7 reported by Himwich *et al.* (1955). Dashes indicate that no complaints were reported.

DISCUSSION

The original objective of this study was to find, among a relatively stable population, a number of subjects who were adversely affected by ingestion of reasonable amounts of MSG in order to investigate the mechanisms of these adverse effects. This objective has not been reached in the present study. However, this study has brought forth results that are important in defining the meaning of adverse effects to MSG.

The classical description of adverse reactions to MSG or GA is found in the literature of the time when these chemicals were in wide clinical use. Hyperglycemia and hypotension have been reported in adults (Himwich *et al.*, 1955) after administration of 15 g of MSG. Oral administration of GA has caused complaints of fullness in the stomach, vomiting, and diarrhea, while iv injection has caused flushes, vomiting, dizziness, and general malaise (Albert *et al.*, 1946). A review of the clinical literature also reveals that

in many studies, large amounts of MSG or GA were administered for 2- to 6-month periods of time without causing any adverse reaction (Asten and Ross, 1960).

More recently, reports by Schaumburg *et al.* (1969) have appeared which are at variance with the clinical literature. In a study involving 56 subjects, they reported that a syndrome existed which consisted of a burning sensation radiating out to forearms and thorax, facial pressure and chest pain. The syndrome could be produced with oral MSG in 55 of the male and female subjects. The same symptoms could be evoked with iv MSG or with oral GA. The range of oral dosage of MSG was 2–12 g with the majority, 23 of 36 subjects, reacting to 5 g or less of MSG. These authors proposed MSG as the cause of the Chinese Restaurant Syndrome. An additional complaint, headache, occurred consistently in 2 of the 56 subjects. This triad of symptoms, burning, chest pain, and facial pressure, following ingestion of MSG, had not been described in the older literature of MSG or GA.

In the present study, 98 subjects were given 5 mg of MSG orally and in no case were all 3 symptoms of the Chinese Restaurant Syndrome observed or reported by the subjects. Of the symptoms mentioned above, only tightness in the face was reported in 12% (adjusted for controls) of the subjects. A burning sensation radiating out to forearm and thorax was never reported, nor was chest pain. In this study, heart palpitation occurred in only 3% of the subjects.

Subsequently, selected subjects from the large group were given 8 g and later 12 g of MSG, in a double blind study, but these studies also failed to reveal a single subject who had experienced the triad of symptoms suggestive of the Chinese Restaurant Syndrome. One subject who received 8 g of MSG did not continue on the study because he believed that he had been affected adversely. This subject reported feeling facial pressure and chest pains which make up 2 of the 3 symptoms reported by Schaumburg *et al.* (1969), but he also reported nausea, flushing, and dizziness. However, since no significant changes in blood pressure or pulse or in the clinical chemistry values were found, it was not considered an example of the Chinese Restaurant Syndrome.

With regard to the adverse responses of hypotension (Himwich *et al.*, 1955) or hyperglycemia (Himwich *et al.*, 1955) reported in the older literature of MSG and GA, neither response was more common after 8 or 12 g of MSG than after NaCl. Of the symptoms reported in the clinical literature, nausea, flushing, or fullness in the stomach (either heartburn or "sour stomach") occurred with low frequency after 5 g of MSG. However, vomiting and diarrhea were never seen at any dose of MSG or NaCl in the present study. If dizziness can be equated with lightheadedness, then this complaint was also observed in the present study, as its most common finding (18% when adjusted for controls).

The results obtained in this study with MSG demonstrate the difficulties encountered in extrapolating from one clinical study to another. From the report of Schaumburg *et al.* (1969), it had been expected that administration of 5 g of MSG would uncover a large number of reactors so that the phenomenon of the Chinese Restaurant Syndrome could be studied in depth. This expectation was not achieved, but a clearer viewpoint about responses to MSG was obtained. At 5 g of MSG, the two most common complaints are lightheadedness and tightness in the face. With larger doses of 8 or 12 g of MSG, nausea can become more common than with the lower dose, but it is not followed by vomiting.

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Quantitative Determination of Glutamic, Aspartic, and Cysteic Acids by Ion Exchange Chromatography[1]

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In 1952 Hirs, Moore, and Stein described an ion exchange chromatographic method for the isolation of the amino acids from protein hydrolyzates (1). In this procedure, which used volatile buffers, a separate column of an anion exchange resin was employed for the isolation of the dicarboxylic amino acids. Separation of aspartic and glutamic acid was not complete, however. Since a quantitative method for the determination of these substances alone was needed, the procedure of Hirs *et al.* has been modified so as to provide increased resolving power, sensitivity, and speed. With the aid of this procedure, the glutamic and aspartic acid content of plasma and urine have been determined after acid hydrolysis.

MATERIALS AND METHODS

Preparation of the Resin and Columns

A volume of 200 ml of a Rohm and Haas 100–200 mesh CG-4B weakly basic anionic resin is suspended in 600 ml of 2 *N* acetic acid, stirred for 2 hours to remove air bubbles, and then filtered by suction. The cake is washed consecutively with (*1*) about 600 ml of 2 *N* acetic acid until the wash solution has a pH less than 3, (*2*) 600 ml of distilled water, and (*3*) sufficient 0.2 N sodium acetate buffer, pH 4.5, until the filtrate fluid is at pH 4.5. The resin is suspended in 0.2 *N* sodium acetate buffer pH 4.5 and allowed to stand for 2–3 hours to release trapped air bubbles.

After resuspension of the resin slurry by gentle stirring, the resin is poured, in three equal sections, into a 10 × 300 mm or 1 × 30 cm column supplied with a sintered disc, to a height of 26 cm. Each layer is packed with 10–15 mm of mercury air pressure. The resin slurry must contain

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sufficient fine particles so that three distinct bands can be seen when the column is completed. The column is equilibrated with the eluting buffer, 0.2 N sodium acetate, pH 4.5, for 2–3 hours before use. The column is operated by gravity flow controlled by adjusting the height of the reservoir. After each use of the column, the discolored upper surface of the resin is removed and replaced with an equal quantity of fresh resin.

Regeneration of the Resin

The column retains good resolution for five analyses of plasma or urine before regeneration is necessary. For regeneration, the resin is removed from the column, suspended in 2 N sodium hydroxide, and filtered by suction. The resin cake is washed with 2 N sodium hydroxide until the pH of the wash fluid is greater than 8, and is then converted to the acetate form with 2 N acetic acid, and re-equilibrated with acetate buffer.

Collection and Analysis of the Effluent Samples

Originally, 100 one-ml fractions were collected by a Technicon Drop-counter fraction collector[2] and analyzed by the ninhydrin method of Moore and Stein (2, 3). Currently, the analysis of the column effluate is automated by means of the Technicon AutoAnalyzer (4). Three columns are run simultaneously using three colorimeters at 570 mμ, each with a 15-mm tubular flow cell. The results are recorded on a 3-Point Bristol recorder. Samples are placed on the columns about 10 minutes apart to avoid superimposition of the amino acid curves.

The ninhydrin reagent employed for the AutoAnalyzer is prepared as follows: To 750 ml of peroxide-free methylcellusolve (reagent grade), containing 20 gm of ninhydrin and 1.5 gm of hydrindantin, is added 250 ml of 4.0 N sodium acetate buffer, pH 5.5. One liter of ammonia-free water[3] and one liter of methylcellusolve are added bringing the total volume to 3 liters. Nitrogen is then bubbled through the reagent for 4 hours. It is stored under nitrogen in a dark bottle and is stable for about 2 weeks under these conditions.

Standard Solutions

Glutamine, leucine, glutamic, aspartic, and cysteic acid standards[4] were originally 1 mM in 0.1 N sodium citrate buffer, pH 5.0. However,

[2] Technicon Corp., Chauncey, New York.

[3] Obtained by using De-Ionizer cartridge, Illco Way research model.

[4] The amino acids were purchased from Nutritional Biochemical Corp., Cleveland, Ohio.

with the increased sensitivity of the AutoAnalyzer, standard amino acid solutions are 0.25 or 0.50 mM, and a 1-ml aliquot is analyzed.

Analysis of Blood and Urine

Preparation of Plasma. Blood is collected in a heparinized syringe; hemolysis must be avoided. After separation, the plasma is recentrifuged to remove any residual cellular material.

For the determination of glutamic acid, 10 ml of a 1:6 picric acid filtrate is filtered through a 2-3 cm Dowex 2 × 8 resin bed as described by Stein and Moore (5). The column is washed three times with approximately 10 ml of 0.02 N HCl. All of the effluent is concentrated to a volume of 1-2 ml on a rotary evaporator at 40°. A few milligrams of Celite (analytical filter aid) are added and the mixture is filtered through Whatman No. 40 filter paper previously washed with 1 N HCl. The filtrate is evaporated to dryness and the residue is dissolved in 3 ml of 0.2 N sodium acetate, pH 4.5. A 2-ml aliquot is used for analysis.

For the determination of glutamine and asparagine, 5 ml of a 1:6 picric acid filtrate is treated similarly except that the final residue is dissolved in 4 ml of 6 N hydrochloric acid for hydrolysis. The flasks are evacuated through securely clamped and lightly greased stopcock attachments and placed in an oven at 110° for 16-22 hours.

The sample is diluted with distilled water, rinsing down the stopcock unit and flask walls. The resulting volume is concentrated to 1-2 ml. To ensure removal of hydrochloric acid, the dilution with water and concentrates are repeated at least two additional times. The residue is dissolved in 3 ml of 0.2 N sodium acetate buffer, pH 4.5. A 2-ml aliquot is analyzed.

Preparation of Red Cells

After removal of the plasma, the red cells are washed three times with lactate Ringer's solution. The packed cells (1 ml) are added to 3 ml distilled water and the mixture is quickly frozen and thawed. A 1-ml sample of the hemolyzate is added to 5 ml of picric acid. The picric acid filtrate is filtered through a Dowex 2 × 8 resin bed, etc. The technique previously outlined for the preparation of plasma for glutamic and aspartic acid analyses is followed.

Analysis of Urine

For the determination of glutamic and aspartic acids, a 0.5 ml of fresh urine is placed directly on the column. To determine glutamine and asparagine, 0.5 ml of urine is hydrolyzed either by the method of

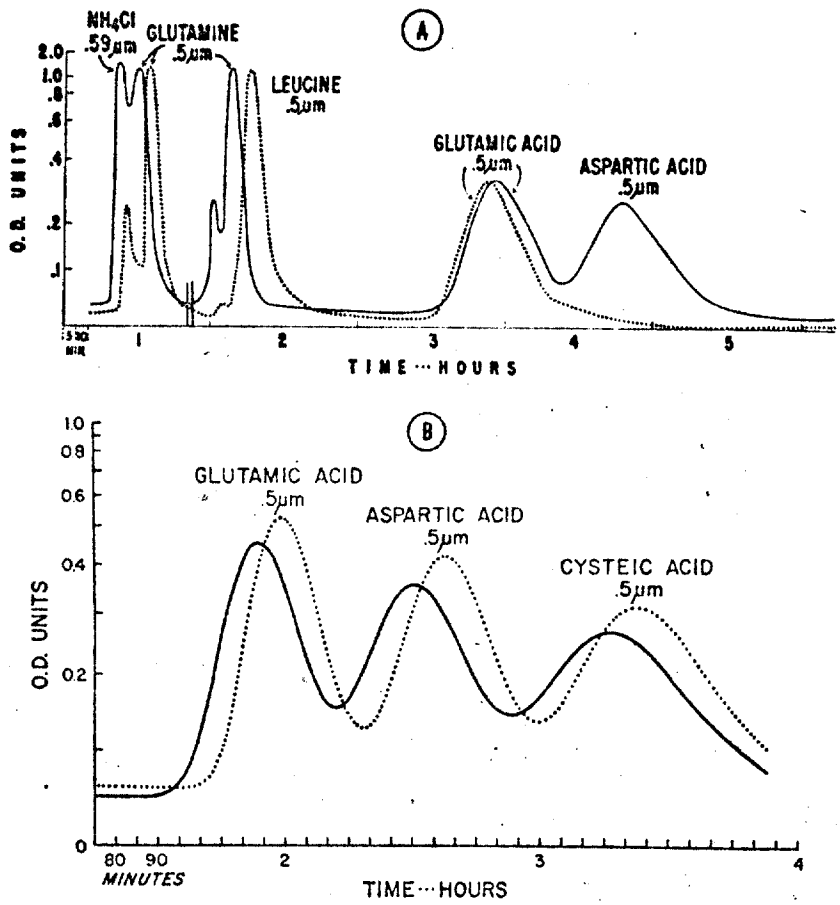

FIG. 1. (A) Standard amino acid curves obtained by the AutoAnalyzer employing two separate columns as represented by the dotted and solid lines. (1) To the left of the black parallel lines (1 hour, 20 min) the separation of ammonia from glutamine is demonstrated by comparison of chromatograms of glutamine (dotted line) and glutamine and ammonium chloride (solid line). (2) To the right of the black parallel lines a new series of analyses demonstrating the separation of the neutral amino and dicarboxylic amino acids. (B) Standard amino acid curves recorded by the AutoAnalyzer demonstrating the separation of the three amino acids. The integration constants for glutamic acid are 25.5, 25.0; for aspartic acid, 26.2, 25.6; and for cysteic acid 26.6, 26.9.

Stein (6) or as described above. If the urine contains a significant amount of protein, a 1:6 picric acid filtrate is prepared as outlined for plasma and a sample is analyzed.

RESULTS AND DISCUSSION

The resolution of glutamic and aspartic acids obtained under the present conditions is shown in Fig. 1A.

The adaptation of the method to the AutoAnalyzer has improved the sensitivity so that 0.06 to 1 μg of glutamic and aspartic acids are determined routinely.

Duplicate integration constants (14) for standard amino acid solutions on a given resin column agree to $\pm 2\%$ (Fig. 1B). Standard solutions of leucine, glutamine, glutamic, and aspartic acids added to plasma filtrate were recovered $100 \pm 5\%$. The conversion of glutamine to glutamic acid after 16–22 hours of acid hydrolysis is $95 \pm 5\%$ complete. Glutamic acid is stable as a picric aicd filtrate under the following conditions: (*a*) stored at 4°; (*b*) at −70° or (*c*) at 4° before separation of the supernatant from the protein precipitate (Table 1).

Physiologic Data

The results obtained on analysis of a sample of plasma are shown in Fig. 2. All neutral amino acids and ammonia form one large peak but glutamic, aspartic, and cysteic acids are separated and can be determined quantitatively. For the determination of glutamine after hydrolysis of plasma, no correction is made for the small quantity of glutamic acid present in the unhydrolyzed specimen. Duplicate determinations of glutamic acid, glutamine and asparagine in plasma agree to ± 6, ± 5, and $\pm 7\%$, respectively.

Plasma filtrates were analyzed both by the manual method and the automated technique. The values differed by less than $\pm 5\%$. All of the results reported in Table 2 are averages of both methods. The results obtained are similar to those reported by others who have used chromatographic, chemical or enzymatic procedures (5, 8–10). A small but constant amount of glutamic acid is detected in human plasma but aspartic acid is not detectable. Too few observations have been made so far to warrant comment as to the physiologic occurrence of cysteic acid. Fewer determinations of asparagine are presented because quantitative measurements were only obtainable by the more sensitive automated method. There is no significant difference in the plasma concentrations of glutamic acid, glutamine or asparagine in normal or cardiac subjects regardless of the severity or chronicity of the heart failure.

TABLE 1

VARIATIONS WITH TIME AND METHOD OF STORAGE OF THE GLUTAMIC ACID CONCENTRATION IN PLASMA

No.	Date specimen obtained and prepared	Dates of analysis	Method of storage	Glutamic acid conc. (mg/100 ml)
1	July 17	July 17		0.635
2	Normal plasma	August 3	4° filtrate	0.620
3		August 3	−70° filtrate	0.635
4		September 28	4° filtrate	1.55
5		September 30	4° Picric acid	1.07
6		October 20	4° filtrate	1.12
7		October 26	−70° filtrate	1.23
8		October 27	−70° filtrate	1.53
1	November 13 Normal plasma	November 18	4° filtrate	0.95
				0.95
2		November 20	4° filtrate	1.27
				1.18
3		November 23	4° filtrate	1.02
				1.04
4		November 24	4° filtrate	1.06
				0.92
5		November 30	4° Picric acid	0.85
				0.85
6		December 3	4° filtrate	1.37
7		December 3	4° Picric acid	1.21
8		December 4	4° filtrate	1.22
9		December 4	4° Picric acid	1.07
10		December 7	−70° filtrate	0.94
11		December 8	−70° filtrate	1.09
1	December 16 Normal plasma	December 16		0.92
				0.84
2		December 17	4° filtrate	0.95
				0.85
3		December 18	4° filtrate	0.87
		December 22	4° filtrate	1.03
				1.01
5		December 23	4° filtrate	1.14
				1.03

CONTENT OF RED CELLS

Three values for the glutamic acid are listed in Table 2. The high concentration of glutathione as well as glutamine in cells emphasizes the importance of using plasma free of cells and hemolysis for the analysis

TABLE 2

PLASMA AND URINE VALUES OF GLUTAMIC ACID, GLUTAMINE, AND ASPARAGINE

	Site of specimen								
	Antecubital vein (mg % ± SD)			Artery (mg % ± SD)			Urine (mg/24 hours)		Red blood cells (mg/100 ml)
	Glutamic acid*	Glutamine	Asparagine	Glutamic acid	Glutamine	Asparagine	Glutamine	Asparagine	Glutamic acid
Pooled plasma	1.06 ± .17[a] (16)	11.6 ± .35 (9)	3.2 ± .29 (9)						
filtrate	.96 ± .10[b] (9)								
Normal subjects	.91 ± .073 (16)	9.67 ± 1.60 (15)	2.25 ± 1.13 (8)				373 ± 91 (9)	170 ± 49 (5)	2.8 3.1 4.7
Patients with heart disease	1.05 ± .11 (3)	9.08 ± 1.60 (3)	1.42 ± .63 (3)	1.22 ± .12 (18)	8.71 ± 1.02 (16)	1.91 ± .85 (12)			

* *a* and *b* correspond to averages of Normal Plasma in Table 1. Numbers in parentheses indicate number of determinations.

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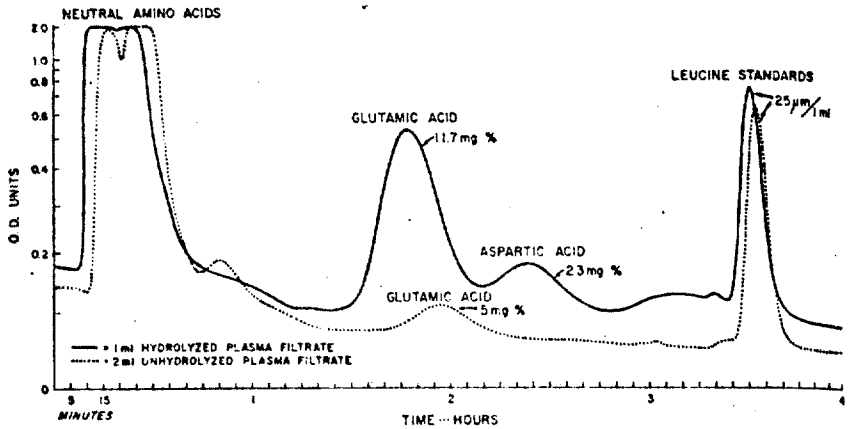

FIG. 2. Plasma filtrate amino acid curves analyzed by the AutoAnalyzer demonstrating the increase in glutamic acid (glutamine) and aspartic acid (asparagine) after acid hydrolysis.

of plasma glutamine (11). The concentration of glutamic acid in red cells is only slightly higher than in plasma.

Urine contains no aspartic or glutamic acid (6). The daily urinary excretion of glutamine ranges from 192 to 450 mg/24 hours and the excretion of asparagine ranges from 118 to 244 mg/24 hours (6) (Table 2).

SUMMARY

A modified ion exchange of chromatographic method for the separation of glutamic, aspartic, and cysteic acids has been presented. Glutamine and asparagine are determined as glutamic and aspartic acids after 16–22 hours of acid hydrolysis. The method is rapid, simple, quantitative, and can be automated.

ACKNOWLEDGMENTS

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editorial

The many-faceted mystique of monosodium glutamate

M. E. Rubini

The use of seaweeds, sea tangles, and soy sauces as condiments has a history in the Orient of at least a thousand years. All contain monosodium glutamate and to this substance is ascribed their palate-pleasing properties. In the student home economics class and in the Cordon Bleu demonstration course, the gourmet cook-to-be is taught that freshness and flavor are enhanced by adding monosodium glutamate to his choice recipe. The food processor is similarly inclined, and tons of this chemical have found their way into canned and frozen foods to make them more tasty. It is on the GRAS list (the FDA's Generally-Regarded-As-Safe designation). The chemical that is utilized as a food additive is now usually prepared by fermentation, but extraction of beet residues is still used by some manufacturers and accounts for 10% of production. As might be expected, the greatest production capacity is in Japan.

Now in the wake of cyclamate publicity, monosodium glutamate came to new attention when several major baby food manufacturers (Gerber, Beech-Nut, and Heinz) announced their intention to desist adding this compound to processed baby food, at least until more is known about its safety.

Presumably, the manufacturer has become concerned that the taste of baby food may be less critical than its safety. Although monosodium glutamate is known to be a toxic material when given intravenously, the basis of action is inconclusive that oral glutamate poses a threat to human infants because it was reported toxic to infant rodents. Others, especially the chemical companies making glutamate and the competing baby food packers, but also some scientists, have questioned the scientific basis of such action. They have pointed to its long use as a food additive, its presence in natural protein, and even to the testimonial of the Far East cultures which, because they are ancient, must be wise. They argue that glutamate is nearly innocuous when fed orally and because it means better taste to food some infinitesimal and nebulous hazard can be neglected until more data are available. How much pleasure can be equated with how little safety or how much risk is not defined. The first step in doing so might be to try to quantitate the pleasure obtained from adding glutamate to food.

Despite the wide public acceptance that glutamate enhances the pleasure of eating, it is difficult to prove that glutamate improves flavor, or changes taste or really does anything at all. Despite the many millions of dollars spent on this substance, the mechanism of its action in making eating more pleasurable, as is true of most physiologic properties, is unknown. Most commercial usage depends upon taste analysis, and the subjective enhancement of flavor is said to be distinctly different from alteration or accentuation of taste. Efforts to demonstrate changes in taste threshold by the addition of monosodium glutamate to dilute solutions of test substances have been generally unsuccessful—(shades of the Emperor's new clothes . . .).

Then there is the so-called "Chinese Restaurant Syndrome" that has been recently brought to medical attention (1). The burning, chest pain, headache, and abdominal distress that some people experience after eating Chinese food seem to be due to the monosodium glutamate content of their din-

ner. But even this is not a clearly predictable drug effect. Different individuals react differently to monosodium glutamate. Some persons react vigorously to 1- or 2-g doses, but many people develop no symptoms even if given 12 g. In a previous era, when glutamate and glutamine were thought to be beneficial in individuals with mental disease, and were even ascribed an intelligence-raising capacity, some patients were apparently given as much as 45–75 g/day without symptoms. The L isomer of glutamates evokes symptoms, but the D isomer does not. Previous ingestion of foods appeared to block symptomatology, but no known drug is effective—sympathetic blockades, parasympathomimetics, vasoactive compounds, et cetera.

Thus far the biochemists' knowledge of the metabolism of glutamates is of little help in explaining the basis of toxicity. Glutamate is a nonessential amino acid with a function as an amino carrier between L-ketoglutarate and glutamine. It is an important precursor of glutathione and insulin, as well as a critical source of urinary ammoniam. Curiously, glutamate is unique among amino acids by virtue of its direct utilization as an energy source by cerebral tissue.

Real experimental evidence that glutamate is toxic orally is nebulous. Turgull reported that even small amounts of monosodium glutamate fed orally to rabbits for 1 month induced fetal resorption or malformations. But other studies in which rabbits were given glutamate in amounts up to 100 times this quantity did not duplicate these findings.

Even in the Chinese Restaurant Syndrome it is difficult to incriminate glutamate specifically and there is sound reason to believe that at least in some persons a food allergy is operative. Over 20 years ago, Randolph and Collins demonstrated that headaches, leg and abdominal cramps, urticaria, pruritus, coughing, heartburn, mental confusion, and fatigue may occur in individuals ingesting 2–5 g monosodium glutamate prepared from the sugar beet residue following sugar extraction. Because monosodium glutamate prepared from wheat gluten did not evoke the syndrome, they considered the symptoms to be allergic. Some individuals sensitive to wheat protein had similar sensitivity to monosodium glutamate prepared from wheat. A high protein diet contains more natural glutamate than the 1–2% added to food. Beets and borscht have been the focus of an amusing defense by Bessman and Hochstein (3). For a compound ubiquitous in protein-containing natural foods, this late discovery of a pharmacologic activity by oral feeding is surprising.

It seems unequivocal that glutamic acid, monosodium glutamate, and glutamates are toxic compounds when given parenterally. Aspartate acts similarly at equivalent doses, although apparently aspartate is benign when fed. Ketoglutarate, given intravenously, is benign, at least in regard to certain enzymatic protein hydrolysates. Perhaps it is just as well that these proved clinically unpopular, because young mice injected with glutamate develop brain lesions, obesity, sterility, and are stunted in growth (2). Chicks given glutamate parenterally develop striking skeletal malformations. Rabbits consistently develop retinal lesions when given only modest amounts of glutamate systemically. Fortunately, there are no chronic toxicity data on man, although acute intravenous administration of glutamate makes men and dogs vomit.

It is difficult to understand how a simple substance that is readily absorbed is so much more toxic when given by vein than by mouth. Some authors have tried to correlate plasma levels with toxicity but these data are difficult to interpret, as plasma clearance is rapid. Blood levels of glutamate vary substantially in different persons after oral ingestion and basal levels are generally reached after 3 hr even after high doses. Injected or fed glutamate is thought to be readily metabolized to bicarbonate while adding its amino nitrogen to the labile transamination pool. Some have suggested that high levels of glutamate may inhibit glutaminase activity and cause glutamate to accumulate intracellularly. Little is known about the specific metabolism of glutamate and especially the inhibition of formation or metabolism of glutamate-containing compounds when glutamate is available in excess. Although glutamate-aspartate transaminase activity is increased when glutamate is administered,

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may not be protective at some biochemical sites where transamination is not available.

More knowledge is needed for a rational understanding of what monosodium glutamate offers, both as advantage and as risk. Although it can be argued that the removal of glutamate from baby foods was premature and not based on sound scientific evidence, perhaps one should also ask why it was added in the first place. There seems to be reasonable evidence that changes in the taste of food (according to an adult taster) do influence infant behavioral response, but whether this is taste in the same sense that older child experiences taste is uncertain. If the baby can taste, its taste must be atrocious. Was the taste or flavor of a slurry of pureed apricots and minced veal—or a gruel of powdered bone cereal and sieved liver—really improved by the addition of monosodium glutamate? For the infant? For its mother? Ugh.

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Effect of Glutamic Acid on the Central Action of the Ammonium Ion.

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In 1936, in a study of the effect of various stimulants and depressants of the central nervous system on the aftercontraction in humans, it was noted that small amounts of ammonium salts (.5 g ammonium), taken by mouth, caused a marked increase in this phenomenon. We have considered the aftercontraction a measure of the excitability of the human cerebral cortex.[1,2,3] It has been repeatedly affirmed in the literature that the concentration of ammonium ion increases in the brain tissue and cerebrospinal fluid in states of increased excitability of humans and animals. The fact that oral and parenteral administration of ammonium salts can produce convulsions in animals is well known.

Since ammonium convulsions so closely simulate spontaneous seizures, it seemed that any substance which would prevent these seizures, might be useful in other types of convulsive activity. The work of Krebs on the metabolism of amino acids in brain tissue slices offered an important clue.[4] Of many substances tested he found that glutamic acid specifically caused a marked reduction in the ammonium ion concentration present in the medium surrounding the tissue slices, due to the action of the enzyme glutaminase which synthesized glutamine from glutamic acid and ammonia. The enzyme system was reversible, was stimulated by the physiological *l* (+) glutamic acid and inhibited by *d* (—) glutamic acid. Under this concept, it should be possible to increase or decrease the ammonium ion concentration of the brain and thus control its convulsive activity. Experiments to test this hypothesis were begun in July, 1942.

In our studies on the role of ammonium ion in the convulsive state, we developed a uniform method of production of seizures in rabbits by the slow intravenous injection of 2½% ammonium chloride solution. One cc per kilo of this solution is administered every 5 minutes until a generalized convulsion results. All 12 of the rabbits (2 kg) used in these experiments had convulsions after 15-25 cc of the solution had been injected. The observations were

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repeated at weekly intervals to check the thresholds.

We have employed the natural *l* (+) glutamic acid by intravenous injection in an attempt to prevent ammonium convulsions. In a series of preliminary experiments, we have found *l* (+) glutamic acid to give complete protection from the convulsant effects of the ammonium chloride solution. In 8 experiments 50 cc of *l* (+) glutamic acid (5% glutamic acid and 3% sodium bicarbonate) was given intravenously shortly before the injection of ammonium chloride (also dissolved in sodium glutamate solution) and no convulsion resulted in any instance even after 50 cc of the 2½% solution of ammonium chloride was injected. As a control, similar experiments with equimolar amounts of *l* aspartic were performed in the same rabbits. Aspartic acid fails to protect from the convulsant activity of ammonium chloride, indicating the specificity of the glutamic acid reaction.

If *l* (+) glutamic acid is capable of preventing ammonium convulsions, it should be effective in other types of convulsive activity, which are associated with disturbances in ammonium metabolism, perhaps including human epilepsy. We have injected intravenously as much as 500 cc of 5% *l* (+) sodium glutamate into humans within a few hours without any apparent toxic effects. We are now investigating its action in human epileptic seizures.

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CHINESE-RESTAURANT SYNDROME

To the Editor: It is with some relief and delight that I rea of the Chinese-Restaurant syndrome in the April 4 issue For the past two years I have on three occasions exper enced a tightening of my masseter and temporalis muscle lacrimation, periorbital fasciculation, numbness of the nec and hands, palpitation and syncope. Each episode occurre within 20 minutes after I ate in my favorite Chinese resta rant. On one occasion it was experienced by two of m companions but not the third. On relating my experience t my colleagues, I have usually been exposed to ridicule an shouts of "crock". I have always attributed it to an allerg reaction, although I have no known allergies. The sensatio will usually disappear within five minutes and does not recu in spite of my continuance of the meal.

I don't think the cause is soy sauce or monosodium glut mate, for we use the same ingredients in our home. Th theory of transient hypernatremia sounds reasonable; how ever, on one of the occasions we had consumed 24 ounc of beer immediately before the meal.

I have always thought that the experience was "restaurat dependent" since I notice it at only one place and I am chronic consumer of Chinese food. I should be delighted t submit a grant, perhaps a career development award, to th NIH for an intensive study (with foreign travel fundsy o this problem.

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THE UPTAKE OF GLUTAMIC ACID AND GLUTAMINE BY BRAIN AND OTHER TISSUES OF THE RAT AND MOUSE*

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Recently we reported studies of the absorption of glutamic acid and glutamine from the gut of the cat by analysis of the portal blood, and of the concentration changes of the two amino acids in the peripheral blood plasma after oral administration of glutamic acid to human subjects (3). In a further study of the fate of glutamic acid and glutamine in the animal body, the uptake of the two amino acids by brain, muscle, liver, and kidney after intravenous administration to rats and mice has now been determined. Changes in total amino acid nitrogen have been estimated previously in brain and other tissues (4, 5) after the intravenous administration of glutamic acid to rats and dogs, but this unspecific measure of amino acid concentration cannot give evidence regarding the movement of particular amino acids, especially since it has been found that a high blood level of glutamic acid causes an increased uptake of other amino acids by the tissues (3, 6).

EXPERIMENTAL

Animal Experiments—Rats (160 to 180 gm.) and mice (16 to 18 gm.) were kept without food for about 18 hours prior to the experiment. Glutamic acid (as the sodium salt), or glutamine, was injected in 7 per cent aqueous solution into the tail vein in amounts roughly corresponding to 1.2 to 1.3 mg. per gm. of body weight. Care was taken to excite the animals as little as possible during the experiment. After different time intervals, the animals were killed by decapitation and the blood was caught in weighed bottles containing 5 per cent trichloroacetic acid, which were then weighed again. In the experiments on rats the head was dropped directly into liquid nitrogen. The organs to be analyzed were dropped into liquid nitrogen within 1 minute after the death of the animal. The frozen organs were crushed (7). Muscle tissue could be obtained in

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† Fellow of the Children's Hospital Research Foundation, Washington, D. C. Under contract with the Office of Naval Research.

powdered form only with difficulty. The removal of the brain was greatly facilitated by the fact that in liquid nitrogen the rat skull including the skin cracked open longitudinally in such a way that the crack usually separated the brain into two equal halves which could be easily lifted out of the skull with the aid of a small chisel.

In the experiments on mice, the brain and other organs were removed as quickly as possible and ground in a mortar precooled with dry ice. The layer of tissue was frozen within 2 minutes after the death of the animal. The frozen rat and mouse tissues were weighed in trichloroacetic acid, as described for blood.

Preparation of Extracts and Determination—The tissue suspensions were transferred quantitatively to centrifuge tubes and centrifuged. The residues were extracted repeatedly with trichloroacetic acid, with vigorous stirring before each centrifuging. The combined extracts were transferred to volumetric flasks and diluted with water to about 90 per cent of the final volume, neutralized to pH 7, and diluted to the mark. The final concentration of trichloroacetic acid was less than 2.5 per cent. The volumes were selected so that the final concentration of glutamic acid or glutamine would be 10 to 20 γ per ml.

Glutamic acid and glutamine were determined as described previously with the modification introduced for the removal of glutathione (8, 9, 3). The fact that the ratio of glutamine to glutamic acid is much lower in the tissues than in the blood necessitated the concentration of the filtrates before hydrolysis. 2 ml. of neutralized filtrate from the "acid alumina" column (8) and 2 ml. of wash water were collected in a graduated centrifuge tube (12 ml.) and concentrated to 0.5 ml. in a boiling water bath with the aid of a gentle stream of filtered air. After the addition of 0.25 ml. of 6 N HCl, the sample was hydrolyzed for 1 hour in a boiling water bath and neutralized, and water was added to a final volume of 5 or 10 ml., according to the expected glutamic acid concentration. The glutamic acid derived from glutamine after hydrolysis was not determined directly, but was first passed through an alumina column to avoid interference by aspartic acid liberated from its amide by the hydrolysis. With tissues containing a large excess of glutamic acid over glutamine (kidney and brain), it was necessary to start with an extract which contained a multiple of the glutamic concentration suitable for the colorimetric method. This was possible, since the capacity of the adsorption column for glutamic acid exceeds by far the amounts which are within the range of the colorimetric method. Therefore, a more concentrated extract was subjected to adsorption, but only an aliquot of the acetic acid eluate was used for the ninhydrin treatment after adjustment of the aliquot to 4 ml. with 0.5 N acetic acid. By using concentrated tissue extracts and in addition concentrating the glu-

tamine-containing filtrate (see above), the glutamine values were brought within the range of the colorimetric method.

The recovery of glutamic acid and glutamine was tested after the addition of the two compounds and of glutathione to trichloroacetic acid extracts of liver and brain (Table I). Large amounts of glutathione do not interfere with the determination of the amino acid and its amide, a fact particularly important when the method is applied to liver extracts in which a concentration of glutathione at least equal to the sum of glutamic acid and glutamine is to be expected.

All determinations were carried out in duplicate with a glutamic acid standard included in every set. All values (Tables II and III) are based on

TABLE I

Recovery of Added Glutamic Acid (Acid) and Glutamine (Amide) in Extracts of Rat Liver and Brain

Values expressed as mg. per 100 gm. of tissue.

Organ	Added			Found		Calculated	
	Acid	Amide	Glutathione	Acid	Amide	Acid	Amide
Liver I				48	67		
	26	31	132	75	101	74	98
" II				47			
	36	41	180	89		83	
" III				26	55		
	47	53	235	71	106	73	108
	94	106	470	126	167	120	161
Brain				163	67		
	157	89	210	322	134	320	156
	314	178	420	490	232	477	245

determinations carried out on the tissue extracts of the pooled organs of three animals.

RESULTS AND DISCUSSION

Concentration in Organs of Fasting Animals—The glutamic acid and glutamine concentrations found in the organs of the control animals (Tables II and III) are of considerable interest, since they represent, as far as we know, the first data on the content of the two amino acids in the non-protein fraction of brain, muscle, kidney, and liver of the rat and mouse. In brain and kidney a considerable excess of the amino acid over its amide was found. The ratio in the brains of these two animals is very similar to that found with our method in cattle, rabbits, cats, and pigeons.[1] In

[1] Unpublished.

the liver, the glutamine concentration equals or exceeds that of glutamic acid, and the limited data on skeletal muscle also indicate an excess of the amide. Krebs and associates (11) recently reported on the concentration of glutamic acid and glutamine in a variety of tissues of pigeons, sheep, and cats. The determinations which have been reported are in agreement in

TABLE II

Glutamic Acid (Acid) and Glutamine (Amide) Concentration in Rat Organs after Intravenous Injection of Glutamic Acid or Glutamine

Values expressed as mg. per 100 gm.

Min.	Brain		Liver		Muscle		Kidney		Blood	
	Acid	Amide	Acid	Amide	Acid	Amide	Acid	Amide	Acid	Amide
0*	152 ±16†	57 ±8.1	49 ±14	55 ±3.7	18 ±2.6	40 ±12	96 ±4.5	22 ±11	3 ±0.7	6.1 ±1.4
Glutamic acid administered										
10	120	65	94	38	40	35				
10	144	52	99	31	86	58	400	29	109	5.4
15	122	58	63	69	18	42	490	38	63	7.5
15	111	34	111	65	54	55	520	43	90	7.1
20	140	56	72	45			424	30	85	6.6
20	158	62	114	44			510	47	68	6.2
30	116	37					260	40	4.6	4.3
60	131	67								
Glutamine administered										
10	120	68	210	210	18	68				
15	161	78	370	92			219	99	5.1	32
15	165	85					146	239	6.7	86
20	140	84	200	73	13	53				
30	136	36	157	128			130	185	4.4	34
30	155	83					274	174	6.1	36
60	147	78					94	21	3.6	7.5

* Each average in this row represents six groups of three animals each, except for liver and muscle for which five and three groups were used, respectively.

† Standard deviation. The bold-faced values differ from the control values significantly ($P < 0.05$) (10).

showing that the brains of cattle,[1] sheep (11), cats (11),[1] dogs (12), rabbits,[1] pigeons (11),[1] rats, and mice contain about 0.01 mole of glutamic acid and about 0.004 mole of glutamine per kilo. Sheep (11) kidney contains a large excess of glutamic acid over glutamine, just as does the kidney of the rat and mouse. In the blood of rat and mouse, as in that of other mammals

studied (man, ox, and dog), a considerably higher concentration of glutamine than of glutamic acid was found.

Effect of Administration of Glutamic Acid or Glutamine on Their Concentrations in Blood—The amino acids were injected in amounts calculated to yield an initial blood concentration of 1 to 1.5 per cent, but only a small

TABLE III

Glutamic Acid (Acid) and Glutamine (Amide) Concentrations in Mouse Organs after Intravenous Injection of Glutamic Acid or Glutamine

Values expressed as mg. per 100 gm.

Min.	Brain		Liver		Kidney		Blood	
	Acid	Amide	Acid	Amide	Acid	Amide	Acid	Amide
0*	169 ± 14†	72 ± 18	19	35	78	10	3.1 ± 0.6	7.2 ± 1.7
Glutamic acid administered								
15	153	80	70	32				
15	171	72					45	5.9
15	204	81			574	23	172	11
15	178	61					153	11.5
20	195	66					14	8
30	184	60					98	6.8
30	166	61					32	8.0
45	134	70					38	10.2
Glutamine administered								
15	140	116	300	90				
15	158	83			104	83	13.4	51
15	137	79					7.1	37
30	200	78			92	41	4.7	16
30	198	105					4.8	45
45	172	77					3.9	13.3

* The number of groups of three animals represented by the averages were brain (five), liver (one), kidney (one), blood (three).

† Standard deviation. The bold-faced values differ from the control values significantly ($P < 0.05$) (19).

fraction of these concentrations was found 10 or 15 minutes after the injection. The rapid disappearance of amino acids from the blood, known since the studies of Van Slyke and Meyer (13), was observed in every experiment.

In the previous study an increased concentration of glutamic acid in portal or peripheral blood was found to depress the concentration of glutamine in the plasma (3). This finding was interpreted as the result of an

increased uptake of the amide by the tissues under the influence of the elevated glutamic acid level in the blood. The phenomenon was not observed in the present experiments. The discrepancy between the earlier (3) and the present findings is not necessarily real, for glutamine was measured in the whole blood in this study and in plasma in the one reported earlier. Furthermore, the concentrations of glutamic acid were far higher in this than in the previous investigation.

The administration of glutamine led to an increase in glutamic acid concentration, presumably because of a return to the blood of the acid liberated in tissues from the amide.

Effect of Administration of Glutamic Acid and Glutamine on Their Concentrations in Brain, Liver, Muscle, and Kidney—No significant change in the concentration of either compound in the brain was found after the administration of glutamic acid, but in several experiments the concentration of the amide increased significantly after it had been given.

After glutamine injection, large increases in both glutamic acid and glutamine were found in the liver, and in some of the experiments the acid rose far more than the amide. It is reasonable to assume that the glutamic acid was liberated from the amide and that the sum of the increments of the two compounds represents the actual amount of the amide taken up and retained by the liver.

After the administration of glutamic acid the increased concentration in liver and in muscle may be explained by equilibration of the plasma concentration with that of the extracellular fluid phase. Under the conditions of the injection experiments, liver, like brain, showed an uptake of glutamine but not of the parent amino acid. It should not be overlooked that the injection of large amounts of a single amino acid creates unphysiological conditions and that different results might be obtained if glutamic acid or glutamine were administered together with and in a naturally occurring ratio to other amino acids.

After the administration of either of the two compounds, large increases in the corresponding fraction were found in the kidney. In the glutamine experiments, the increase in amide concentration was accompanied by a rise in glutamic acid.

It is probable that an inability of the brain to absorb glutamic acid is responsible for the absence of a significant increase in the concentration of either the amino acid or its amide after the administration of glutamic acid. The possibility must be recognized, however, that glutamic acid is absorbed by the brain but is metabolized at such a rapid rate that it does not increase above the normal level. Inasmuch as our analyses were carried out on whole brain without differentiation of gray and white matter, it is possible, furthermore, that a small uptake of glutamic acid by gray matter was not detected.

The high concentration of glutamic acid in brain raises the question as to the source of the amino acid. Its concentration is about twice, and together with glutamine, 3 times that of glucose. While it cannot be concluded that under different experimental conditions or in other species glutamic acid may not be found to enter the central nervous system, our experiments carried out on rats and mice suggest that the glutamic acid of the brain may have its origin partly in the amide and partly through synthesis in the brain itself. Recently Stern and associates (14) reported that glutamic acid accumulates against the concentration gradient in slices of guinea pig brain cortex suspended in a saline medium containing glucose and glutamic acid. While it cannot be excluded that species differences account for the uptake of glutamic acid by cortex slices (guinea pig) and the lack of uptake by the brain in the intact animal (rat and mouse), it is more likely that the difference in results can be ascribed to basic differences in permeability between whole organs in intact animals and tissue slices.

It may be of considerable biological significance that, as shown in our experiments, glutamine enters the liver and probably the brain with greater ease than does the parent amino acid. While glutamic acid or its keto acid takes part in many of the basic metabolic reactions (oxidative deamination, entrance of ammonia into the amino acid pool, transamination, etc.), the amide is inert in all these processes. It represents therefore a store of the metabolically highly active amino acid and keto acid from which they can be liberated easily by enzymatic action. While a change in glutamic acid or ketoglutaric acid may influence the rate of such basic metabolic mechanisms as the tricarboxylic acid cycle or transamination, a change in glutamine concentration will not affect the rate of these processes.

SUMMARY

The determination of glutamic acid and glutamine in the protein-free filtrates of brain, muscle, liver, and kidney of rats and mice with the aid of a microchemical method is described. In the whole brain of these animals, as well as in the brain of cattle, sheep, rabbits, cats, and pigeons, free glutamic acid occurs in a concentration of about 0.01 mole and glutamine of about 0.004 mole per kilo. Kidney contains a large excess of the amino acid over the amide, while in liver the concentrations of the two compounds are about equal.

The changes in concentration of glutamic acid and glutamine in blood, liver, muscle, kidney, and brain were determined at various intervals up to 1 hour after the intravenous administration of the two compounds to rats and mice.

Both amino acids disappear rapidly from the blood. After the administration of the amide, large amounts of it are taken up by the liver. The

rise in amide concentration in the liver is accompanied by the liberation of glutamic acid. After glutamic acid administration, its increase in liver and muscle may be explained by the equilibration of the blood plasma concentration with that of the extracellular fluid phase.

There was a considerable accumulation in the kidney of both compounds after the administration of either one; when an excess of glutamine was present, glutamic acid was liberated.

No significant increase in the glutamic acid or glutamine concentrations of the brain followed the injection of glutamic acid. The results of the experiments in which the amide was administered suggest that glutamine may enter the brain.

The significance of the results with respect to brain metabolism and the function of glutamine is discussed.

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NEPHROTOXIC EFFECT OF MSG (MONOSODIUM GLUTAMATE) IN THE CHICKEN

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SUMMARY: In order to prove sodium toxicity of MSG, baby chicks known to be the most susceptible one to sodium chloride were chosen as the experimental animal. Almost isotonic solution of MSG, NaCl or monopotassium glutamate was given *ad libitum* as the sole source of drinking water. Two-day old chickens given MSG died of gout within a few days and showed higher mortality and lesions severer than those given physiological saline, while none died nor was weakened among those receiving monopotassium glutamate. Very rapid development of kidney lesions and a large amount of urate deposits were the two main features resulting from MSG ingestion, and primary histological changes were tubular degeneration and tophi obstruction in collecting ducts. Even at half concentration, MSG ingestion caused lobar atrophy in the kidney and some died of gout. Besides sodium toxicity, glutamate counterpart is suspected of contributing to uric acid synthesis.

INTRODUCTION

In recent years MSG has been noticed as the causative agent of hypothalamic and retinal lesion in new born animals (Olney, 1969 a,b). Amino acid portion of MSG has been considered as the main causative agent in explaining the mechanism of these lesions with a relatively little attention paid to the sodium part (Olney, 1970; Freedman and Potts, 1962). However, it is an established fact in veterinary science that various sodium salts cause nervous symptoms in animals and especially in poultry they are nephrotoxic (Smith and Jones, 1966). Further, it has recently been found that rats and hamsters administered MSG and NaCl by gastric intubation develop marked nervous symptoms and LD_{50} values in both species turned out to be quite parallel to the concentration of sodium ions (Shimizu et al., 1971). To observe sodium toxicity of MSG we chose the chicken as the most susceptible experimental animal to NaCl with its characteristic development of nephrosclerosis (Selye, 1942). A 2.6% MSG solution given to baby chickens as the exclusive source of drinking water showed higher toxicity than 0.9% saline which had been reported to be fatal in a few days (Doll et al., 1946). In the present paper we report macroscopic and microscopic findings of the chickens given MSG, NaCl or monopotassium glutamate added to the drinking water.

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MATERIALS AND METHODS

Solutions of MSG and NaCl were prepared to contain the same amount of sodium ions, and the solution of equivalent monopotassium glutamate (abbreviated to KG) was adopted in order to eliminate sodium toxicity. Osmotic pressure of solutions of 2.6% MSG, 0.9% NaCl and 2.8% KG were 243, 280 and 258 mOsm, respectively.

Two-day old hybrid chickens were divided into five groups and started to receive each solution as drinking water (*ad libitum*); test group A 2.6% MSG, test group B 1.3% MSG, test group C 0.9% NaCl, test group D 2.8% KG, and control group distilled water; all these groups were fed with commercial chick food containing 0.1–0.3% of sodium chloride. Observation continued for 4 weeks.

On post mortem examinations of dead and sacrificed birds, joints were carefully observed. All organs were fixed in Bouin's fluid and 10% neutral buffered formalin, embedded in paraffin, and sections were cut at 5 μ and stained with haematoxylin and eosin.

RESULTS

Mortality and weight gains are shown in Table 1 and Fig. 1, respectively.

Most of the chickens given 2.6% MSG (test group A) died within 3 days. They had neither eaten nor drunk for half to a full day before death, stood still with their eyes closed, then succumbed on the floor chirping weakly, and often lost balance due to stiff joints of the legs. At necropsy marked deposits of white substance were recognized on the pericardium, air sacs, serous surface of internal organs, fascial membranes of muscles and in joints and kidneys (Figs. 2, 3, 4). In severe cases it covered all the surface of the abdominal wall both inside and outside accompanying visceral adhesion and deposited in the spleen and gall bladder (Figs. 5, 6). In all cases the kidneys were pale, swollen and diffusely studded with minute white substance and chalky substance often plugged the dilated ureters (Fig. 4).

Table 1. Mortality in chickens given various salts in drinking water (*ad libitum*)

Group	Solution	Post hatching date														Death rate
		1	2	3	4	5	6	7	8	9	10	11	12	13	14–30	
A	MSG 2.6% o.p. 243 mOsm			0	6	16	3	5								30/30
B	MSG 1.3%			0	0	1	0	0	0	0	0	1	0	0	0	2/30
C	NaCl 0.9% o.p. 280 mOsm			0	0	2	0	1	1	2	1	0	0	1	0	8/20
D	KG 2.8% o.p. 258 mOsm			0	0	0	0	0	0	0	0	0	0	0	0	0/19
Control (distilled water)				0	0	0	0	0	0	0	0	0	0	0	0	0/31

The tophaceous substance was Murexid reaction positive, showed needle shaped birefringent crystals under polarized microscopy (Fig. 10), and disappeared by the action of uricase. It also revealed maximum ultraviolet absorption at 292 mμ, pH 9.4.

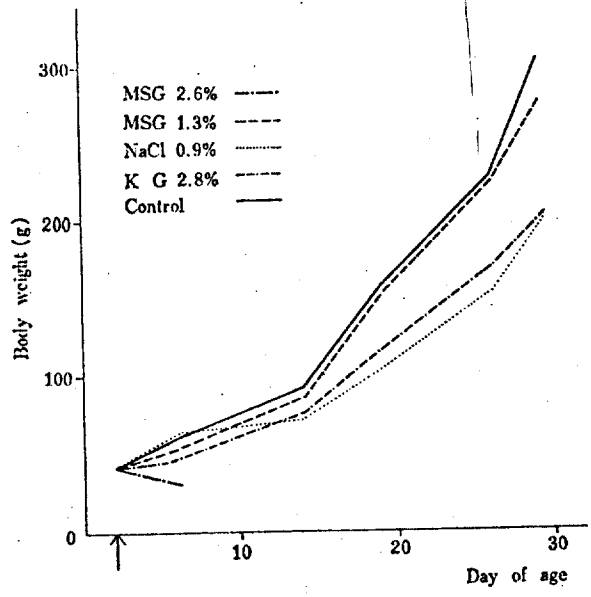

Fig. 1. Weight gains of chickens receiving various salts in drinking water.

On the other hand, sodium was detected in the tophi, suggesting the presence of sodium urate.

In test group B (which received 1.3% MSG in drinking water), only 2 chickens died of gout on the 3rd (visceral type) and the 9th (articular type) days of experiment. Clinical features of the two were identical to those of group A. All other chickens showed apparently normal growth, but when they were sacrificed at 35 day of age, 10 of 28 birds revealed various degree of renal atrophy in some lobes of the kidney (lobar atrophy), associated with compensatory hypertrophy (Fig. 7).

Soon after the start of the experiment in both test groups C and D the feces became very watery. In a few days diarrhea became milder in the chickens receiving KG but it persisted or was rather aggravated for more than a week in those given NaCl. However, the chickens did not lose their appetite and especially those of group C ate and drank voraciously.

Eight of the 20 chickens of group C died by the 11th day of experiment. During the 3rd to the 7th days of the experiment they revealed subcutaneous oedema attributable to renal failure and subsequently died. Pericardial oedema and fluid in body cavity were common findings (Fig. 8). The affected kidneys were pale in colour, often hardened and sometimes swollen but no partial deformity was observed, nor the slightest deposition of tophi (Fig. 9). The average weight of the surviving 12 birds was light. They were dirty and looked miserable.

All the chickens having received KG showed no sign of weakness and survived till the end of the experiment. Although they were smaller than normal in size, no macroscopic or microscopic change was found in their internal organs.

No abnormal finding was observed in the control group.

On histological study, obstruction of collecting ducts with urate deposits was

Fig. 3

Fig. 5

Fig. 2

Fig. 4

Fig. 7

Fig. 9

Fig. 6

Fig. 8

Fig. 11

Fig. 13

Fig. 10

Fig. 12

EXPLANATION OF FIGURES

Fig. 2. Group A. Heavy urate deposits on the pericardium and left knee joints (lower middle); lower degree on the serous surface of the gizzard.

Fig. 3. Group A. Heavy urate deposits on the knee joint.

Fig. 4. Group A. Swollen pale kidneys studded with minute white spots. The serous surface of the right cranial lobe is encrusted with urate. Dilated ureters are plugged with chalky substance.

Fig. 5. Group A. Severe urate deposits and subsequent visceral adhesion.

Fig. 6. Group A. Urate deposits in the spleen and gall bladder.

Fig. 7. Group B. Atrophy of left cranial lobe and compensatory hypertrophy of right lobes.

Fig. 8. Group C. Marked pericardial oedema.

Fig. 9. Group C. Pale, swollen and a little hardened kidneys and oedematous swelling testes.

Fig. 10. Needle shaped crystals under polarized microscopy. ×400

Fig. 11. Group A. Intraductal tophi accumulation surrounded by heterophils and giant cells; thin and thick limbs are intact. ×100

Fig. 12. Group B. A large tophus surrounded by giant cells (left) and atrophic kidney tissue (right). ×10

Fig. 13. Group C. Epithelial crescent in a renal corpuscle and vacuolar degeneration of proximal tubules. ×100

prominent in gouty kidneys of groups A and B. Being surrounded by heterophils or giant cells, intraductal tophi seemed to stop the urinary flow and ruinate associated tubules (Fig. 11). Colloid retention or tubular dilatation was not uncommon. Oedematous and fibrous tendency was observed in the interstitial tissue. The glomerular lesion known to occur in NaCl poisoning was not encountered. No particular lesion was recognized in the brain or retina. Only tophaceous deposits surrounded by giant cells were sometimes seen in other organs. The atrophic lobe of the kidney in group B proved to be once ruinated shrunken kidney tissue (Fig. 12). Poisoned kidney of group C revealed nephrosclerosis and diffuse vacuolar degeneration of proximal tubules (Fig. 13), while collecting ducts remained intact.

Discussion

Many sodium salts are reported to be goutgenic to the fowl (Bokiri, 1966). In the present experiment a strong nephrotoxic effect of MSG in baby chickens was demonstrated as intraductal tophi accumulation, while NaCl mainly affected the upper part of nephron. The gouty lesion caused by MSG ingestion is distinguishable for its rapidity and accuracy. Elimination of sodium toxicity by the use of monopotassium glutamate was successful, but marked retardation of weight gain, probably attributable to initial diarrhea, occurred.

The causes of rapid obstruction in collecting ducts are inferred as follows: (1) Uric acid is synthesized in too large amount to drain. (2) Reabsorption of water in renal tubules is activated. It is well known that glutamine works as a precursor in purine synthesis. According to Katsunuma et al. (1970), in birds the presence of glutamate strongly inhibited glutaminase and the feedback inhibition of PRPP-amidotransferase by AMP, IMP or GMP was less than that in mammals. Suspicion is strengthened that the acid part of MSG may contribute to the uric acid overproduction. In fact, most of the gouty chickens revealed heavy urate deposition on visceral surface and in joints at the same time, which was rare in both spontaneous cases (Siller, 1959) and artificially produced gout (Bokiri, 1965, 1966).

As chickens excrete very concentrated urine, water reabsorption in renal tubules must be of high efficiency. Immaturity of tubular membrane may conduce to higher permeability. ADH (anti-diuretic hormone) may participate in its mechanism to activate facultative water reabsorption in collecting ducts and distal tubules. Such speculations need further inquiry into urine.

Sodium not collaborating with chloride may bring up a little different effects to the kidney. Tubular degeneration caused by MSG ingestion was focal, whereas diffuse in NaCl intoxication, and glomerular lesion typical of the latter was absent in the former. Investigation of the blood may be necessary to clarify hypernatremia and hyperuricemia.

Membranous transportation of Na^+ and glutamate is assumed to require a common carrier (Curran et al., 1967), and owing to the renal portal system, the kidney of the bird may receive direct influence of MSG in the gut. So, the coodination of sodium and glutamic acid may be the key to clarify this phenomenon. Though avian gout is not directly connected with human gout, further study will be required on the mechanism of nephrotoxicity of MSG.

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The Enzymic Estimation of Glutamate and Glutamine

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A method of estimating glutamic acid is described, based on its dehydrogenation by glutamate dehydrogenase coupled, by means of *N*-methylphenazine methosulphate, to the reduction of tetrazolium salts. The method is suitable for the estimation of 0·0-0·3μmole of glutamic acid. The response is linear, but not stoicheiometric: possible reasons for this are discussed. If suitable precautions are taken, the use of a partially purified preparation of glutaminase makes it possible to estimate glutamine also.

No convenient and specific micro-method for the estimation of glutamate and glutamine exists, except that developed by Holzer & Söling (1962) which uses glutamate dehydrogenase and the rather expensive acetyl-NAD. The present paper describes a method in which the oxidation by glutamate dehydrogenase is coupled to the reduction of suitable tetrazolium salts. Glutamine can be estimated after hydrolysis to glutamate. Preliminary reports of the method have appeared (Sowerby & Ottaway, 1961, 1962).

EXPERIMENTAL AND RESULTS

Materials

Glutamate dehydrogenase as a solution in 50% (v/v) glycerol was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. It was stored at −15°.

Glutaminase I (L-glutamine amidohydrolase, EC 3.5.1.2) was prepared from pig-kidney mitochondria by the method of Klingman & Handler (1958). Purification was taken only to the stage of the second precipitation with 4M-phosphate, since further purification gave poor yields of enzyme which very quickly lost activity, even at −15°. The precipitate from the second phosphate treatment was dissolved in a small volume of 0·03M-borate–0·02M-phosphate buffer, pH 8·1, which stabilizes the enzyme (Klingman & Handler, 1958), to give a solution containing about 12mg. of protein/ml. This was dialysed in the cold against the same borate–phosphate buffer to remove bound glutamate and dispensed into 5ml. vials, which were stored at −15°. This preparation was stable for 6-12 months at this temperature and for several weeks at 0°. Occasional preparations were even stable for 24hr. at room temperature, much longer than the time reported by Klingman & Handler (1958).

Glutamic acid and glutamine were obtained from Roche Products Ltd., Welwyn Garden City, Herts. Stock solutions (0·05M) are stable for 1-2 weeks in the refrigerator.

NAD was obtained from Mycofarm, Delft, The Netherlands. A stock solution containing 0·5% was stable for several weeks in the refrigerator.

PMS* was obtained from Sigma Chemical Co. Ltd. (London). A 1% (w/v) stock solution was kept in the refrigerator in an opaque bottle. It was stable indefinitely in such conditions but it, and any dilute solution prepared from it, must be rigorously protected from light. *N*-Ethylphenazine methosulphate was prepared by the method of Dickens & McIlwain (1938). MTT was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and INT from British Drug Houses Ltd., Poole, Dorset. Some samples of INT from this and other sources could not be reduced at all by the assay system, even after recrystallization from hot water. Solutions (0·4%) of the compounds were stable indefinitely at room temperature when kept in the dark. The INT solution was filtered before use.

The formazans corresponding to INT and MTT were most conveniently prepared by the enzymic reduction of the tetrazolium salts, with glutamate as hydrogen donor. Alternatively, they could be reduced by ascorbate in the cold at pH 8·0 if PMS was also added. The insoluble formazans were filtered off, washed well with water, recrystallized from aqueous acetone or methanol and dried to constant weight *in vacuo*. Fig. 1 shows that the absorption spectra of the formazan produced by chemical reduction of INT with more vigorous reagents, e.g. dithionite, was not identical with that of the enzymically produced compounds. Their millimolar extinction coefficients were: in aq. 40% (v/v) acetone, INT 12·5 at 460mμ, MTT 11 at 560mμ; in ethyl acetate, INT 8·1 at 490mμ and MTT 8·8 at 560mμ.

Acetone was purified by distillation from $KMnO_4$. An efficient vapour trap was necessary since the addition of acetone containing traces of manganese salts to INT dissolved in phosphate buffer gave rise to formazan even in the absence of other hydrogen donors.

Tris was purified by recrystallization from methanol.

Phosphate was purified by preparing a saturated solution at room temperature, of NaH_2PO_4 in water contain-

* Abbreviations: PMS, *N*-methylphenazine methosulphate; MTT, 3-(4,5-dimethylthiazol-1-yl)-2,5-phenyltetrazolium bromide; INT, 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride.

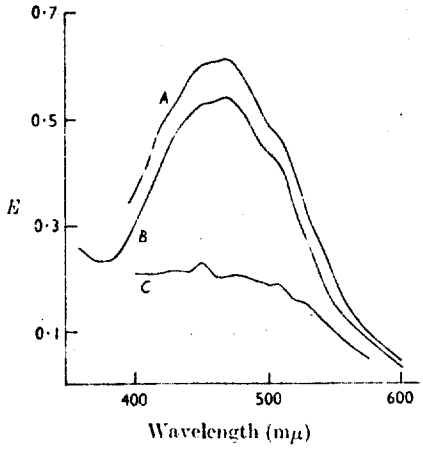


Fig. 1. Absorption spectra of formazan produced by the reduction of INT. The solvent was aq. 40% (v/v) acetone. *A*, Enzyme reduction with glutamate as hydrogen donor; *B*, reduction with ascorbate, pH 9, in the presence of PMS; *C*, reduction with dithionite.

0·1% (w/v) of EDTA or 8-hydroxyquinoline, warming slightly, and adding the calculated quantity of NaOH to form Na_2HPO_4. The precipitated salt was washed with ethanol–water (1:1, v/v) and then with ethanol, and dried *in vacuo*. Phosphate analysis showed the product to be $Na_2HPO_4,2H_2O$.

All other chemicals were A.R. grade. Glass-distilled water was used throughout.

Deproteinization of tissue samples

Tissues were frozen in an acetone–solid CO_2 mixture and pulverized in a percussion mortar cooled to −70°. Either of the methods of deproteinization described below could then be used.

(1) A weighed amount of the still-frozen powder was homogenized with 2ml. of cold acetone/g. of tissue in a centrifuge tube, by means of a loose-fitting power-driven Teflon pestle, which was subsequently washed with 1ml. of water/g. of tissue. The solution was immersed in a water bath at 70° for 3min., care being taken not to let the mixture boil over. Most of the acetone was removed by a stream of air, and 2ml. of water/g. of tissue was added, together with a few drops of N-acetic acid to bring the pH of the solution to the isoelectric point of the proteins. The mixture was left for at least 15min., then centrifuged, and the supernatant removed. The precipitate was washed with water and recentrifuged, and the combined supernatants were made up to a suitable volume.

(2) The frozen powdered tissue (0·2–0·5g.) was homogenized with 2·5ml. of cold ethanol–N-HCl (1:1, v/v), the pestle was washed with 1ml. of water and the suspension heated in a water bath at 60° for 3min. After cooling, the solution was neutralized with 1·25m-moles of KOH and allowed to stand at 4° for 3hr. The volume was then made up to 10ml., the protein centrifuged down and the supernatant used for analysis.

Estimation of glutamate

The enzyme solution, made up on the day of use, consisted of: 3ml. of 0·2M-tris buffer, pH 7·6, containing 0·025% of EDTA, 2ml. of 7mM-NAD and 0·1ml. of glutamate dehydrogenase solution (i.e. 2mg. of protein). The coenzyme was added to the enzyme solution, since dilute solutions of glutamate dehydrogenase rapidly lose activity in the absence of NAD (Frieden, 1959). The dilute enzyme solution is stable for at least 24hr. at 0°.

To a test tube were added the following reagents: up to 1ml. of sample containing 0–0·3μmole of glutamate, 1ml. of 0·2M-phosphate buffer, pH 7·6, 0·5ml. of 8mM-INT or 4mM-MTT, 0·5ml. of enzyme solution and water to make a total volume of 3·5ml. The reaction was started by adding to each tube 0·1ml. of a 1:100 dilution of the stock PMS solution. (Alternatively, stock PMS could be added to INT, or MTT, solution to give a 1:500 dilution of PMS. In this case 0·5ml. of freshly made up PMS–INT reagent was added to start the reaction.)

The tubes, including a blank containing all reagents except glutamate, were incubated for 30min. at 37° and, if INT was being used, each tube then received 0·5ml. of 2N-HCl, which prevents turbidity due to precipitation of protein and salt on the addition of acetone, and also inhibits the slow production of formazan induced by slightly impure acetone in the presence of phosphate. The formazan of MTT is decolorized even in slightly acid solutions, so that HCl could not be added when this reagent was used. When INT was used, 5ml. of purified acetone was then added to each tube; with MTT turbidity could be avoided by using the same volume of aq. 60% (v/v) acetone. The contents of the tubes were mixed and allowed to stand for 5min. to reach room temperature. The colours, which are stable for at least 12hr., were then read against the reagent blank at 460mμ (INT) or 560mμ (MTT). Beer's law is obeyed to about 0·2μmole of glutamate with INT and up to about 1μmole with MTT. The final mixture of acetone and water dissolves only about 0·3μmole of INT formazan; samples containing more than 0·3μmole of glutamate could be estimated by diluting the formazan suspension with aq. 50% (v/v) acetone.

The reaction could be made about twice as sensitive by extracting the formazan into 3–4ml. of ethyl acetate; it was not necessary in this case to add acid. The absorption maximum of INT in ethyl acetate is at 490mμ, and that of MTT at 560mμ. Extraction was almost quantitative, but it was usually necessary to centrifuge the tubes to remove water droplets from the upper layer. The ethyl acetate technique was advantageous with very small amounts of glutamate, or when there was so much salt in the sample that it was precipitated on the addition of acetone.

Estimation of glutamine

Up to 1·5ml. of the sample was added to 1ml. of 0·2M-phosphate buffer, pH 7·6, containing 1–10μmoles of Mg^{2+} and 0·05ml. of glutaminase I solution. The total volume was made up to 2·5ml. with water. The mixture was incubated at 37° for 30min., by which time hydrolysis was complete. It was desirable to boil the samples at this stage, to destroy enzymes associated with the glutaminase preparation. Then 0·5ml. of INT or MTT solution containing 20μg. of PMS/ml. was added, followed by 0·5ml.

of glutamate dehydrogenase–NAD solution. The samples were subsequently treated as described for the glutamate assay. It was necessary to run a blank containing all reagents since the glutaminase contains some tetrazolium-reducing substances, even after dialysis to remove bound glutamate (Klingman & Handler, 1958). Carrying out the estimation sequentially, as described above, gave better yields of formazan than if the GAD and glutaminase were added at the same time. A separate glutamine standard curve was prepared. If the sample contained preformed glutamate, a duplicate set of tubes without glutaminase was run and the glutamine content was calculated by difference. We have found no evidence for inhibition of glutaminase by glutamate in amounts up to 0·3μmole/tube. Also, NH_4^+ does not inhibit the reaction in amounts up to 1μmole/tube.

Factors affecting the estimation

Substrate specificity. At pH 9·6 acids other than glutamate are oxidized by glutamate dehydrogenase, notably norvaline and L-α-aminobutyrate (17 and 3% as fast as glutamate respectively). At pH 7·6, however, Struck & Sizer (1960) found that no amino acid other than glutamate is oxidized, thus confirming the original report of Olson & Anfinsen (1953). Under our conditions only 1% of 1μmole samples of norvaline and L-α-aminobutyrate were oxidized in 30min. All other amino acids tested, including aspartate and glutamine (1μmole), produced no detectable amount of formazan in 30min. Alanine was tested in amounts up to 37μmoles.

Suppression of non-enzymic reduction of PMS. Direct reduction of PMS by substances present in tissue extracts may occur. This could be prevented by a preliminary oxidation of interfering substances by oxygen (incubation for 10–30min. of the sample in buffer with PMS but without glutamate dehydrogenase–NAD and final hydrogen acceptor). The assay was then completed by adding fresh PMS, INT and glutamate dehydrogenase–NAD in that order. Fresh PMS must be added because it is oxidized in air to phenazine, 2-oxophenazine and other products. Under these conditions direct non-enzymic reduction of PMS is negligible.

Effect of pH. The optimum pH for the estimation is 7·6. Five buffers were tested: tris, imidazole, phosphate, glycylglycine and *N*-ethylmorpholine. Formazan production was poor with both glycylglycine and *N*-ethylmorpholine, satisfactory with tris if cyanide was also added, and satisfactory with phosphate (with this buffer the presence of cyanide did not affect formazan production) and with imidazole (addition of cyanide depressed formazan formation). Since, when assaying glutamine, it is necessary to activate glutaminase with phosphate, this buffer was chosen for routine assays of glutamine; it has the added advantage that cyanide need not be used. Better results were obtained when A.R.-grade phosphate was recrystallized from a solution containing a metal-sequestering agent.

Effect of temperature on the yield of formazan. The optimum temperature is between 30 and 37°. The enzyme is rapidly inactivated at temperatures above 50°. The amount of formazan formed at temperatures lower than 30° could not be increased by lengthening the incubation time; at all temperatures tested, formazan formation practically ceased after 20min. incubation. The low percentage formation of formazan at low temperatures did not appear to be due to inactivation of the enzyme before all the glutamate had been dehydrogenated. Table 1 shows that the amount of formazan formed from a known amount of glutamate after 30min. was much less at 22° than at 37°. However, continued incubation at 22° after the addition of more glutamate dehydrogenase or PMS only increased the yield slightly, and not up to that obtained from a 30min. incubation at 37°. Other experiments of this type, in which a second addition of glutamate was made after a 30min. incubation, showed that all the components of the reaction were still active at this time. These observations are further discussed below.

Concentration of reagents. The percentage production of formazan increased with the concentration of enzyme, and it was not possible to compensate for low concentrations of glutamate dehydrogenase by prolonging the incubation time. Recovery of formazan did not increase further when more than 200μg. of glutamate dehydrogenase was added to each tube. The percentage formation of formazan became independent of NAD concen-

Table 1. *Production of formazan from glutamate under various conditions*

Each tube contained 0·18μmole of glutamate, 1·75μmoles of EDTA, 20μmoles of KCN, 1·4μmoles of NAD, 200μg. of glutamate dehydrogenase, 33mμmoles of PMS and 4μmoles of INT, together with 1ml. of 0·15M-tris buffer, pH 7·6. The total volume was 3·5ml. The reaction was stopped by the addition of 0·5ml. of 1·5N-HCl, and the formazan was estimated spectrophotometrically after the addition of 5ml. of acetone.

Conditions	Formazan produced (% of theoretical amount)
Incubation for 30min. at 37°	84
Incubation for 30min. at 22°	54
Incubation for 60min. at 22°	62
Glutamate dehydrogenase (100μg.) and PMS (10μg.) added after 30min. at 22°, then incubation for further 30min. at 37°	63

tration when this was greater than 1·5 μmoles of NAD/tube. Dilute solutions of glutamate dehydrogenase are said to split into inactive sub-units in the absence of some nucleotides, of which NAD is one (Frieden, 1959). However, a dilute enzyme solution made up as described in the section on 'Estimation of glutamate' was perfectly stable for at least 14hr. when the NAD concentration was as low as 1 μmole/ml.

The optimum concentration of INT was 4 μmoles/tube, and that of MTT 2 μmoles/tube. Fig. 2 shows the optimum concentrations of PMS for use with INT and MTT. These concentrations (33 mμmoles/tube or less) are much lower than those usually employed (see Ells, 1959). When the final hydrogen acceptor is oxygen, the use of high concentrations of PMS is understandable, since the rate constant for the reaction:

$$PMSH_2 + O_2 \rightarrow PMS + H_2O_2$$

is relatively low (see Ottaway, 1966). However, the reaction of $PMSH_2$ (reduced PMS) with tetrazolium salts is fast, and the rate-limiting step in the oxidation of glutamate is the primary dehydrogenation. Since PMS and $PMSH_2$ readily decompose to give, in particular, phenazine and 2-oxophenazine, both of which are reddish compounds, high concentrations of PMS give high and variable blanks, and should therefore be avoided. A concentration of 33 mμmoles/tube was generally used.

Fig. 3 shows that the presence of a small concentration of EDTA or other chelating agent increased the percentage yield of formazan. Higher concentrations depressed the yield almost to zero, possibly because glutamate dehydrogenase contains zinc (Adelstein & Vallee, 1958), which can be removed by EDTA. A concentration of 0·5–2·0 μmoles/tube was used. If the reaction is carried out in non-sequestering buffers, Ca^{2+} and Mg^{2+} interfere; they can be removed by a suitable increase in the concentration of EDTA. When phosphate buffer is used, Mg^{2+} (in amounts up to 1 μmole/ml. of sample) no longer interferes, and Ca^{2+} interferes only slightly. No advantage was gained by including either zinc salts or Zn–EDTA in the reaction mixture.

Effect of cyanide on the reaction. Cyanide (20 μmoles/tube) increased the yield of formazan considerably, but not to 100%, when tris was used (Fig. 4). The effects of EDTA and cyanide were additive (Fig. 3). On the other hand, cyanide was without effect in phosphate buffer, and definitely diminished the yield in imidazole buffer.

Two effects of cyanide, on different stages of the complete reaction, may be responsible for these observations. Spectrophotometric estimation of

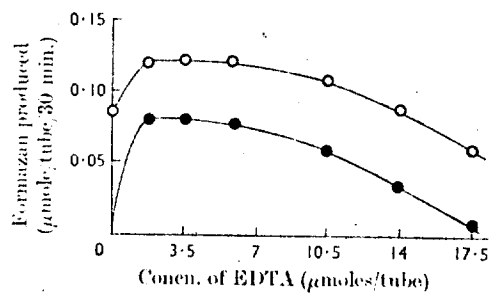


Fig. 3. Effect of EDTA concentration on the production of formazan from glutamate. The amount of EDTA per tube was varied in the absence of KCN (●) and in the presence of 20 μmoles of KCN (○). In other respects the reaction mixture was as given in Table 1 and the procedure was that described in the text.

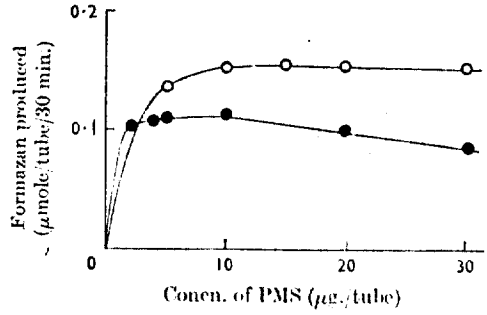


Fig. 2. Effect of PMS concentration on the production of formazan from glutamate. Each tube contained the amount of PMS indicated and 4 μmoles of either MTT (○) or INT (●). Otherwise the reagents were as given in Table 1 and the procedure was that described in the text.

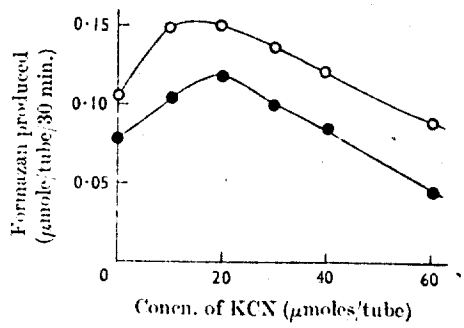
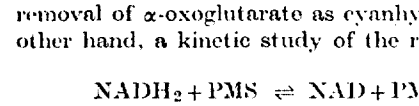


Fig. 4. Effect of cyanide concentration on the production of formazan from glutamate. Each tube contained the amount of KCN indicated and 4 μmoles of either MTT (○) or INT (●). Otherwise the reagents were as given in Table 1 and the procedure was that described in the text.

the equilibrium between glutamate and NAD catalysed by glutamate dehydrogenase showed that cyanide both increased the rate of oxidation of glutamate and shifted the equilibrium in favour of $NADH_2$. These results are compatible with the removal of α-oxoglutarate as cyanhydrin. On the other hand, a kinetic study of the reactions:



$$NADH_2 + PMS \rightleftharpoons NAD + PMSH_2$$
$$PMSH_2 + O_2 \rightarrow PMS + H_2O_2$$

suggests that cyanide both lowers the effective $PMS/PMSH_2$ redox potential and prevents the formation of an intermediate $PMSH_2$–NAD compound, which is not readily oxidized. On this view, the buffer–cyanide interactions would reflect the differing tendencies of the buffer ions to stabilize the non-reactive intermediate. The varying percentage yields of formazan from glutamate found with the different buffers with and without cyanide were very similar to the yields from $NADH_2$ added directly to similar reaction systems, which suggest that in practice the effects of cyanide on the $NADH_2 \rightarrow PMS$ reaction are more important than those on glutamate oxidation itself. This is also suggested by the very large effects of cyanide on yield of formazan in the succinate-oxidizing system described by Nachlas, Margulies & Seligman (1960).

Interfering substances. Hydrazine, hydroxylamine and to a smaller extent ascorbate interfere by reducing PMS directly, and hence INT or MTT. Semicarbazide, cysteine and glutathione do not reduce PMS at this pH. α-Oxoglutarate and NH_4^+ decrease the yield of formazan slightly at concentrations of 1 μmole/tube; below this their effect is negligible. Interference from ammonia can be removed by making the phosphate buffer 0·01M with respect to Mg^{2+}. No interference has been found from any amino acid other than norvaline or α-aminobutyrate, or from citrate, fumarate, succinate, β-hydroxybutyrate, pyruvate or lactate at a concentration of 1 μmole/tube. Oxalate at this concentration decreases the yield slightly.

Deproteinization of tissue extracts. Most protein precipitants such as tungstic acid, molybdic acid and trichloroacetic acid, even when neutralized, inactivate glutamate dehydrogenase. Saturated (0·06M) potassium perchlorate does not do so, but forms an insoluble salt with INT. Two methods of deproteinization with organic solvents have been described in the present paper. That with acid ethanol is the less satisfactory, since Grisolia, Fernández, Amelunxen & Quijada (1962) have reported that glutamate dehydrogenase is inhibited by high concentrations of salts, including potassium chloride. We found that formazan formation from glutamate was inhibited by about 10% when 1ml. samples of the supernatant (containing about 120 μmoles of potassium chloride) were used for analysis. With 0·5ml. samples there was no inhibition. The concentration of ethanol in the final supernatant was about 12%; this had no effect on the reaction, even with 1ml. samples.

Table 2. *Effect of oxygen concentration on yield of formazan from INT*

Each tube contained 0·18 μmole of glutamate, and the other reagents were identical with those reported in Table 1. The anaerobic experiments were carried out in evacuated Thunberg tubes; the reaction was started by the addition of enzyme from the side arm.

Concn. of O_2 (μmole/ml. of reaction mixture)	Formazan produced (% of theoretical amount)
0·0 (anaerobic)	99·5
0·0 (reagents made up in freshly boiled water)	96
0·2 (in air)	70·5
1·0 (moist O_2 gas bubbled through)	48·5

The samples should not be heated too long during deproteinization, since glutamate and glutamine (the latter very rapidly) form pyrrolidonecarboxylate on being heated in aqueous solution at neutral pH. Re-formation of glutamate from this compound is very slow. Under the standard assay conditions the loss of glutamate or of glutamine is less than 5%.

Effect of oxygen. The yield of formazan from glutamate or $NADH_2$ could be increased to the theoretical amount by carrying out the reaction anaerobically in Thunberg tubes. Yields of about 95% could be obtained by using reagents made up in freshly boiled water and stored under nitrogen. Conversely, the yields were much lower than usual if the reagents were saturated with oxygen (Table 2). Direct measurement with an oxygen electrode in using air-saturated reagents failed, however, to show any significant disappearance of oxygen to account for the less-than-theoretical yield of tetrazolium salt.

A mitochondrial factor increasing the yield of formazan. When the glutaminase preparation from kidney mitochondria was present in the assay system the yield of formazan from INT or MTT was theoretical, with glutamate as hydrogen donor. The effect was independent of the concentration of oxygen, and independent of cyanide when tris buffer was used. Glutaminase activity could be lost from the extract without simultaneous loss of ability to enhance the yield of formazan. There is reasonable evidence that the active factor is a protein (Sowerby & Ottaway, 1962), but it is not clear that it is truly enzymic, since it withstands boiling for 5min. in dilute (but not c

centrated) solution. It does not replace PMS, and is therefore not a tetrazolium reductase (Slater, 1959). Scaife (1964) has described a coupling factor, with very similar properties to our material, which is lost from thymus mitochondria on irradiation. We were unable to separate the formazan factor from glutaminase by chromatography or electrophoresis.

Since oxygen depresses the yield of formazan in the assay, but does not disappear in equivalent amounts, it seemed possible that its effect is due to the production of hydrogen peroxide, which might, for example, inactivate glutamate dehydrogenase. The glutaminase preparation is very rich in catalase, but its property of enhancing the yield of formazan is not due to this enzyme, because it is effective in the presence of cyanide. Further, added catalase does not affect the yield of formazan.

The mitochondrial factor does not affect either the position, or the rate of attainment, of equilibrium between glutamate and NAD. Kinetic studies show that, in the presence of EDTA, the mitochondrial factor increases the rate of reduction of PMS by $NADH_2$. In the presence of 1mM-cyanide this capability is largely lost, and catalase and the mitochondrial factor now have very similar effects on the reaction $NADH_2 \rightarrow PMS \rightarrow O_2$. The ability of the mitochondrial factor to increase the yield of formazan is not, however, inhibited by cyanide. These results may be interpreted to mean that $PMSH_2$, and possibly also PMS, are bound to a non-haem site on a protein, to form essentially the prosthetic group of an enzyme catalysing the transfer of hydrogen from $NADH_2$ to tetrazolium salts. The binding need not be very specific, since boiled mitochondrial factor appears to be at least partially effective as an 'apo-enzyme'.

Use of phenazine ethosulphate as carrier. The N-methyl group of PMS is very readily oxidized to formaldehyde, but other N-alkylphenazines are much more stable. Most of the present experiments were repeated with N-ethylphenazine ethosulphate, but no differences were found in the rates of reaction or in percentage yields of formazan.

Enzyme specificity of the glutaminase preparation

The glutaminase preparation contains no α-oxoglutarate-specific transaminase activity (at least in the absence of added pyridoxal phosphate), no L-amino acid-oxidase activity and no detectable butate-, succinate-, malate-, β-hydroxybutyrate- or ethanol-dehydrogenase activity, but it does contain some so far unidentified NAD-requiring dehydrogenase. Most biological samples do not contain the substrate(s) for this enzyme or enzymes, but it was found advisable to check this. Alternatively, interference from dehydrogenase can be removed by placing the samples in a boiling-water bath for 2min. after incubation with glutaminase. This treatment causes about 10% of the glutamate present to cyclize, and for accurate estimations internal standards should be used.

DISCUSSION

The methods described above have been in routine use in this Laboratory for over 2 years and have given no trouble other than occasional decrease of sensitivity caused by a batch of impure INT. In view of the unpredictable variations in this tetrazolium salt, and the difficulty of purifying it, we recommend the use of MTT, although the molar extinction coefficient of its formazan is rather smaller than that from INT. We have not used the factor enhancing the yield of formazan described in the test, because of the danger of incomplete inactivation of glutaminase. Although the assay could be carried out under anaerobic conditions, we have preferred aerobic conditions and have compensated for the failure to obtain quantitative yields of formazan by using internal standards, particularly when estimating glutamine.

The incomplete yields of formazan in the absence of the mitochondrial factor may be interpreted in terms of a $PMSH_2$ derivative that does not react with tetrazolium salts. The amount of reducing equivalents in the intermediate could not itself be great enough to account for the apparent loss of formazan, but if its breakdown to free PMS were assisted by $NADH_2$ (see Ottaway, 1966), and particularly if the intermediate were stabilized by some of the ions present, the concentration of free PMS might fall to very low levels after the first few minutes of the reaction. This would mean that the rate of oxidation of $NADH_2$ would become immeasurably small, i.e. a false equilibrium would be reached. Such an explanation would account for the fact that, particularly at room temperature (see Table 1), the reaction comes to a halt before completion, and yet, on the addition of more glutamate, all the components of the system are found to be still active. It would also provide an explanation for the effects of cyanide on the reaction.

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7C 7013

Infusion of protein hydrolysates in the newborn infant: Plasma amino acid concentrations

Protein hydrolysate infusions commonly administered to young infants unable to sustain adequate protein intake contain large amounts of glutamate and aspartate. Neurotoxic effects have been reported in other species and were attributed to high doses of these amino acids. Plasma glutamate and aspartate levels in infants treated with such preparations were within normal limits, but other amino acids were markedly below fasting levels. Plasma amino acid levels quickly reflected the amino acid composition of the hydrolysate and some degree of amino acid imbalance resulted when either of the 2 products employed in this study constituted the sole source of protein intake.

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THE ADMINISTRATION of high-calorie solutions by central venous catheter to infants who are unable to sustain an adequate oral intake has recently become popular.[1-3] Infants benefiting from such therapy include those severely ill and debilitated with intractable diarrhea from a variety of causes or those with surgically correctable congenital malformations of the gastrointestinal tract. The intravenous preparations supply adequate calories in the form of dextrose and sufficient quantities of a protein hydrolysate to achieve a positive nitrogen balance and net protein synthesis. Despite common use of such preparations, no studies are available to our knowledge concerning the effect of such infusions on plasma amino acid levels.

In addition, a number of investigators have reported acute and irreversible degenerative changes in the infant mouse retina following subcutaneous injection of either monosodium glutamate or aspartate.[4-9] Adult mice were much more resistant to glutamate-induced lesions than newborn animals,[4] and glutamate injection of pregnant mice produced no observable abnormalities in the offspring.[4] Olney and Sharpe[9-11] have reported that the arcuate nucleus of the hypothalamus is particularly vulnerable to monosodium glutamate injected subcutaneously at dose levels ranging from 0.5 to 2 Gm. per kilogram of body weight in the infant mouse, rat, rabbit, and a single immature rhesus monkey; Adamo and Ratner[12] were unable

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to reproduce such effects in the infant rat. Olney and Ho[13] recently reported that oral ingestion of high levels of glutamate, aspartate, or cysteine by the infant mouse also produces such lesions.

McLaughlan and associates[14] reported plasma glutamate levels were increased 3 to 3.5 times and aspartate levels doubled at peak absorption (20 minutes) after ingestion of 0.2 Gm. of monosodium glutamate per kilogram by the young rat who had been fasted overnight. A slower rise in glutamate levels was noted when the glutamate was administered in the presence of food proteins; 30 minutes after administration, plasma glutamate was 3 times normal values and still increasing. Brain glutamate concentrations were not affected in these animals. Similar results have been reported by Schwerin and associates[15] for the mouse. However, subcutaneous injection of glutamate bypasses 2 important organs controlling plasma glutamate levels: the liver and the gut. Neame and Wiseman[16,17] and Matthews and Wiseman[18] have shown that substantial quantities of ingested glutamate are absorbed as alanine. The alanine is undoubtedly formed by glutamate dependent transamination of pyruvate (produced either by glycolysis, or from glutamate itself via α-ketoglutarate, succinyl-coenzyme A, fumarate and malate by Kreb's cycle enzymes, and conversion of malate to pyruvate via nicotinamide adenine dinucleotide phosphate [NADP+] malate dehydrogenase). Our studies with the newborn pig indicate that the normal high levels of glutamate in the portal blood (48 μmoles per 100 ml.) are drastically reduced in peripheral blood (8 μmoles per 100 ml.) upon passage through the liver even in absence of glutamate load.[19]

Large amounts of glutamate and aspartate, normally found at low levels in the plasma, are contained in the 2 most commonly used intravenous preparations: Amigen and Aminosol. In view of the reported neurotoxic effects of monosodium glutamate in the newborn animal, the use of protein hydrolysate infusions to treat young infants is of considerable concern. Infants on such therapy usually receive 120 ml. of hydrolysate per kilogram per 24 hours by constant infusion. This amounts to 288 mg. of glutamate and 73 mg. of aspartate in casein hydrolysates and 80 mg. of glutamate and 105 mg. of aspartate in beef fibrin hydrolysates per kilogram per day. Since these preparations are infused into the superior vena cava bypassing hepatic and gut control, and in view of the reported neurotoxic effects of injected glutamate and aspartate, it is imperative to know what effect, if any, such infusions have on plasma glutamate and aspartate concentrations.

METHODS

Heparinized blood samples from patients and normal control infants, drawn at 2 P.M. to avoid circadian rhythm influences, were immediately centrifuged to remove the erythrocytes and deproteinized with sulfosalacylic acid.[20] The deproteinized plasma was either analyzed immediately or stored at −80° C. until assay (never more than 24 hours) to minimize conversion of glutamine to glutamic acid and pyrrolidone carboxylic acid and avoid the loss of cystine.[21,22] Amino acid analyses were carried out on Technicon NC-1 amino acid analyzers using the buffer system described by Efron[20] and the automatic temperature control system developed in this laboratory.[23] The free amino acid composition of the protein hydrolysate solutions was determined after treating 2 ml. of the solution with 100 mg. solid sulfosalacylic acid. Replicate aliquotes were analyzed at 3 concentrations (0.01 ml., 0.05 ml., and 0.10 ml.) to obtain accurate estimations of amino acids present in both large and small amounts.

The hydrolysate preparations studied were Amigen, (Baxter Lab., Morton Grove, Ill.) and Aminosol (Abbott Lab., North Chicago, Ill.) Patients treated with these protein hydrolysate infusions receive 120 ml. of hydrolysate per kilogram per 24 hours by constant infusion via the superior vena cava. These protein hydrolysates differ in their source of protein; Amigen is prepared from casein and Aminosol from beef fibrin. The amino acid

Table I. Free amino acid composition of protein hydrolysate preparations for infusion

Amino acid	Amigen (casein)	Aminosol (beef fibrin)
	(micromoles/100 ml.)	
Taurine	Not detected	Not detected
Cysteic acid	Not detected	112
Hydroxyproline	Not detected	Not detected
Methionine sulfoxides	117	15
Aspartic acid	500	660
Threonine	922	860
Serine	1,900	1,398
Asparagine	Not detected	Not detected
Glutamine	Not detected	Not detected
Glutamic acid	1,960	358
Proline	1,280	1,020
Citrulline	5	7
Glycine	758	5,630
Alanine	1,109	1,150
Valine	1,686	404
1/2 Cystine	Not detected	75
Methionine	754	476
Isoleucine	1,365	580
Leucine	2,960	3,950
Tyrosine	234	343
Phenylalanine	1,182	381
Ornithine	40	80
Lysine	2,493	1,190
1-Methylhistidine	Not detected	32
Histidine	631	326
Arginine	165	690
Tryptophan	165	110

Table II. Composition of protein hydrolysate infusion (content per liter)

Infusion	Amount
Protein	25 Gm.
Glucose	225 Gm.
Lipid	—
Sodium	30 mEq.
Potassium	20 mEq.
Chloride	20 mEq.
Phosphate	15 mEq.
Calcium	500 mEq.
Magnesium	50 mg.
Folic acid	37 mg.
Ascorbic acid	500 mg.
Vitamin A	10,000 U.S.P. units
Vitamin D	1,000 U.S.P. units
Thiamine HCl	50 mg.
Riboflavin	10 mg.
Niacinamide	100 mg.
Pyridoxine HCl	15 mg.
Panthenol	25 mg.
Vitamin E	5 International Units
Heparin sodium	1,000 U.

composition of these 2 preparations determined in our laboratory by replicate analysis at several concentrations is shown in Table I. The free amino acid content of the products analyzed in our laboratory varied considerably from the composition stated on the label and also differ from each other. Both preparations differ considerably in composition from plasma amino acid levels and contain large amounts of glutamate and aspartate, normally found at low levels in plasma.

Intravenous lipid preparations are not available for clinical use at this time, thus the solutions commonly used do not contain a source of lipid. In order to supply essential fatty acids, a plasma infusion is given each week. Calories, minerals, electrolytes, and vitamins are supplied in adequate amounts (Table II). Because of high solute concentration these solutions must be given into a vessel with high blood flow, and the superior vena cava is most frequently chosen.

Eight plasma free amino acid determinations from 6 infants receiving such preparations by central venous catheter were carried out. Three infants were debilitated because of severe chronic diarrhea, one was debilitated with recurrent postoperative bowel fistulae, one had giant cell hepatitis with hypoglycemia, and one was well nourished but required surgical repair of a large omphalocele. Six samples of plasma were drawn while the infants were receiving a casein hydrolysate and 2 while a beef fibrin hydrolysate was being infused. The duration of hydrolysate administration before plasma amino acid analysis was performed ranged from 2 to 54 days. Patient K. C. was receiving a casein hydrolysate orally in addition to the parenteral feeding. Patient B. D. was studied with each of the protein hydrolysate preparations.

RESULTS

Plasma amino acid levels in infants treated with these preparations are shown in Table III, along with normal plasma amino acid levels for 1 month old infants, 2 hours postprandially, and for 1- to 2-year-old children

Table III. Plasma amino acid levels in young infants treated with protein

Amino acid	Plasma amino acid l				
	Patients				
	C. E.	*W. W.*	*K. C.*	*C. P.*	*R. S.*
	Age (days): 50 *Diagnosis: Chronic diarrhea* *D*: 2* *P†: Casein* *N‡: Debilitated*	*Age (days): 13* *Diagnosis: Chronic diarrhea* *D: 2* *P: Casein* *N: Debilitated*	*Age (days): 68* *Diagnosis: Hepatitis* *D: 28* *P: Casein + oral* *N: Fair*	*Age (days): 92* *Diagnosis: Chronic diarrhea* *D: 54* *P: Casein* *N: Debilitated*	*Age (days): 1* *Diagnosis: Fi* *D: 6* *P: Casein* *N: Debilitated*
Taurine	2.22	3.05	1.00	0.60	1.40
Hydroxyproline	0.30	0.40	1.64	1.71	0.90
Aspartate	1.70	1.67	0.42	1.43	0.62
Threonine	11.9	6.94	15.4	15.6	11.2
Serine	12.5	15.6	18.6	8.03	11.2
Glutamine	47.7	25.5	41.5	36.9	38.5
Glutamate	9.40	7.78	8.00	1.34	5.18
Proline	24.4	11.4	16.9	9.36	12.2
Citrulline	1.94	0.83	1.47	1.21	1.69
Glycine	22.8	20.3	33.9	16.0	24.8
Alanine	17.5	13.9	35.0	22.5	21.1
α-Aminobutyrate	0.60	0.65	0.60	0.70	0.53
Valine	20.3	9.99	10.3	9.48	13.4
1/2-Cystine	1.67	1.94	5.18	2.34	1.37
Methionine	3.05	1.95	2.55	1.89	2.95
Isoleucine	7.50	4.44	2.80	2.72	4.91
Leucine	10.8	8.05	4.41	6.54	9.45
Tyrosine	1.40	1.45	2.99	4.00	3.68
Phenylalanine	8.05	5.30	6.67	3.90	9.06
Ornithine	4.15	3.61	4.50	6.00	5.77
Lysine	14.8	15.0	9.70	17.1	15.0
Histidine	6.39	7.22	5.96	3.23	5.50
Arginine	3.33	1.40	2.55	2.36	3.78

*Days on preparation.

†Protein based of infusion.

‡Nutritional status.

after an overnight fast, as determined in this laboratory.

Plasma glutamate and aspartate levels were within normal nonfasting limits in all infants studied. Substantial conversion of infused glutamate and aspartate to glutamine and asparagine, respectively, was apparent, since these latter amino acids are not found in the hydrolysate preparations. In our experience, infants treated with these 2 preparations remain in relative positive nitrogen balance, grow, and show no recognizable neurotoxic symptoms. Infant K. C. was known to have giant cell hepatitis. Despite a moderate to severe degree of liver damage demonstrated by hypoglycemia, abnormal liver function tests, and biopsy, plasma levels of glutamate and aspartate were within normal limits. It is difficult to imagine glutamate-induced neural damage without substantial elevation of plasma glutamate levels at least of the order noted by McLaughlan and associates.[14]

Certain plasma amino acid concentrations in these patients were, however, well below normal fasting levels observed in full-term infants. The plasma amino acid levels closely reflected the amino acid composition of the protein hydrolysate. Infants infused with casein hydrolysates had subfasting plasma concentrations of hydroxyproline, valine, arginine, tyrosine, and cystine. In contrast to normal plasma aminograms where phenylalanine to tyrosine ratios are near unity, the

drolysate infusions and in normal subjects

icromoles per 100 ml. plasma)

			Normal subjects	
B. D.	B. D.	B. D.	N = 15	N = 15
t (days): 6 *gnosis: Omphalo-* *cele* *3* *Fibrin* *Good*	*Age (days): 10* *Diagnosis: Omphalo-cele* *D: 7* *P: Fibrin* *N: Good*	*Age (days): 12* *Diagnosis: Omphalo-cele* *D: 2* *P: Casein* *N: Good*	*Age (mo.): 1* *Diagnosis: Normal* *D: —* *P: Normal diet* *N: 2 hr. postprandial*	*Age (yr.): 1-2* *Diagnosis: Normal* *D: —* *P: Normal diet* *N: Overnight fast*
2.75	12.8	3.10	12.2 ± 4.22	10.3 ± 4.14
0.30	1.25	2.30	9.99 ± 2.47	5.36 ± 2.06
0.20	0.63	0.53	2.12 ± 2.27	0.47 ± 0.44
20.3	36.0	23.2	20.4 ± 3.43	12.1 ± 4.15
11.5	19.3	13.2	23.6 ± 5.34	14.3 ± 2.02
45.3	57.3	47.8	70.3 ± 14.6	54.9 ± 3.71
3.25	5.75	5.01	7.70 ± 2.53	2.73 ± 1.53
11.8	15.0	22.9	33.6 ± 6.75	16.5 ± 6.46
1.25	0.88	0.77	4.23 ± 1.91	3.20 ± 3.28
30.5	57.0	27.8	34.8 ± 5.26	23.0 ± 5.40
7.50	10.8	7.94	40.9 ± 7.17	27.7 ± 6.26
0.15	0.43	0.17	2.46 ± 2.71	1.70 ± 2.80
3.00	4.30	12.9	34.6 ± 7.06	24.5 ± 4.74
4.00	6.00	3.14	9.93 ± 3.00	9.45 ± 2.72
2.25	4.00	2.64	4.91 ± 1.45	2.72 ± 1.71
3.00	5.25	4.62	13.9 ± 3.29	7.62 ± 2.41
7.50	12.5	7.00	21.8 ± 4.96	13.2 ± 2.09
1.50	2.75	2.39	12.6 ± 3.40	6.91 ± 1.80
2.12	2.75	6.01	11.2 ± 1.59	6.12 ± 1.80
2.50	4.76	2.14	13.9 ± 5.76	5.81 ± 2.21
9.00	14.8	9.29	24.3 ± 4.66	16.3 ± 2.56
4.00	5.75	5.28	10.4 ± 2.35	8.18 ± 1.34
4.00	6.00	3.21	12.9 ± 4.12	7.48 ± 1.52

ratio of plasma phenylalanine to tyrosine remained at the elevated ratio found in the hydrolysate (5:1). The one exception to this unusual ratio was Infant C. P. This infant had been treated with the casein hydrolysate infusion for a considerable length of time, and induction of enzymes necessary to handle both phenylalanine and glutamate may have occurred since the phenylalanine to tyrosine ratios were normal, and glutamate was at the fasting level. Induction and suppression of enzymes to handle increased levels of amino acids would be consistent with data presented by Daniel and Waisman[24] after long-term feeding of excess dietary methionine in the rat. Plasma cystine levels are particularly low in infants fed casein hydrolysate preparations, reflecting the absence of this amino acid in the hydrolysate, a factor of some concern in the immature infant (see Discussion). The one exception was Infant C. P. who was receiving some oral intake.

Certain plasma amino acid levels were also below fasting in Infant B. D., who was treated with the beef fibrin hydrolysate preparation. Although plasma cystine and arginine levels more closely resembled normal (when compared to levels in infants treated with casein hydrolysate preparations), hydroxyproline, valine, and tyrosine were below fasting levels with both preparations; alanine and phenylalanine values were especially low in this infant, although the phenylalanine to tyrosine ratio approached unity.

DISCUSSION

Although concern about the composition of protein hydrolysate preparations, particularly in regard to glutamate toxicity, is reasonable, our data indicate that the young infant is able to adequately control plasma glutamate levels at the infusion rates used in this study. We have followed several patients from our center for 18 months or longer postinfusion and have been unable to detect any demonstrable evidence of central nervous system damage or growth change. Although the side effect of nausea and vomiting occuring with the use of such infusions is usually attributed to monosodium glutamate levels, Vinnars and associates[25] report the occurrence of these symptoms in patients infused with Intramin Novum which contains no glutamate.

Our data demonstrate that the amino acid composition of the protein hydrolysate preparations directly affect plasma amino acid levels, and indicate that each preparation produces a characteristic plasma amino acid pattern. A comparison of the amino acid composition of the 2 preparations studied (Table I) demonstrates that the plasma amino acid changes which occur are logical. Beef fibrin hydrolysates contain equal molar quantities of phenylalanine and tyrosine, accounting for the 1:1 ratio found in the plasma of infants treated with such preparations, but contain a lower absolute amount of phenylalanine when compared to the casein hydrolysate preparation, resulting in decreased plasma phenylalanine levels. The considerably lower valine content of the beef fibrin preparation is reflected in the lower plasma levels of this amino acid. Since the beef fibrin hydrolysate contains larger quantities of glycine, arginine, and cystine than the casein hydrolysate, plasma levels of these amino acids would be expected to be higher in the infant treated with this preparation. A comparison of Patient B.D.'s plasma amino acid levels after a change from the beef fibrin to the casein hydrolysate preparations (10 and 12 days of age) support this concept. Glycine, cystine, arginine, and threonine levels were decreased, valine and phenylalanine levels increased, and the phenylalanine to tyrosine ratio was changed from unity to 5:2.

The reason for the lower alanine levels in Infant B. D. is not clear. Both preparations contain approximately equal amounts of this amino acid, and no substantial change in alanine levels occurred when the infant was shifted from the beef fibrin to the casein hydrolysate preparation.

A priori, it would seem that the amino acid content of the protein infusion should resemble that of normal human plasma as closely as possible, since the infusion enters directly into circulation without passage through the gastrointestinal tract and liver. Although considerable care has been taken to supplement these preparations with adequate amounts of "essential amino acids," little concern appears to have been given to matching plasma amino acid levels. The terms "essential" and "nonessential" amino acid relate only to dietary requirements when oral feeding is used, and have no meaning with respect to the relative importance of each amino acid metabolically. We feel that serious consideration should be given to possible subacute states of amino acid imbalance, antagonism or toxicity resulting from the present formulation of these products. The basis for the adverse effects attributed to these conditions has not been established, but a number of investigators have reported striking changes in plasma amino acid levels under conditions of dietary amino acid imbalance (see review in reference 26). Studies in experimental animals indicate that the homeostatic response to an altered balance of amino acids in the blood and body fluids seems to inhibit the animal from ingesting an excessive amount of a severely unbalanced diet. Forced feedings of such a severely unbalanced diet have resulted in degeneration of certain organs and even in early death.[27, 28]

The absence or low levels of cystine or cysteine in these preparations is of special concern in the treatment of the premature and full-term newborn infant. Although cysteine is readily formed from methionine

via cystathionine in the human adult, Sturman and associates[59] have recently provided strong evidence that cysteine (or cystine) may be an essential amino acid in the human fetus, premature, and full-term newborn infant. In view of the low plasma cysteine values noted in these studies, especially with Amigen, it appears that some supplementation with cysteine or cystine is desirable.

Our data indicate that the amino acid composition of the hydrolysate has a profound effect on plasma amino acid levels and suggest a degree of amino acid imbalance in both products. Most of the studies which have defined the conditions of amino acid imbalance, toxicity, or antagonism have utilized diets administered by way of the gastrointestinal tract, although some studies of amino acid toxicity have utilized the injection of amino acid supplied in excess. This and the paucity of data in the literature concerning the effects of intravenous feedings on plasma amino acid levels make it difficult to generalize directly as to the extent of imbalance present in our patients. It seems obvious that a better physiologic response of the patient could be achieved if such preparations were formulated to more closely match plasma amino acid levels. Studies evaluating such preparations are currently in progress.

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CHROMATOGRAPHIC DETERMINATION OF THE AMINO ACID COMPOSITION OF PROTEINS

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There can be little doubt that the present extensive and fruitful use of chromatographic methods for the separation of water-soluble substances has been stimulated, to a very large degree, by the pioneer investigations of Martin and Synge and their co-workers. They developed the silica gel column for acetyl amino acids, the now famous paper chromatograms, and introduced the use of starch columns (Elsden and Synge, 1944; Synge, 1944). Our own work with chromatographic methods, like that of many others, has been a logical outgrowth of these earlier investigations.

In our studies, the first objective was to ascertain whether chromatographic methods could provide a convenient and accurate means to determine quantitatively the amino acid composition of proteins. For accurate work of this kind, column chromatography appeared to be more promising than paper chromatography. In addition, a column possesses the advantage that it can be operated on a scale sufficiently large to permit, where necessary, isolation of 20 mg. or more of a component. Accordingly, the starch column was chosen for development (Moore and Stein, 1948, 1949; Stein and Moore, 1948).

In Figure 1 is shown the result which may be obtained when a complex mixture of amino acids is fractionated on a starch column. In this case the mixture was made up to simulate an acid hydrolysate of bovine serum albumin and contained 18 components—17 amino acids and NH_4Cl. For this type of experiment, the effluent is divided into a regular series of very small fractions of known volume. These fractions are analyzed quantitatively. The data obtained permit the construction of effluent curves, such as the one shown, which reveal the detailed behavior and full resolving power of the column.

In the laboratory, the packed column is mounted over an automatic fraction collector which has been designed to collect continuously small fractions of specified volume (Stein and Moore, 1948). The basic parts of the machine are a turntable holding the receiving tubes and a photo-electric drop counting circuit which controls the amount of effluent delivered per tube. The curve shown in Figure 1 required the collection of about 400 0.5 cc. fractions, over a period of seven 24 hour days, and it is thus apparent that automatic machinery is essential before any considerable number of experiments of this type can be undertaken.

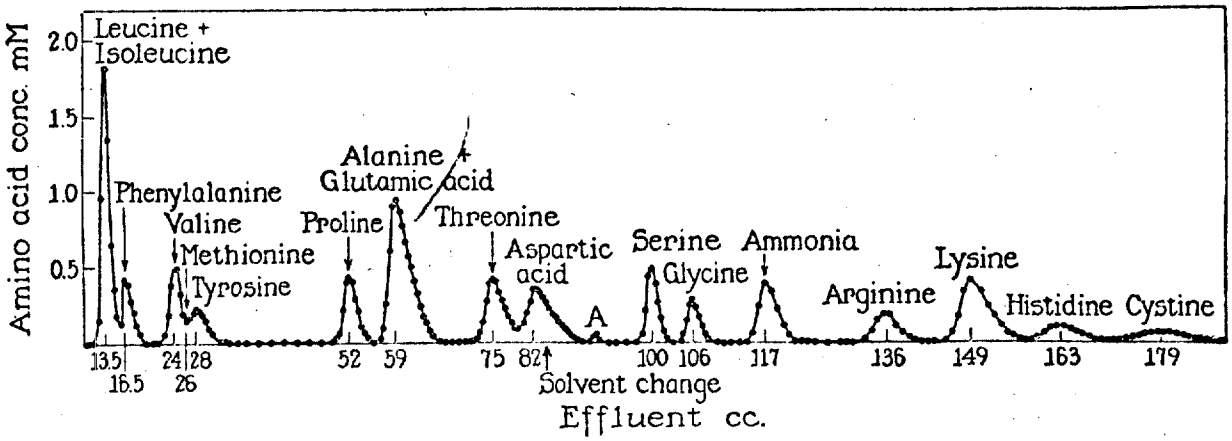

FIG. 1. Separation of amino acids from a synthetic mixture containing seventeen amino acids and ammonium chloride. Solvent, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1N HCl, followed by 2:1 *n*-propyl alcohol-0.5N HCl. Column, 13.4 gm. of starch (anhydrous); diameter, about 0.9 cm.; height, about 30 cm. Sample, about 3 mg. of amino acids. The effluent was collected in 0.5 cc. fractions. *A* is a small artifact peak.

At the beginning of the experiment the sample containing the amino acid mixture is added in a few tenths of a cc. to the top of the column. The sample is allowed to drain into the starch, and fresh solvent, containing no amino acids, is added and run through under slight pressure. The load on a column one cm. in diameter is about 2.5 mg. of an amino acid mixture. The apparatus and the procedures have been designed so that, when necessary,

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the load can be increased about 100 fold on columns 8 cm. in diameter for experiments on a preparative scale.

The next step in the procedure is the analysis of the numerous fractions collected for their amino acid content. The amino acid concentration is determined by a simple photometric ninhydrin method which has been developed for this purpose (Moore and Stein, 1948). It is necessary for the analytical method to be highly sensitive, accurate, and readily adapted to the handling of hundreds of individual analyses. The ninhydrin method is sensitive to one part per million of amino acid in aqueous or alcoholic solution, and is accurate to ± 2 percent for two to three micrograms of amino acid nitrogen.

After the mechanics of the procedure were worked out, it became necessary to investigate the behavior of various solvent mixtures. The immediate objective in these studies was the development of quantitative methods for the determination of the amino acid composition of protein hydrolysates. For this purpose a great many combinations of solvents were investigated before the most convenient systems were established. It was found that a wide variety of neutral, acidic, and buffered solvent mixtures can be employed with starch columns. Dilute HCl mixtures, however, have proved to be the most useful for separating the common amino acids. In the curve shown in Figure 1, the chromatogram was started with a solvent composed of 1 part normal butyl alcohol, 2 parts normal propyl alcohol, and 1 part aqueous 0.1N HCl. After the emergence of aspartic acid, the solvent running through the column was changed to a mixture of 2 parts of propyl alcohol and 1 part 0.5N HCl, which accelerates the movement of the remaining amino acids. In this experiment, and all others in which known mixtures were employed, the positions of the various amino acid peaks were first established by a series of chromatograms performed with mixtures of increasing complexity.

It is important to note that in the experiment shown in Figure 1 the position on the effluent curve of a given amino acid is constant to ±5 to 10 percent, and is not influenced by the amino acid composition of the mixture fractionated. Proline, for example, emerges at the same place whether it is alone on the column, or whether it is originally admixed with 18 other components. Even more reproducible than the absolute position of a peak on the abscissa, is the position of a peak relative to its neighbors. This constancy of relative position leads to a pattern of peaks which is characteristic for each solvent, is readily recognized, and is, therefore, extremely useful when dealing with unknown mixtures.

The curve in Figure 1 illustrates the high resolving power of which the starch column is capable. The synthetic mixture of amino acids chromatographed in this instance contained 18 components and yielded 14 peaks. Nevertheless, the resolution is not satisfactory in all cases. Leucine, isoleucine,

TABLE 1. RECOVERY OF AMINO ACIDS FROM KNOWN MIXTURE CONTAINING 18 COMPONENTS

Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1N HCl followed, after the emergence of aspartic acid, by 2:1 *n*-propyl alcohol-0.5N HCl (*cf.* Fig. 1).

Constituent	Amount present	Percent recovery			
		Chromatogram 456	Chromatogram 457	Chromatogram 520	Average
	mg.				
Leucine-isoleucine	0.364	99.4	99.5	101.5	100.3
Phenylalanine	0.165	94.8	96.1	94.8	95.2
Valine-methionine-tyrosine	0.354	99.6	101.0	100.1	100.2
Proline	0.136	99.7	97.8	100.0	99.2
Glutamic acid*-alanine	0.515	95.2	94.6	96.8	95.5
Threonine	0.201	97.5	101.0	102.0	100.2
Aspartic acid*	0.267	93.5	94.1	94.7	94.1
Serine	0.118	100.0	99.8	101.2	100.3
Glycine	0.051	99.1	100.5	101.0	100.2
Ammonia	0.024	102.0	99.5	104.5	102.0
Arginine	0.143	97.7	102.8	105.0	101.8
Lysine	0.302	96.3	103.0	99.5	99.6
Histidine	0.094	99.7	104.6	97.4	100.6
Cystine	0.133	89.5	102.7	101.5	97.9
All constituents	2.867	97.3	99.3	99.6	98.7

* When the value for glutamic acid is corrected for the 7 percent low recovery due to esterification, the recoveries for glutamic acid plus alanine become 100.2, 99.7, and 101.7 percent. The aspartic acid recoveries, which run 6 percent low, may be similarly corrected to yield the figures 99.4, 99.9, and 100.8 percent. The total recoveries, on this basis, become 98.6, 100.8, and 101.0 percent

and phenylalanine are not well separated. The same is true of valine, methionine, and tyrosine. Glutamic acid and alanine emerge together. A satisfactory degree of resolution has been obtained for the remaining ten components—proline, threonine, aspartic acid, serine, glycine, NH_4Cl, arginine, lysine, histidine, and cystine. Integration of the curves gives quantitative recoveries of most of the components, as can be seen in Table 1. The overlapping amino acids are integrated as a group. For most of the single peaks, the average recoveries are accurate to ±3 percent. A low yield is obtained of aspartic acid and of glutamic acid + alanine. The low recoveries result from the loss of about 6 percent of the aspartic and 7 percent of the glutamic acid by esterification in the acidic alcoholic solvents. The degree of esterification is independent of the amino acid concentration, and hence, with unknowns, accurate aspartic acid figures may be obtained by incorporating a 6 percent correction factor into the final result. It may be noted that a single chromatogram requires about 3 mg. of total amino acid mixture, corresponding to between 5 and 30 hundredths of a mg. of each component. The recovery for the sum of the components in these experiments is 99 percent. For many investigations, this single type of chromatogram sometimes provides sufficient information in spite of the overlaps.

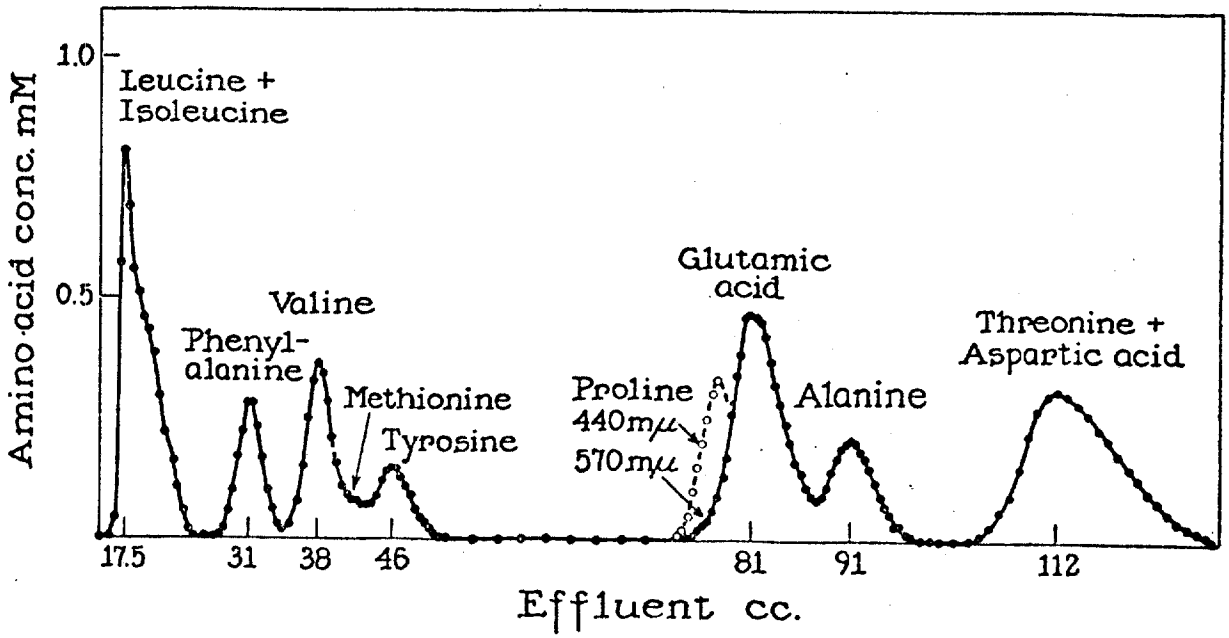

FIG. 2. Separation of glutamic acid, alanine, and other amino acids from a synthetic mixture containing 18 components. Solvent, 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1N HCl. In other respects, the experimental conditions were the same as those described in Fig. 1.

It is possible to resolve the overlaps present in the curve shown in Figure 1 by the appropriate choice of solvents. Thus, glutamic acid and alanine, as may be seen in Figure 2, are separated by the use of a mixture of *tert*- butyl alcohol, *sec*- butyl alcohol and 0.1N HCl in the proportions of 2:1:1.

TABLE 2. RECOVERY OF GLUTAMIC ACID, ALANINE, AND OTHER AMINO ACIDS FROM SYNTHETIC MIXTURE
Solvent, 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1N HCl (*cf.* Fig. 2). The mixture contained 18 components (*cf.* Table 1).

Constituent	Amount present	Percent recovery			
		Chromatogram 474	Chromatogram 543	Chromatogram 481	Average
Leucine-isoleucine	0.373	99.0	100.8		99.9
Phenylalanine	0.169	101.6	103.6		102.6
Valine-methionine-tyrosine	0.363	100.6	104.4		102.5
Glutamic acid	0.426	96.3	97.8	100.2	98.1
Alanine	0.102	97.3	101.3	97.5	98.7

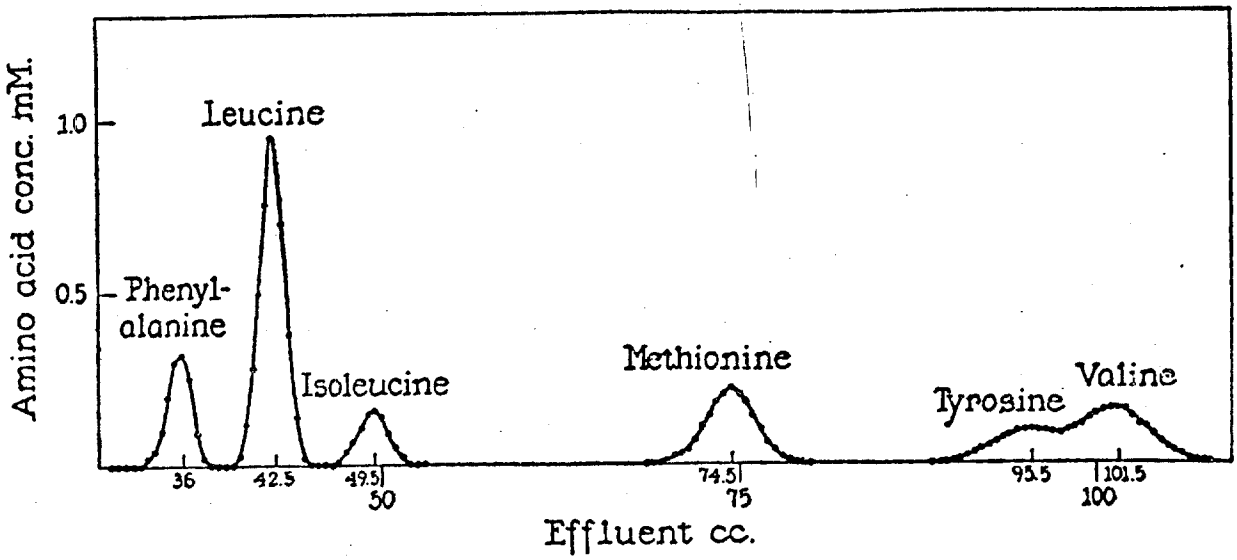

FIG. 3. Separation of amino acids from a synthetic mixture containing 18 components. Solvent, 1:1:0.288 *n*-butyl alcohol-benzyl alcohol-water, containing 0.5 percent thiodiglycol. In other respects, the experimental conditions were the same as those described in Fig. 1.

In addition to glutamic acid and alanine, this experiment yields a determination of phenylalanine. The quantitative data obtained from a chromatogram of this type are shown in Table 2. Glutamic acid is not esterified in this solvent, as evidenced by its quantitative recovery.

The overlaps still remaining from the chromatogram shown in Figure 1 can be resolved by the use of a third solvent composed of butyl and benzyl alcohol in equal proportions, 12 percent water and 0.5 percent thiodiglycol (Fig. 3). Thiodiglycol protects the sulfur of methionine from oxidation by traces of impurities present in the benzyl alcohol. It may be noted that in this solvent phenylalanine, leucine, and isoleucine emerge as well defined peaks. The separation of tryrosine and valine is not optimum, but is sufficiently good to permit calculation of the amount of each present. The quantitative data obtained from experiments such as the one shown in Figure 3 are given in Table 3.

Thus, by the use of three chromatograms, it is possible to obtain quantitative values for all of the 18 components of the synthetic mixture. The three columns are run with the butyl-propyl alcohol-0.1N HCl solvent, the butyl-benzyl alcohol mixture, and the tertiary butyl-secondary butyl alcohol solvent.

It should be mentioned that D, L, and DL amino acids have identical rates of travel on starch columns. This fact has been established in experiments with the L and DL forms of 12 of the amino acids discussed.

Attention can now be focussed on the application of the starch column technique to the determination of the quantitative amino acid composition of *purified* proteins. It should be noted that the word is "purified" not "pure." For analysis the protein

TABLE 3. RECOVERY OF AMINO ACIDS FROM KNOWN MIXTURE CONTAINING 18 COMPONENTS

Solvent, 1:1:0.288 *n*-butyl alcohol-benzyl alcohol-water (*cf.* Fig. 3). The composition of the mixture was similar to that given in Table 1.

Amino acid	Percent recovery				
	Chromatogram 270	Chromatogram 293	Chromatogram 338	Chromatogram 390	Average
Phenylalanine	103.6	101.8	98.4	99.4	100.8
Leucine	101.0	101.6	102.0	101.0	101.4
Isoleucine	102.0	103.6	100.0	102.8	102.1
Methionine			100.0*	100.6*	100.3
Tyrosine	106.4	100.6	101.1	102.7	102.7
Valine	96.7	99.0	100.1	100.8	99.2

* The solvent contained 0.5 percent thiodiglycol.

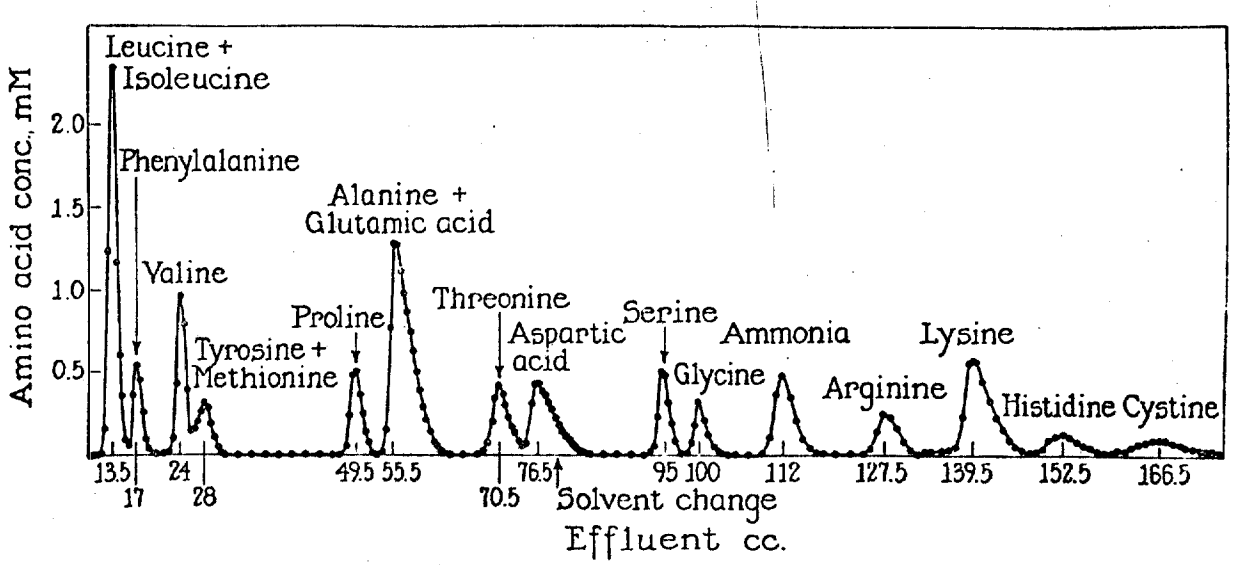

FIG. 4. Chromatographic fractionation of a hydrolysate of bovine serum albumin. Sample, an amount of hydrolysate corresponding to about 2.5 mg. of protein. The experimental conditions were the same as those described in Fig. 1.

preparations were hydrolyzed for 16 hrs. with 200 volumes of boiling 6N HCl, the excess HCl concentrated off, and the residue made up to a known volume. The chromatographic analyses were carried out on aliquots of the hydrolysate in exactly the manner described for the known amino acid mixtures. β-Lactoglobulin and bovine serum albumin have been analyzed by this procedure (Stein and Moore, 1949). The present discussion will be restricted to the latter. An effluent curve obtained with a hydrolysate of crystalline bovine serum albumin is given in Figure 4.

In the application of the chromatogram to unknown mixtures, the most important question concerns the correct identification of the peaks on the curve. In order to render the identification absolutely certain, it would be necessary to isolate the material responsible for each peak and to identify each substance by elementary analysis, optical rotation, or other means. The starch column can offer a means of accomplishing this end. But the isolation of all the components presents a formidable task under the best of circumstances. For analytical work it is worth while to consider whether a reason-

TABLE 4. CHROMATOGRAPHIC ANALYSES OF HYDROLYSATES OF BOVINE SERUM ALBUMIN

Solvents, 1:2:1 *n*-butyl alcohol-*n*-propyl alcohol-0.1N HCl and 2:1 *n*-propyl alcohol-0.5N HCl (*cf.* Fig. 4).

Constituent	Chromatogram 480		Chromatogram 481		Chromatogram 488	
	Gm. amino acid per 100 gm. protein	N as percent of protein N	Gm. amino acid per 100 gm. protein	N as percent of protein N	Gm. amino acid per 100 gm. protein	N as percent of protein N
Leucine-isoleucine	14.4	9.58	14.75	9.80	14.3	9.51
Phenylalanine	6.32	3.34	6.44	3.40	6.52	3.44
Valine-methionine-tyrosine		7.42 *ca.*		7.37 *ca.*		7.20 *ca.*
Proline	4.65	3.52	4.75	3.60	4.85	3.67
Glutamic acid-alanine		14.90 *ca.*		15.25 *cc.*		15.40 *ca.*
Threonine	5.44	3.98	5.72	4.19	5.50	4.05
Aspartic acid	10.91	7.15	10.86	7.12	10.96	7.19
Serine	3.91	3.24	3.76	3.12	3.77	3.13
Glycine	1.85	2.15	1.75	2.03	1.85	2.15
Ammonia	1.03	5.28	1.08	5.54	1.06	5.44
Arginine	5.96	11.92	6.03	12.07	5.72	11.45
Lysine	12.70	15.15	12.62	15.05	13.15	15.68
Histidine	4.29	7.24	3.67	6.18	4.04	6.82
Cystine	5.83	4.23	5.81	4.22	6.08	4.41
Total nitrogen recovery		99.1		98.9		99.5

ably sound answer can be arrived at without isolation. We believe that, in favorable cases, certain considerations and certain experiments make it possible to identify correctly the peaks on the curve with a very high degree of probability. The analysis of an acid hydrolysate of protein material is a favorable case. So much work has already been done with proteins, that it is possible to have a fair idea of what to expect. Moreover, the material analyzed has been subjected to exhaustive acid hydrolysis, thus virtually eliminating the possible presence of peptides.

TABLE 5. AMINO ACID COMPOSITION OF BOVINE SERUM ALBUMIN

The values for phenylalanine, leucine, isoleucine, tyrosine, and valine are from chromatograms carried out with 1:1:0.288 *n*-butyl alcohol-benzyl alcohol-water containing 0.5 percent thiodiglycol. Glutamic acid and alanine were determined with 2:1:1 *tert*-butyl alcohol-*sec*-butyl alcohol-0.1N HCl. The remaining chromatographic values are the average figures from Table 4. The nitrogen content of the protein was 16.07 percent, on an ash- and moisture-free basis.

Constituent	Gm. amino acid per 100 gm. protein	Gm. amino acid residue per 100 gm. protein	N as percent of protein N
Phenylalanine	6.59	5.87	3.48
Leucine	12.27	10.58	8.17
Isoleucine	2.61	2.25	1.74
Methionine	0.81*	0.71	0.47
Tyrosine	5.06	4.56	2.44
Valine	5.92	5.01	4.41
Proline	4.75	4.00	3.60
Glutamic acid	16.50	14.49	9.78
Aspartic acid	10.91	9.44	7.15
Alanine	6.25	4.99	6.12
Threonine	5.83†	4.95	4.27
Serine	4.23†	3.51	3.52
Glycine	1.82	1.38	2.11
Arginine	5.90	5.29	11.80
Lysine	12.82	11.25	15.30
Histidine	4.00	3.54	6.75
Cystine+cysteine	6.52‡	5.54	4.73
Tryptophan	0.58§	0.53	0.50
Amide-NH_3	0.95‖		4.87
Total		97.9	101.2

* The value for methionine is that determined by Brand *et al.* (1944, 1946). The chromatograms gave a figure of about 0.92.

† The average threonine and serine values of 5.55 and 3.81 from Table 4 have been divided by 0.95 and 0.90, respectively, in accordance with the estimates of Rees (1946) for decomposition of these amino acids during hydrolysis.

‡ The cystine+cysteine value is that determined by Brand *et al.* (1944, 1946). The chromatograms gave an average value of 5.91.

§ The tryptophan value is that determined by Brand *et al.* (1944, 1946).

‖ This figure is a maximum value for amide-NH_3 calculated from the total NH_3 of the hydrolysate corrected for the approximate amount of NH_3 formed on the decomposition of serine and threonine.

First of all, it will be noted that the curve (Fig. 4) obtained with the hydrolysate is similar to that obtained with a known mixture (Fig. 1). Careful inspection reveals that for both curves, the absolute positions of the peaks are the same (±5 percent) and the relative positions of the peaks are identical. The pattern is thus a familiar one. There are no new peaks on the curve. Hence there is no evidence for the presence in the hydrolysate of unsuspected amino-nitrogen containing substances.

Specifically, the proline peak is one which can be identified without question spectrophotometrically because of the reddish-yellow color which proline gives with ninhydrin. Other color tests can be used to help in the identification of amino acids such as tyrosine, histidine, arginine, and cystine. A more crucial experiment, however, is to add two or three amino acids to a sample of the hydrolysate and repeat the chromatogram. When threonine, serine, and histidine were added to this particular hydrolysate, the designated peaks rose without loss of symmetry and the added amount of amino acid was recovered quantitatively in each case. With these precautions, therefore, we feel that a curve, such as the one given in Figure 4, obtained on a hydrolysate of a purified protein can be interpreted with reasonable certainty.

The average values obtained by integration of the curve shown in Figure 4, and duplicates thereof, are given in Table 4. It can be noted that nearly all the nitrogen of the hydrolysate, 99 percent, has been accounted for. Individual determinations have been achieved for 10 of the components of the hydrolysate and the triplicate determinations are in good agreement. To complete the analysis for phenylalanine, leucine, isolueucine, methionine, tyrosine, and valine, butyl-benzyl alcohol columns were run. A third type of chromatogram run with the tertiary butyl alcohol-secondary butyl alcohol solvent yielded figures for glutamic acid and alanine. The amino acid composition of bovine serum albumin thus derived is given in Table 5.

The values in the first column are the average figures obtained chromatographically, with three exceptions. The values for tryptophan, methionine, and cystine are those of Brand and his associates (1944, 1946). Tryptophan is completely, and cystine partially destroyed under the hydrolytic conditions which we have used. Special hydrolytic methods are required before these amino acids can be determined, a problem with which we have not dealt. In the case of methionine, Brand's value is probably more accurate than the chromatographic figure, which was not determined under conditions optimum for a component present to less than one percent.

The figures at the bottom of the table are perhaps the most useful ones to emphasize at this time. In the third column it is seen that the total nitrogen

recovery is about 101 percent. In the second column, the total recovery in terms of amino acid residues, accounts for about 98 percent of the protein. Thus it is possible, primarily by chromatographic means, to obtain for a protein preparation a balance sheet which accounts for essentially all the amino acid components of the protein.

In the case of bovine serum albumin, Brand and his associates have previously estimated the amino acid composition, using microbiological assays for the largest part, and have determined all the amino acids except alanine. Other investigators have determined a good many of the components by various chemical, microbiological, and isotopic methods. A detailed comparison of these data with the results obtained by chromatography has already been given (Stein and Moore, 1949) and need not be repeated here. It is heartening to find that, for the most part, the analyses by the chromatographic and other methods are in good agreement. In analytical chemistry it is always advantageous and reassuring to have more than one way of getting the answer.

The experiments discussed thus far have dealt with purified protein preparations. Some times, however, it is desirable to determine the amino acid composition of the proteins of a whole tissue or a tissue extract. The application of the chromatographic techniques to the analysis of unfractionated biological material can be exemplified by the following experiments on tubercle bacilli. Through the generous cooperation of Dr. Gardner Middlebrook and Dr. Rene Dubos, it has been possible to chromatograph, for comparative purposes, hydrolysates of virulent and avirulent tubercle bacilli (Fig. 5). For these experiments the simplest possible procedure was followed, namely, the whole bacilli after being filtered and washed, were suspended in 6N HCl and refluxed for 18 hours. The humin and insoluble fat were filtered off and an aliquot of the complete hydrolysate chromatographed. The presence of non-amino nitrogen containing components offered no interference on the chromatogram. An alternative procedure, which has been employed on tissue proteins and bacterial cells, is to hydrolyze a trichloracetic acid precipitate.

The top curve in Figure 5 was obtained from the avirulent strain, the lower curve from the virulent strain. Within the accuracy of the method, no differences in the amino acid composition of the two strains are observed. The experiment is primarily an example of the applicability of the chromatographic technique to quantitative studies on the comparative chemistry of bacterial cells. It is sometimes useful to be able to look for differences by means of parallel experiments of this type.

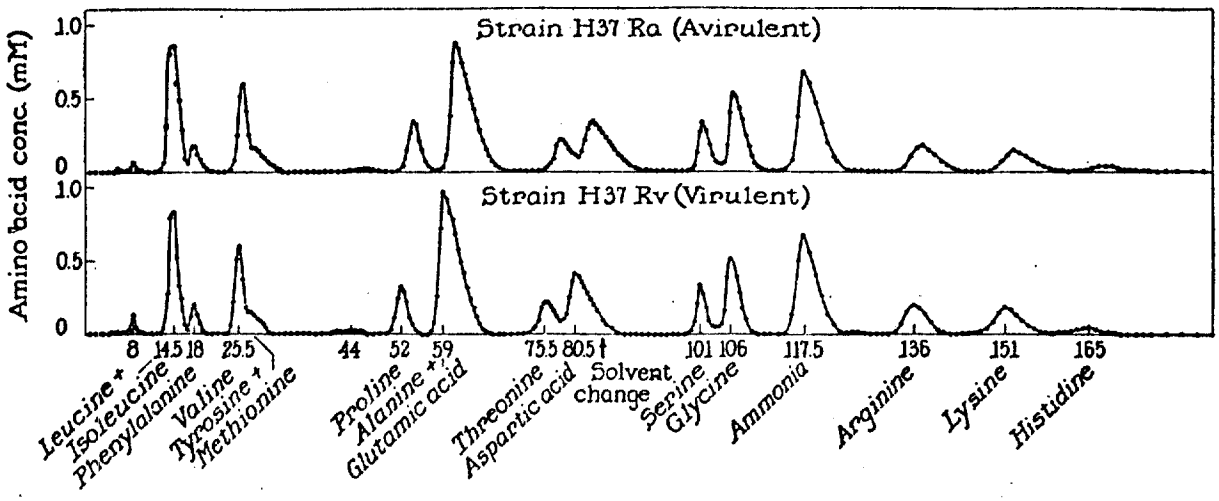

FIG. 5. Chromatographic fractionation of hydrolysates of virulent and avirulent strains of tubercle bacilli. The experimental conditions were the same as those described in Fig. 1.

Before proceeding to further applications of the starch column, it may be of interest to discuss some of the general implications of the work already outlined. The results have been achieved primarily because of two attributes possessed by the starch columns—their reproducibility and their high resolving power. These two attributes are related to one another and to certain mechanical considerations connected with the preparation of the column.

To the extent that the flow of solvent through a column is channeled, or non-uniform, the zones will be broadened and two adjacent peaks, instead of being separated by a clear valley, will merge into one another. For maximum efficiency and reproducibility, numerous experiments have shown that a chromatograph tube should be tightly packed with a material of small particle size, operated at a slow flow rate, and not overloaded with respect to the material being fractionated. It has been found that, with starch, the particles should be at least as small as 0.04 mm. in diameter, corresponding to about

300 mesh material. Potato starch granules average about this size. Canna starch, with larger granules, is less satisfactory, and rice starch, which is much finer than potato starch, gives flow rates that are inconveniently slow, even with automatic machinery.

The desired tightness of packing, which is important, can be achieved by utilizing the imbibition pressure of starch. If a slurry of starch in dry butanol is used in pouring the column, and a wet organic solvent is subsequently run through, the resultant swelling of the starch granules creates within the column an evenly distributed internal pressure capable of reducing the inter-granule interstices. It has thus been possible to devise a simple laboratory procedure which yields tightly packed, fully reproducible columns from experiment to experiment.

Starch columns are operated at the very slow flow rate of 2.0 to 3.0 cc. per hour per sq. cm. cross sectional area of the column. Under these conditions, it has been observed that the width of a slow moving amino acid peak is equivalent in effluent cc. to about 10 percent of the effluent volume at which the peak emerges. Thus, a peak emerging at 50 effluent cc. spreads over a narrow zone covering about 5 cc. (*cf.* Fig. 1, for example). Doubling the flow rate causes a slight but perceptible broadening and flattening of the peaks, which becomes intensified at markedly increased rates. High rates of flow result, therefore, in a loss of resolving power. Reducing the rate to one half of the recommended value does not alter the shape of the peak. The flow rate which has been found to be optimum for starch may not be so for other adsorbents. It seems clear, however, that chromatograms run at the excessively high flow rates frequently reported are, in many cases, operated at far from their maximum efficiency.

The amount of material fractionated, that is the load, on a column also markedly influences the resolving power attainable. On starch, the optimum load has been found to be about 0.3 mg. of an individual amino acid per sq. cm. of cross sectional area of the column. At such a level sharp symmetrical peaks are obtained. If the load is increased markedly, say by a factor of 5 to 10, the peak broadens and skews so that there is observed a sharp fronted peak emerging ahead of its normal position, followed by a long tail. This behavior represents a loss in resolving power, since the single broad peak will engulf any peak which, at a more moderate load, would immediately precede it. The shape of the peak suggests that, at the higher amino acid concentration, the adsorption isotherm is no longer linear.

Another factor affecting the resolving power of the chromatogram is the degree of retardation of the substances being fractionated on the column. For example, at the point of emergence of the advancing front of solvent, which is about 6 cc. after the sample has been added to a column 0.9 × 30 cm., there will emerge all the substances which are not adsorbed at all by starch. A peak at this position, therefore, would, in all likelihood, be a mixture of substances. It is only when the starch has considerable affinity for the components, resulting in slow band rates, or R_F values, that the maximum resolving power of the column is attained. It can be shown, using the mathematical treatment of Mayer and Tompkins (1947), that for starch columns 30 cm. in height, maximum efficiency is attained after four or five column volumes of solvent have passed through the column, or after about 30 effluent cc. in the curve shown in Figure 1. The maximum plate-efficiency of the column is realized for the proline peak and those emerging thereafter. In order to obtain the same efficiency for peaks emerging at small effluent volumes, theoretically it would be necessary to add the sample in an extremely small volume and to cut much smaller fractions.

In order to get the best results from a chromatograph column, therefore, it is necessary to find a combination of adsorbent and solvent which gives sufficient retardation of the substances being fractionated. As larger effluent volumes are reached, however, the peaks become increasingly broad and eventually a point is reached where the concentration of solute in the effluent becomes too low to be measured accurately. After 10 to 20 column volumes of solvent on a 30 cm. starch column, it is usually necessary to change the solvent to one which gives faster rates of travel for the remaining zones on the column, as in the shift of solvent after aspartic acid in the experiment shown in Figure 1. The new solvent sharpens up the remaining peaks.

In the work discussed thus far, a satisfactory rate of travel on the column has been achieved for nearly all the amino acids by the use of appropriate solvent combinations. Many of the solvent mixtures employed are miscible with water. Indeed, the number of solvents potentially useful in chromatographic work with starch columns is increased greatly when it is realized that these columns are not liquid-liquid (partition) chromatograms. A detailed treatment of this theoretical question cannot be undertaken at this time. A study of distribution coefficients has revealed, however, that the new term "partition chromatography" is not applicable to the starch column. Starch appears to act as a solid, polar adsorbent, which can be used with a variety of solvents.

Amino acids are not the only substances for which suitable band rates on starch have been obtained. Reichard (1948) has fractionated the nucleosides, and Daly and Mirsky (1949), and Hammarsten and his co-workers (1949) have chromatographed successfully the purines and pyrimidines. There are, however, many mixtures of substances, such as the high molecular weight peptides, which we have been unable to fractionate efficiently on starch. Di-, tri-, and tetrapeptides can be handled, since their chro-

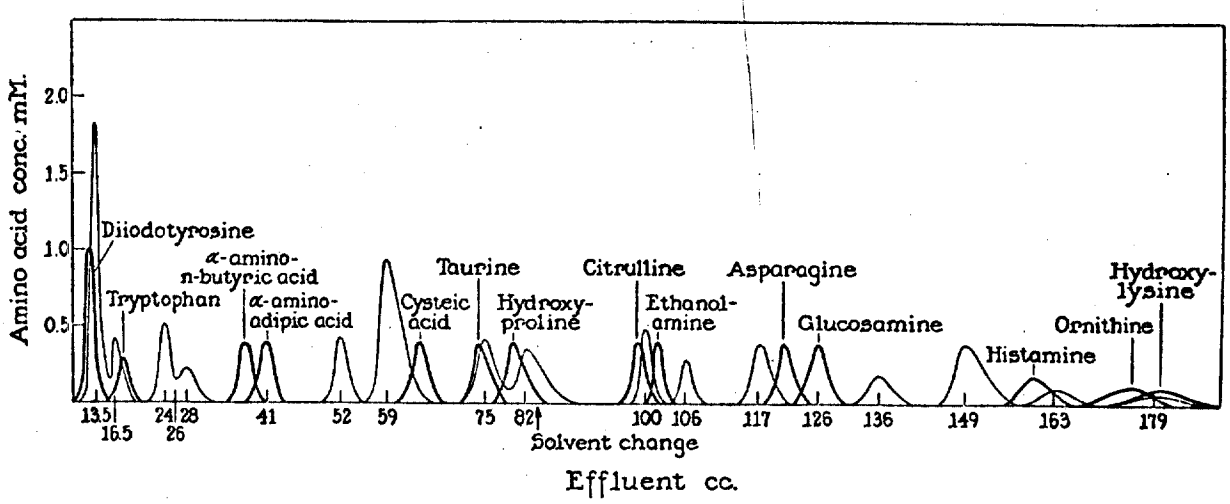

FIG. 6. Chromatographic behavior of a number of amino nitrogen containing substances. The background curve (thin line) is the same as the curve shown in Fig. 1.

matographic behavior appears to be similar to that of the amino acids. With polymyxin, or bacitracin, or other large peptides, however, we have not been able to obtain a suitable rate of travel. Craig, Gregory and Barry (1949) have brought peptides of this type within the range of the counter-current distribution method, but a satisfactory chromatographic system has not as yet been obtained. The peptides tend to run rapidly through the column and emerge with the solvent front, or else move scarcely at all on the column. At the present it would appear, therefore, that starch is not a good adsorbent for the higher peptides, and a search is currently being made for other adsorbent materials which might be applied effectively to this class of substances.

There remain two interesting applications of the starch column which warrant mention. They concern the chromatographic analysis of urine and of blood plasma. Obviously, urine may be expected to contain many ninhydrin positive compounds other than the common amino acids discussed thus far. Accordingly, the chromatographic behavior of a number of additional compounds has been investigated. The results are shown in Figure 6. The background curve (thin line) is the familiar amino acid

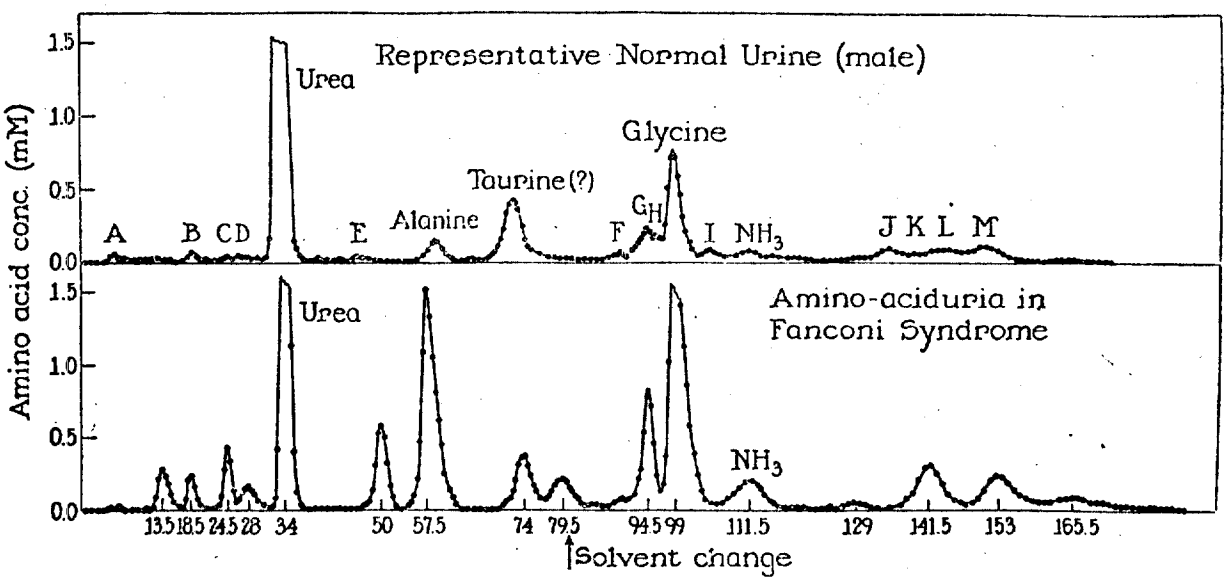

FIG. 7. Chromatographic fractionation of urine. The samples were withdrawn from a 24 hour specimen and desalted prior to chromatography. The amount of desalted urine chromatographed corresponded in each case to about 0.17 percent of the 24 hour specimen. The experimental conditions were the same as those described in Fig. 1.

pattern shown in Figure 1. Placed on this curve are a variety of other ninhydrin-positive substances. This picture indicates the necessity for the maximum resolving power attainable, and emphasizes the problems concerned with the correct identification of peaks when complex mixtures are chromatographed.

Preliminary curves obtained with samples of normal and pathological urines are illustrated in Figure 7. Most of the inorganic salts are removed prior to chromatography, using the electrolytic desalting device of Consden, Gordon and Martin (1947). The upper curve was obtained from a normal adult. There are at least 14 well defined, reproducible peaks on this curve. Ammonia, which was originally present in great amount, has largely been removed during the desalting process. The major amino acid peaks have been *tentatively* identified as alanine, taurine, and glycine. Many of the small peaks probably can be ascribed to other amino acids which are known to be excreted in small amounts. There appear to be at least four substances in the basic amino acid range, two of which are probably lysine and histidine. Glutamic and aspartic acids seem to be absent from urine, but appear after acid hydrolysis;—one would like to know from what source. The major peaks shown are resistant to acid hydrolysis.

The second curve was obtained from an infant exhibiting the Fanconi syndrome. We are greatly indebted to Dr. E. A. Park and Dr. Paul Hardy of the Johns Hopkins Hospital for the urine specimen. The curve is remarkably reminiscent of the ones obtained on protein hydrolysates. The starch column data are, therefore, in accord with the observations of others, notably Dent (1947), that the disease is associated with a marked amino aciduria. It should not be inferred, however, that all the peaks on this curve have been identified. Preliminary satisfactory curves have also been obtained with desalted dialysates of blood plasma.

The work with urine and plasma has been mentioned primarily to emphasize the fact that in chromatographing complex biological mixtures, such as these, particularly if they have not been subjected to acid hydrolysis, the identification of the peaks on the effluent curve becomes a major part of the research. Many substances other than the common amino acids are to be expected, including peptides, and in many cases even the most careful checks and controls cannot lead to more than tentative conclusions. It has seemed to us for some time that isolation of the components responsible for the peaks would be the only way to accumulate unequivocal data.

Work on convenient methods for the isolation of 25 to 50 mg. quantities of pure compounds from large scale columns is now in progress. Isolation experiments, however, present several major problems which are not encountered with chromatograms run on an analytical scale. As mentioned earlier, the capacity of a starch chromatogram is not great under conditions which maintain its high resolving power. To work up one gram of a protein hydrolysate on starch requires the use of at least two columns 8 cm. in diameter and about 25 liters of solvents. Furthermore, the effluent from a starch column is not carbohydrate-free, particularly when acidic solvents are used. Unless measures are taken to remove carbohydrate impurities, it is extremely difficult to isolate crystalline products from the effluent. Finally, in biochemical work there is frequently the problem of acquiring enough starting material to permit isolation. It is true that urine is not normally a scarce commodity, but only rarely would it be possible to obtain enough of a pathological plasma, for example, to warrant an attempt at isolation.

These considerations have led to several further avenues of experimentation. It has been found that the carbohydrate can be removed conveniently from the amino acids in the effluent by using a short column of an ion exchange resin. The fractions from a starch column comprising an amino acid peak can be pooled, concentrated to a small volume, and run through a resin bed. The carbohydrate passes through and the amino acids are retarded. In principle, the procedure seems to be sound. Analytically pure samples of amino acids have been obtained in mg. quantities from protein hydrolysates. The best conditions remain to be worked out, however.

A logical extension of this approach has been the examination in detail of the ability of some of the ion exchange resins themselves to fractionate mixtures of amino acids. Resins have been widely used as a means of separating the constituents of protein hydrolysates into groups of amino acids, and for the determination of a few of the individual components. Applying the principles and techniques which have been worked out to attain the best resolving power from starch, it has been found that some of the newer ion exchange resins now available commercially are capable of giving individual peaks for essentially all the common amino acids. This work is in a preliminary stage, but Dowex-50, for example, appears to possess fractionating power rivalling that of starch for some problems.

In Figure 8 is shown a result obtained recently upon passage of a complex mixture of amino acids through a column of Dowex-50, a nuclear sulfonic acid polymer. It will be noted that in a single chromatogram it has been possible to obtain individual peaks for all 18 components of the mixture, although the serine-threonine resolution is only partial. The column was 55 cm. in height. The first peaks were eluted with 1.5N HCl, followed by 2.5N and 4.0N acid. It is apparent from this curve that the resin is not functioning exclusively as an ion exchange medium. The separation of the monoamino acids, glycine, alanine, valine, etc., must result from non-ionic adsorption by the resin, which coupled with ion exchange, gives a very useful result.

The investigations with Dowex-50 are still in

their early stages. There is doubtless much yet to learn. For example, it is not known how reproducible the results will be with different batches of the resins. Nevertheless, some of the attributes of Dowex-50 columns warrant mention. In comparison with starch, the effluent from Dowex-50 is clean, the capacity is higher, and the performance of the column is not as sensitive to the presence of inorganic salts. The use of strong HCl (Fig. 8) as an eluent is disadvantageous. Experiments in progress indicate that it may be possible to employ Dowex-50 with a variety of buffered systems which would be much more convenient to use.

One of the most interesting features of the curve in Figure 8, is the relative positions of the various amino acid peaks. The pattern on Dowex-50 is completely different from that obtained with starch. Thus in the aliphatic series of amino acids, glycine, alanine, valine, isoleucine, and leucine, the order of emergence is completely reversed. On starch glycine is last, whereas on Dowex-50, it is first. The other amino acids are also shifted to new relative positions. This reversal of the order arises in large part from the fact that starch is essentially a polar adsorbent, whereas Dowex-50 possesses non-ionic adsorptive properties characteristic of a non-polar adsorbent.

The sequential use of Dowex-50 and starch should yield a fractionating power of an exceptional order. It would be a rare coincidence indeed if two substances showed identical rates of travel on both types of columns. It also follows that the use of starch and Dowex-50 in conjunction should go far in identifying a peak in an effluent curve obtained from plasma, for example. Consider a given peak occurring in the glycine position on starch. If on rechromatographing on Dowex-50 the contents of this peak are recovered quantitatively again in the glycine position, and if, in addition, the peak is resistant to acid hydrolysis, and if, finally, glycine can be added and recovered quantitatively with no loss in symmetry of the peak, the identity of the substance in question would be well established. It is such a combination that may provide identification of components, without the need for isolation, when biochemical materials available in limited supply are being investigated.

Although starch and ion exchange columns used in conjunction appear to offer promise for the future, it is clear that much remains to be done. There is need for a variety of polar and non-polar adsorbents

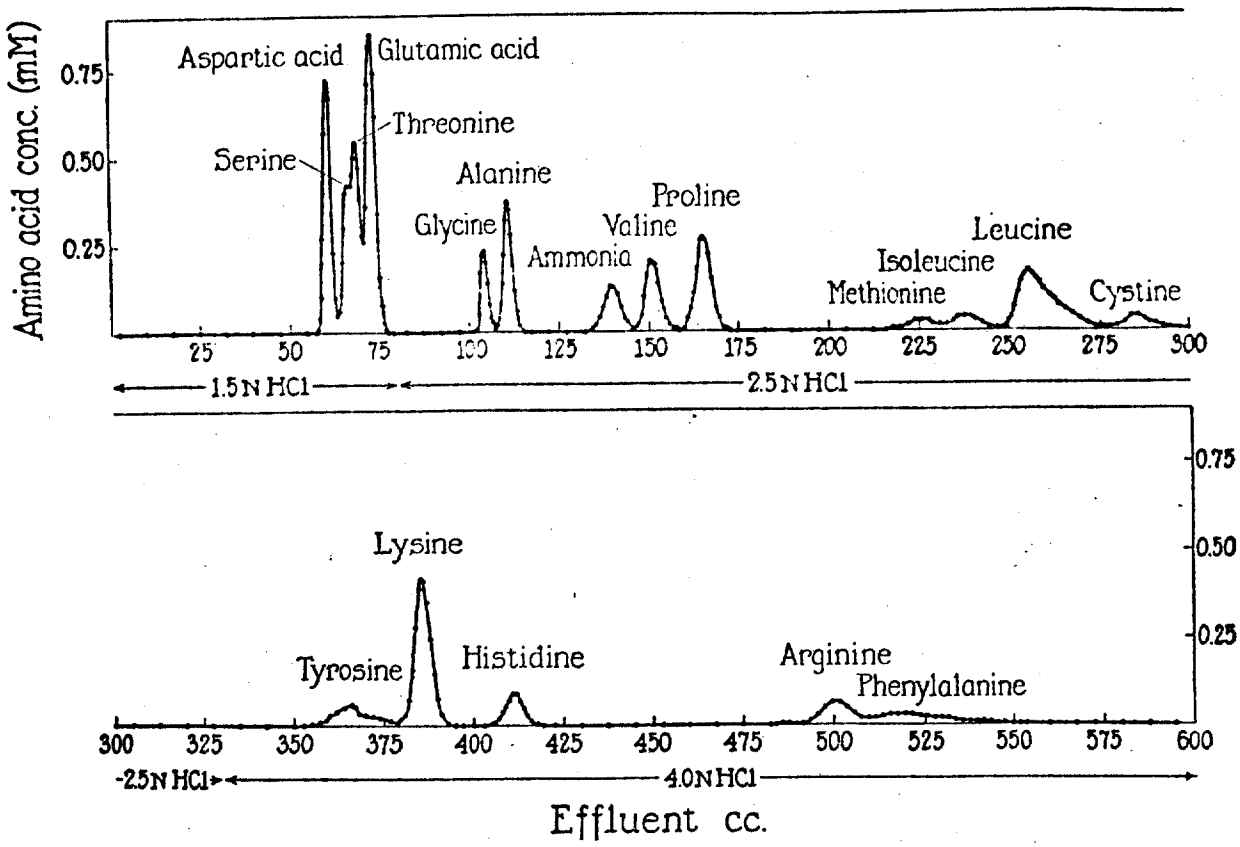

FIG. 8. Fractionation of a known mixture of amino acids on Dowex-50. Solvents, 1.5N, 2.5N, and 4.0N HCl. Column dimensions, 0.9 × 55 cm. Sample, about 3 mg. of amino acids.

that can be utilized in the form of columns of high resolving power. As more information accumulates following the application of these and related techniques to specific problems in the fractionation of mixtures of amino acids and peptides, it is to be hoped that further insight can be gained into some of the architectural mysteries of protein molecules.

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Discussion

Randall: In view of the nature of the processes on which the work of this paper depends it is perhaps rather surprising that more attention has not so far been given to the effect of temperature on the degree of separation of different components of a mixture. This comment can legitimately be applied to starch-resin type columns. It is possible also that the overall efficiency of the method could be improved by a proper choice of temperature.

I understand that promising experiments have been carried out by the Oak Ridge group on the use of resin columns at 80° C. It is clearly a simple experimental matter to provide temperature control of columns and I should like to know the views of the authors on this problem.

Stein: A few preliminary experiments have been carried out which bear upon the question raised by Dr. Randall. Mixtures of amino acids were passed through columns of Dowex-50 maintained at 70° C. Sodium citrate buffers were employed as eluting solvents. From these experiments it appeared that the order in which the amino acids emerged from the column was not markedly altered by the change in temperature from 25° to 70° C, although certain small differences were observed. At the elevated temperature the peaks were notably higher and sharper, however, a finding in accord with the observations of the Oak Ridge group. This definite advantage was offset by the fact that considerable decomposition of certain amino acids was encountered during the course of the experiments performed at 70° C. To minimize decomposition, we currently prefer to carry out chromatographic separations of amino acids at room temperature.

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THE FREE AMINO ACIDS OF HUMAN BLOOD PLASMA

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The presence of amino acid nitrogen in the non-protein nitrogen fraction of blood plasma was first established in 1912 by Van Slyke and Meyer (1). Chemical, microbiological, and chromatographic techniques have subsequently been employed to determine many of the individual amino acids which contribute to the total α-amino nitrogen as currently measured by the ninhydrin-CO_2 method of Hamilton and Van Slyke (2). In the studies reported in this communication, chromatographic methods in which columns of Dowex 50-X4 are employed (3) have been applied to this problem (Fig. 1). It has been possible to identify with a high degree of probability twenty-eight ninhydrin-positive substances in protein-free plasma and to determine most of them quantitatively. The results, taken in conjunction with the values for glutamine obtained by Archibald (4) and Hamilton (5), account for nearly 100 per cent of the total α-amino N of postabsorptive plasma.

EXPERIMENTAL

Unless otherwise specified, 25 or 50 ml. samples of blood were drawn from the cubital vein before breakfast from normal adult males. Each 25 ml. portion was delivered into a 50 ml. centrifuge tube containing 5 mg. of heparin previously dried as a film on the walls. The freshly drawn blood was centrifuged, and the plasma was withdrawn and centrifuged again in a non-heparinized tube. The samples were deproteinized immediately or stored at −20° in plastic vials. It has been found in the present study, however, that changes in the concentrations of some of the amino acids occur if the whole plasma is stored.

The chromatographic analyses were carried out by the Dowex 50-X4 method (3) with columns initially equilibrated at pH 2.2 in order to obtain optimal separation of taurine and urea.

Removal of Proteins—Equilibrium dialysis, ultrafiltration, and precipitation with picric acid were investigated as methods for the deproteinization of the plasma. For each of the dialysis and precipitation experiments, two 10 ml. aliquots of a single sample of plasma were taken. To one aliquot there were added about 2.5 mg. of a synthetic mixture of amino acids (0.05 ml. of a 1:1 dilution of the mixture used in testing the Dowex 50-X4

method (3)). In the ultrafiltration experiments the procedure was similar, but the quantities were halved. After deproteinization, each pair of samples was analyzed on the ion exchange columns and the recoveries of the added amino acids were determined.

The equilibrium dialysis was carried out for 16 hours at 4° according to the procedure of Hamilton and Archibald (6). The dialysate was concentrated to about 6 ml. on a rotary evaporator (7), and the entire sample was placed on the Dowex 50 column for analysis.

Ultrafiltration was carried out for 16 hours at 4° according to the procedure of Prescott and Waelsch (8).[1] After measurement of the volume, the ultrafiltrate was adjusted to pH 2 for chromatography.

Deproteinization with picric acid was effected in general as described by Hamilton and Van Slyke (2). An additional step has been introduced, however, to remove the excess picric acid. For this purpose, a bed of an ion exchange resin is employed under conditions which are known to permit the amino acids to pass through quantitatively. The resin used for this purpose is the strongly basic anion exchanger Dowex 2-X8 in the chloride form.[2] The fines were first removed by settling the resin in water, and the product was thoroughly washed with N HCl. The resin, suspended in water, was poured into a 2 × 20 cm. chromatograph tube, allowed to settle for 5 minutes under gravity, and packed under a pressure of 2 to 5 cm. of mercury. Sufficient resin was used to give a bed 2 × 2 cm. A circle of soft filter paper was placed on the surface of the resin and the bed was washed with 15 ml. of N HCl, followed by water until the effluent was neutral. If not used immediately, the column was stored with a few mm. of water over the resin surface.

The deproteinization of 10 ml. of plasma was carried out in a glass-stoppered flask by the addition of 50 ml. of 1 per cent picric acid solution. After a few seconds of shaking, the suspension was centrifuged for 10 minutes. The supernatant liquid was poured off completely and exactly 50 ml. (corresponding to 8.34 ml. of the original plasma) were passed through the Dowex 2 bed under a pressure of 1 to 4 cm. of mercury. The resin column removed any traces of precipitate remaining in the supernatant solution.

[1] It is a pleasure to acknowledge the cooperation of Dr. H. Waelsch, who placed some of his ultrafiltration equipment at our disposal.

[2] Dowex 2-X8 (200 to 400 mesh) from Technical Service and Development, The Dow Chemical Company, Midland, Michigan. Quantitative tests on columns of the chloride form of the resin have shown that 0.02 N HCl elutes all of the amino acids without retardation, with the exception of tryptophan, which is held back slightly as a result of its affinity for the aromatic groups of the resin phase. Moreover, the amino acid is not fully stable on Dowex 2, the recovery being only about 85 per cent. The fact that the amino acid is also labile on Dowex 50-X4 columns (3) precludes the determination of tryptophan by the present method. The small amount of Dowex 2 used to remove picric acid in each experiment was discarded after use.

The walls of the chromatograph tube and the resin bed were washed with five 3 ml. samples of 0.02 N HCl. The clear, colorless effluent and washings were concentrated on a rotary evaporator to a volume of 5 ml. and a few mg. of Celite (analytical filter aid) were added. The suspension was filtered by gravity through a paper previously washed with N HCl and water, and the filtrate and washings were concentrated to a volume of about 1 ml. The concentrate was washed into a plastic vial, the final volume being 4 to 5 ml., at which point it frequently was convenient to store the tube at −20° overnight. The following day, the solution was brought to pH 7 to 8 (Hydrion paper) by the dropwise addition of N NaOH and allowed to stand at room temperature for 4 hours, conditions which were shown by analysis for —SH groups to convert cysteine (which cannot be determined on Dowex 50-X4) to cystine. The sample was adjusted to pH 2 by the addition of N HCl and the solution was stored at −20° until analyzed.

RESULTS AND DISCUSSION

Deproteinization—Removal of protein by precipitation with picric acid proved to be the most satisfactory of the three methods tested. Difficulty was experienced in obtaining reproducible recoveries of the individual amino acids added to plasma when either equilibrium dialysis or ultrafiltration was employed. Both processes are relatively slow, requiring about 16 hours at 4°, and apparently during this interval secondary changes can take place. In the picric acid procedure, on the other hand, removal of proteins from the plasma can be accomplished within 30 minutes after the blood is drawn. The recoveries of added amino acids were consistently within 10 per cent of theory. Small losses of tryptophan and also of hippuric acid are incurred, however, when Dowex 2 is used for the removal of picric acid.

Interpretation of Effluent Curves—A typical effluent curve obtained from a picric acid filtrate of normal postabsorptive plasma is shown in Fig. 1. Identification of the peaks on the effluent curve was aided by paper chromatography. In a duplicate experiment, alternate fractions were analyzed by the ninhydrin method to locate the peaks. Aliquots from the remaining tubes were desalted on small beds of ion exchange resins and chromatographed on paper in exactly the manner already described in the work with urine (9). Aspartic acid, ethanolamine, and the isomeric methylhistidines, because of their extremely low concentrations, were the only substances in Fig. 1 which could not be subjected to this additional test. For the identification of ornithine, the specific color reaction of Chinard (10) was applied in the manner already outlined (11).

Qualitatively, the picture in Fig. 1 is in general agreement with many of the results obtained previously by microbiological assays, chemical de-

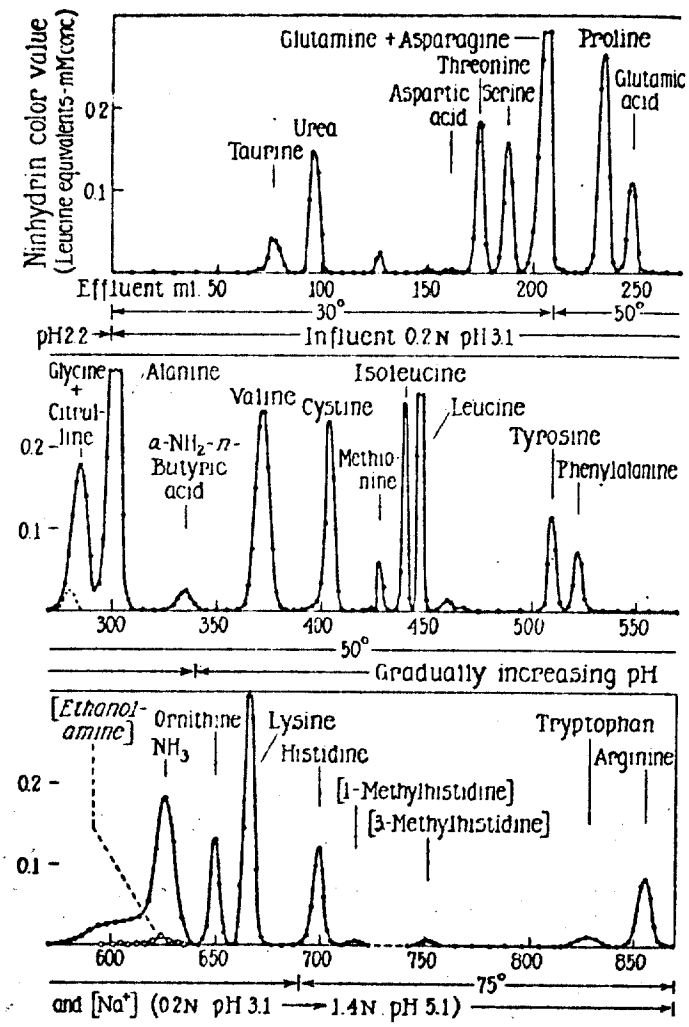

Fig. 1. The ninhydrin-positive components of protein-free human blood plasma obtained from adult males in the postabsorptive state. Chromatography of 8.34 ml. of plasma was carried out on a 150 × 0.9 cm. column of Dowex 50-X4. The terms in brackets refer to substances present in quantities too small to permit paper chromatography to be used as an additional method of identification. The dash curve in the glycine plus citrulline peak represents citrulline. The open circles under the ammonia peak represent the amount of ninhydrin-positive material remaining in the fractions after removal of ammonia. The observed NH_3 is predominantly derived from the decomposition of glutamine during the experiment. The proline and cystine (as half cystine) peaks have been corrected for their relatively low color yields to bring the concentrations (in leucine equivalents) into approximately correct molar proportions relative to those of the other amino acids. The urea peak would require multiplication by about 30 to bring it similarly into scale.

terminations, and paper chromatography. The presence in plasma of taurine and α-amino-*n*-butyric acid has already been revealed by paper chromatography (12, 13). The two compounds have been found in relatively constant amounts in all samples examined by ion exchange chromatography. The existence of asparagine in plasma was detected by Ågren and Nilsson (14) by paper chromatography. The concentration of the compound was determined by Barry (15) employing the Dowex 50-X8 procedure and has been measured in the present study by rechromatography of a sample from the glutamine-asparagine peak (3). The presence of asparagine is not unexpected in view of its occurrence in urine (9)[3] and in tissue extracts (16). The concentration of asparagine in plasma, however, is less than one-tenth that of glutamine. The presence of ornithine in plasma has not been established heretofore. Ågren and Nilsson (14) observed a spot corresponding to ornithine on paper chromatograms of plasma desalted by the procedure of Consden, Gordon, and Martin (17), but this observation was not conclusive proof for the existence of ornithine in plasma, since it has been shown that this amino acid can arise from arginine during the electrolytic desalting procedure (11).

The separation of citrulline from glycine requires a separate experiment in the Dowex 50-X4 method. Archibald (18) demonstrated the presence of citrulline in blood plasma, and its occurrence has been confirmed by rechromatography of a sample from the glycine zone (3). The quantity is indicated by the dotted curve in Fig. 1.

The ammonia peak is primarily an artifact arising as a result of the decomposition of glutamine during the deproteinization and storage of the sample prior to chromatography. The shoulder on the peak comes from ammonia generated from glutamine during the chromatographic process. Identification of the isomeric methylhistidines is not certain because of their very low concentration, but they are the only amino acids known to emerge at the given positions and both are present in relatively large amounts in human urine (9, 19).

The almost complete absence of aspartic acid in plasma and the low concentration of glutamic acid, some of which doubtless results from the decomposition of glutamine, are noteworthy. These two amino acids are absent from freshly voided urine (9), but are found in relatively high concentrations in the extracts of most tissues (16). Even after the ingestion of 50 gm. of casein, the concentration of free aspartic acid is not significantly greater than that shown in Fig. 1. Thus, it is predominantly

[3] In previous studies with normal urine (9), it was not appreciated that the serine + asparagine peak also contained some glutamine, and, therefore, the asparagine values are too high. Experiments performed in connection with the present work have indicated that the asparagine output by a normal adult male is of the order of 20 mg. per day.

the amides of both of these amino acids that are brought to the tissues by the blood stream. These facts may have a bearing on the nausea and vomiting frequently observed to accompany the intravenous administration of protein hydrolysates from which aspartic and glutamic acids have not been removed.

Hydroxyproline has not been found in chromatograms of plasma, indicating that its concentration must be less than 0.2 mg. per cent.

The plasma pattern in Fig. 1 is very different from the curve obtained with urine (9). In urine, taurine is a major component, while the quantities of proline and valine are very low; glycine exceeds alanine in amount and histidine, 1-methylhistidine, and 3-methylhistidine are present to a far greater extent than is lysine. In plasma, the situation is the converse. Moreover, there are only two small unidentified peaks in Fig. 1, one between urea and aspartic acid, the other to the right of leucine, whereas a number of unknown substances render the urine picture less clear-cut.

Quantitative Results—The quantitative data obtained from the analyses of the plasma of five normal adult males are presented in Table I, along with values from the literature. There do not appear to have been previous quantitative determinations of aminobutyric acid, taurine, ornithine, and the 1- and 3-methylhistidines. Since glutamine and tryptophan are not recovered quantitatively from the Dowex 50 columns, the average figures for these amino acids in Table I have been taken from the work of Archibald (4) and Hier and Bergeim (20). When the tryptophan peak in Fig. 1 is corrected for losses, a value similar to the microbiological estimates is obtained. The value for citrulline is that of Archibald (18), with which two chromatographic determinations were in agreement. The glycine values have been corrected for the presence of 0.50 mg. per cent of citrulline in the glycine peak. In almost every instance, agreement can be found between the average results in Table I and one or more of the values from the literature. The variation in the individual values from the literature is frequently considerably greater than that found in the present studies.

The yield of total α-amino N which would be obtained in the ninhydrin-CO_2 method from the individual compounds (taurine excepted) listed in Table I (4.02 mg. per cent) is in close agreement with the average value of 4.1 mg. per cent found by Hamilton and Van Slyke (2). This fact, coupled with the absence of major unknown peaks on the curve shown in Fig. 1, indicates that substantially all of the amino acids of plasma have been accounted for.

The Dowex 50 chromatograms also provide a measure of the urea content of plasma. Since the color yield of urea in the ninhydrin procedure is only 3.14 per cent (35), the peak in Fig. 1 should be multiplied by about 30 to place it on a molar scale comparable to that of the amino acids. The

TABLE I

Free Amino Acids of Human Blood Plasma

Postabsorptive plasma from normal adult males.

Amino acid	Mg. amino acid per 100 ml. plasma						Values from literature*
	Subject A	Subject B	Subject C	Subject D	Subject E	Average	
[Aspartic acid]†	0.03	0.01	0.03	0.02	0.07	0.03	0.8(21)
Asparagine			0.65	0.55	0.54	0.58	1.0–1.4(15), present (14)
Glutamic acid	0.50	0.78	0.43	0.65	1.15	0.70	1.1(8), 3.5(22), 4.4(21)
Glutamine						8.30‡	5.8(22), 7.5(5), 8.8(23), 9.7(8)
Glycine	1.52	1.51	1.61	1.73	1.34	1.54	1.7(24), 1.8(25), 1.1(21), 2.8(26)
Alanine	3.01	3.73	3.51	3.25	3.53	3.41	3.4(24), 4.0(25), 4.2(21), 3.7(27)
Aminobutyric acid	0.33	0.30	0.30	0.22	0.35	0.30	Present(12)
Valine	2.94	2.37	2.84	2.56	3.71	2.88	2.9(20), 2.7(28), 3.4(21), 3.0(29)
Leucine	1.53	1.42	1.65	1.53	2.30	1.69	2.0(20), 1.9(28), 2.4(21), 2.0(29)
Isoleucine	0.76	0.69	0.87	0.87	1.28	0.89	1.7(20), 1.3(28), 1.8(21), 1.5(29)
Serine	1.05	1.20	1.08	1.25	1.01	1.12	1.2(29)
Threonine	1.59	1.21	1.21	1.72	1.21	1.39	2.1(20), 1.7(28), 3.1(21), 1.3(30)
Cysteine + cystine	1.09	1.30	1.20	1.25	1.08	1.18	1.5(28), 1.0(31), 2.0(29)
Methionine	0.33	0.40	0.39	0.36	0.43	0.38	0.52(28), 0.9(21), 0.85(32), 0.29 (29)
Taurine	0.82	0.50	0.56	0.45	0.41	0.55	Present(13)
Proline	3.34	2.17	2.01	2.40	1.84	2.36	2.8(21), 2.9(30)
Phenylalanine	0.76	0.94	0.86	0.69	0.95	0.84	1.4(20), 1.0(33), 1.9(13), 1.6(21)
Tyrosine	0.81	1.11	0.88	0.89	1.45	1.03	1.5(20), 1.0(28), 1.3(21), 1.0(30)
Tryptophan						1.11§	1.1(34), 1.3(28), 1.7(21)
Histidine	1.08	1.48	1.15	0.79	1.23	1.15	1.4(20), 2.1(21), 1.4(30), 1.0(29)
[1-Methylhistidine]†	0.17	0.12	0.04			0.11	
[3-Methylhistidine]†	0.04	0.13	0.07	0.10	0.07	0.08	
Ornithine	0.76	0.62	0.73	0.80	0.70	0.72	
Lysine	3.02	2.51	2.77	2.55	2.73	2.72	3.0(20), 2.2(28), 2.6(30), 2.8(29)
Arginine	1.93	1.64	1.34	1.22	1.43	1.51	2.3(20), 1.6(28), 1.5(30), 2.5(29)
Citrulline						0.50‖	0.50(18), present(14)
Total						37.07	
Calculated yield of α-NH_2-N in ninhydrin-CO_2 method						4.02	
Average α-NH_2-N found by Hamilton and Van Slyke (2)						4.1	

* The values in reference (26, 27, 29) were obtained with serum.

† These compounds were present in too low a concentration to permit unequivocal identification.

‡ Archibald (4).

§ Hier and Bergeim (20).

‖ Archibald (18); chromatographic determinations on two plasmas, 0.45 and 0.48 mg. per cent of citrulline.

plasma urea values for the individuals referred to in Table I were 27, 42, 31, 28, and 30 mg. per cent, all within the normal range. Approximate ethanolamine values of from 0.05 to 0.20 mg. per cent were also obtained in these studies.

In one case, an opportunity was afforded to compare the values obtained with plasma stored for 7 months at −20° with those secured when the plasma was deproteinized and analyzed immediately. With the exception of aspartic acid, glutamic acid, and cystine, the values for all of the amino acids in the fresh and the stored frozen plasma agreed within the experimental error. Upon storage, there was a slight increase in the quantity of aspartic acid and a large increase in the amount of glutamic acid, both doubtless a result of the decomposition of the respective amides. No cystine was found in the stored plasma. If, as seems likely (Fujita and Numata (36)), cysteine rather than cystine is present in blood and tissues, the oxidative formation of intermolecular bridges involving cysteine and the —SH groups of proteins would explain the absence of cystine in the stored plasma. In freshly deproteinized plasma, on the other hand, cysteine-protein linkages are not given an opportunity to form, and oxidation by air of the protein-free filtrates leads only to the formation of cystine. Irrespective of the mechanism, the fact that the apparent cystine or cysteine content decreases with time emphasizes the need for prompt deproteinization of plasma whenever this amino acid is to be determined.

Conjugated Amino Acids—The absence of major quantities of conjugated amino acids in plasma is well established. In recent experiments, Christensen (37) and Dent and Schilling (38) found only a very slight rise in total α-amino N after acid hydrolysis of the deproteinized plasma of the dog. The results of studies showing in detail the effect upon the quantity of each amino acid brought about by the hydrolysis of protein-free plasma are given in Table II. For a fasting subject (E), hydrolysis produces no significant changes in the concentration of most of the amino acids. Only aspartic acid, glutamic acid, glycine, and possibly alanine, serine, and threonine are increased in amount by hydrolysis. Most of the aspartic acid arises from asparagine, and the glutamic acid formed could all come from glutamine. There is evidence for the existence of a small quantity of conjugated glycine in plasma, probably in the form of hippuric acid.

In the absence of conjugated forms of serine and threonine, the values for these two amino acids might be expected to decrease as a result of decomposition during hydrolysis. Actually there is a slight rise, indicating traces of bound forms. There is unequivocal evidence for the decomposition or alteration during hydrolysis of cystine, methionine, proline, and tyrosine. Cystine seems to be converted largely to cysteic acid, and methionine partially to the sulfoxide. The fact that the amount of material in

TABLE II

Amino Acid Content of Protein-Free Plasma after Acid Hydrolysis

The hydrolysis was carried out at 110° for 16 hours in 10 ml. of 6 N HCl. The samples corresponded to the concentrated protein-free filtrate from 8.34 ml. of plasma. For the "post-protein" sample, blood was drawn 2 hours after the ingestion of 50 gm. of casein as Protinal (a 2:1 casein-carbohydrate mixture, The National Drug Company, Philadelphia).

Amino acid	Mg. amino acid per 100 ml. plasma			
	Total after hydrolysis		Increase after hydrolysis	
	Subject E, fasting	Subject F, post-protein	Subject E, fasting	Subject F, post-protein
Aspartic acid*	0.73	1.19	0.66	1.07
Glutamic "	9.48	14.15	8.33	12.35
Glycine†	1.83	1.84	0.28	0.27
Alanine	3.62	4.80	0.09	0.16
Aminobutyric acid	0.40	0.19	0.05	−0.05
Valine	3.72	6.50	0.01	0.12
Leucine	2.23	5.10	−0.07	0.02
Isoleucine	1.37	2.98	0.09	−0.05
Serine	1.06	1.81	0.05	0.01
Threonine	1.26	2.29	0.05	0.03
Cysteine + cystine	<0.03	<0.03	−1.08	−1.49
Cysteic acid‡	1.98	<0.03	1.98	<0.03
Methionine§	0.08	0.45	−0.35	−0.34
Taurine	0.42	0.36	0.01	−0.07
Proline	1.69	5.53	−0.15	−0.20
Phenylalanine	0.97	1.50	0.02	−0.05
Tyrosine	0.98	2.31	−0.47	−0.72
Histidine	1.17	1.72	−0.06	0.12
Ornithine	0.71	1.34	0.01	0.12
Lysine	2.75	5.82	0.02	0.32
Arginine	1.43	2.42	0.00	0.12

* The asparagine content of the plasma before hydrolysis was 0.54 mg. per cent for Subject E and 0.89 mg. per cent for Subject F.

† Not corrected for the presence of citrulline.

‡ The 1.08 mg. per cent of cystine present before hydrolysis in the plasma of Subject E could give rise to a maximum of 1.51 mg. per cent of cysteic acid. The plasma of Subject F stood for 4 months in the deep freeze before being deproteinized and analyzed, and hence contained no free cystine (see the text).

§ In the chromatograms of the hydrolysates, two small peaks appeared ahead of aspartic acid at the positions of the isomeric methionine sulfoxides (3). Assuming that these peaks are the sulfoxides, virtually all of the methionine present before hydrolysis can be accounted for in the hydrolysates as methionine + methionine sulfoxides.

the aminobutyric acid peak is essentially unchanged by hydrolysis eliminates the possibility that the peak might contain glutathione, which also emerges at this position (3). The data on Subject E in Table II confirm the view that, if conjugated amino acids, including peptides, are present in the venous blood of a fasting individual, the amount is extremely small.

A consideration of the data obtained with Subject F (Table II) shows that, even after a meal rich in protein, major quantities of conjugated amino acids do not appear in the plasma. The rise in free amino acids in the plasma of this individual after the protein meal has already been reported (*cf.* (39), Table VI, normal). There is some evidence for the presence of a detectable amount of conjugated lysine, and, as is to be expected, the concentration of asparagine and glutamine rises after a meal. Otherwise, the picture is similar to that observed with the fasting subject.

The possibility still remains that certain forms of conjugated amino acids of unknown nature might exist in plasma and be removed during the deproteinization procedure, either by being bound to the protein-picric acid precipitate or to the Dowex 2 column. Simple peptides and most simple conjugates now known to exist in tissues or urine would be expected to survive the picric acid deproteinization procedure.

Whole Blood—In one instance, the amino acid composition of whole blood was also determined. For this purpose, 5 ml. of blood were laked with 5 ml. of water prior to deproteinization. From the hematocrit and the amino acid composition of the plasma, the amino acid composition of the formed elements of the blood was calculated. The cells were characterized by a high content of glutathione (about 50 mg. per 100 ml. of cells), comparable to that found in tissues. Relative to plasma, the cells contained more taurine, aspartic and glutamic acids, glycine, and ornithine, while the amounts of valine and cystine were less. For the remaining amino acids, the concentrations in the cells and the plasma were quite similar.

Additional Analyses—Examinations have been made of the concentrations of amino acids in the plasma of fasting patients suffering from a number of diseases known or suspected to be associated with aberrations in amino acid metabolism. The results of investigations of patients with Wilson's disease have already been reported (39). In a single cystinuric patient, the concentrations of cystine (0.54 mg. per cent), arginine (0.89 mg. per cent), and alanine (2.58 mg. per cent) were slightly below normal, the concentration of glycine was elevated (2.56 mg. per cent), while the levels of all the other amino acids, including lysine, ornithine, and citrulline, were in the normal range. It would appear, therefore, that the increased excretion of cystine, lysine, arginine, and ornithine (31, 40) by this cystinuric was not a reflection of high blood levels of these amino acids.

In a case of phenylpyruvic oligophrenia, the concentration of phenylalanine in the plasma was 23.5 mg. per cent and the urinary excretion was 334 mg. per day, markedly high values in agreement with previous findings (33, 41–43). All of the other amino acids in the blood and urine were at or near the normal range. In two patients with schizophrenia, one with cirrhosis of the liver, one with tuberculosis, and one with muscular dystrophy, the levels of the amino acids in both the plasma and the urine were in or near the normal range.[4]

The authors wish to acknowledge the technical assistance of Mrs. Gertrude C. Carey, Miss Renate Mikk, and Miss Joyce F. Scheer in the performance of these experiments.

SUMMARY

Deproteinized human blood plasma contains twenty-eight ninhydrin-positive compounds identifiable by chromatography on columns of Dowex 50-X4. The procedure provides quantitative determinations of all of the constituents, except glutamine. The results, taken in conjunction with the previous values for glutamine obtained by Hamilton and Archibald, account for 95 to 100 per cent of the individual amino acids which contribute to the total α-amino N of postabsorptive plasma (4.1 mg. per cent). In agreement with previous investigations, no detectable quantities of peptides have been found in venous plasma, either in the fasting state or after a protein meal.

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[4] The studies on patients were possible through the generous cooperation of Dr. G. A. Jervis of Letchworth Village, Professor Oskar Diethelm and Dr. D. C. Greaves of Cornell University Medical College, Dr. R. E. Weston of the Montefiore Hospital, and Dr. A. G. Bearn and Dr. J. G. Hirsch of the Hospital of the Rockefeller Institute.

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The attached case history from Dr. M.G. Stemmerman concerns a case reported in a paper quoted in the monograph. This paper, "Occult Mechanisms of Brain Damage," was delivered by Dr. John W. Olney at a recent (December 1972) symposium on drugs and developing brain. Dr. Stemmerman has been most cooperative in giving permission to quote his case and in sending a brief case history for inclusion in the monograph. A more detailed report is available from him upon request.

OWEN CLINIC INSTITUTE, INC.
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TELEPHONE 525-6361

NAME: D
BIRTH DATE: 4.2.71

PRESENT ILLNESS On October 14, 1971 (six months) D developed a fever, followed by a rash, and a few days later some sort of seizure. She was hospitalized briefly. EEG blood electrolytes and skull x-rays were normal. SGOT was elevated (140) and since no cause was found believed to be a laboratory error. Her pediatrician placed her on phenobarbital to which mysoline and Dilantin were later added. These had no affect on attacks that continued, 20-30 during a 2-6 hour period each day, usually the afternoon or early evening.

FAMILY HISTORY. Two older male siblings (4 & 5 yr.) are normal and have never had problems similar to the patient's. A young cousin has some type of seizure, type not known.

EXAMINATION. Several seizures were observed during a 15 minute period. The child was holding a doughnut and at no time dropped it. The mouth opened with slight sagging on the right. There was a "shudder" involving shoulders and trunk. The entire episode was one second in duration. Attacks occurred spontaneously and could not be activated by sensory stimulation of any kind. The mother stated all attacks ceased during sleep.

HOSPITALIZATION. Complete work-up at Children's Hospital, Columbus, Ohio, including EEG, brain scan and ventriculogram, revealed no abnormalities. Final Diagnosis was "Seizure Disorder," cause undetermined. It is of interest that several seizures were observed on the first day of hospitalization but none thereafter. Diet consisted of baby foods and milk only.

COURSE. Despite adequate doses of phenobarbital and dephenylhydantoin, with a trial of pyrodoxine, seizures continued, usually in clusters in the afternoon and evening, usually too numerous to count.

Her mother was asked to keep a list of all foods and drink taken. This list suggested that the "clusters" were

more frequent after a meal of hot dogs or spaghetti (both favorites and consumed in large quantities) or other foods containing MSG. The mother was alerted and eliminated all MSG containing foods from the diet. Seizures ceased within less than a week. Anti-convulsants were gradually withdrawn by 11 months of age, five months after onset of seizures.

Following each of the now rare attacks, an offending food has been discovered, usually given to D by a relative or neighbor. The last "clusters" in the meddle of August required considerable detective work and was finally traced to a family hog that was butchered and packaged commercially. D ate large amounts of the sausage that were said to contain only the usual spices and "flavorings". The plant finally admitted the latter was MSG, after the mother insisted that she needed a list of every ingredient.

DEVELOPMENTAL HISTORY. All landmarks were achieved early. Her first tooth appeared at 2½ months and by 5-6 months some of her diet consisted of adult foods, especially hot dogs and spaghetti. Vineland Social Maturity scale yielded a social quotient of 92 at 9 months of age. Sentences began at 17 months.

SUMMARY

4.2.71. Born
10.14.71 (6 months). First seizures. Phenobarbital started.

11.6.71 (7 months). Seizures continue. Dilantin added.

12.30.71 (8 months). Seizures continue. Mysoline and oyridoxine added.

1.27.72 (9 months). Seizures continue. Complete neurosurgical work-up negative.

2.15.72 (10 months). All MSG containing foods eliminated from the diet.

2.20.72 (10½ months). No seizures for past 3 days, first free period since onset. Reduction of anti-convulsants begun.

3.20.72 (11½ months). Off all anti-convulsants. No seizures

5.10.72 (13 months). Few attacks 2-3 hours after surreptitious ingestion of pizza.

August, 1972 (17 months) Several attacks after ingestion locally prepared sausage, later found to contain MSG.

February, 1973. No attacks in 7 months.

M. G. Stemmermann, M.D.

SHORT COMMUNICATION

Permeability of the blood–brain barrier to monosodium glutamate and effects on the components of the energy reserve in newborn mouse brain[1]

(*Received* 20 *October* 1970. *Accepted* 5 *May* 1971)

ALTHOUGH the permeability of the blood–brain barrier to glutamic acid in terms of net increase after glutamate injection has been described for the developing rat (HIMWICH and HIMWICH, 1955), there have been no such studies in newborn mice. In view of the current reports of acute regional neuronal necrosis (inner retinal layers and hypothalamus) after the injection of large doses of glutamate into newborn mice (LUCAS and NEWHOUSE, 1957; COHEN, 1967); OLNEY, 1969*a*; 1969*b*), an investigation of barrier permeability seemed highly relevant. Although the histological lesions produced by glutamate in newborns are regional, the effects of glutamate on cerebral metabolism might not be so sharply localized and hence might be detected in an examination of whole brain. Such is certainly the case in pyrithiamine-induced thiamine deficiency, in which pathological lesions are quite localized and yet striking biochemical deviations are seen in whole brain (HOLOWACH, KAUFFMAN, IKOSSI, THOMAS and MCDOUGAL, 1968). Therefore, we have studied the effect of glutamate on the components of the energy reserve and on other selected metabolites of the newborn mouse brain.

METHODS

Preparation and observation of animals. Six litters of Swiss Webster albino mice (total 60 animals) were used in this study. Although the exact ages of the mice were not known, all of them were less than 24 h old and weighed 0·9–1·6 g. In the four litters used for biochemical studies of the effects of glutamate on brain, one-half of each litter served as control. The dose of monosodium L-glutamate (Sigma Chemical Co., St. Louis, Mo.) was 2 g (11·8 mmol)/kg injected subcutaneously into the flank in a volume of 40–60 ml of water/kg. Controls received an equivalent volume of 0·15 M-NaCl. In these small animals it was impossible to use the intraperitoneal route of injection because of unavoidable leakage of the injected solution. For experimental intervals shorter than 1 h, animals were kept outside the cage under a desk light (25°C) and were stimulated frequently by gentle stroking or moving the body. In longer experiments, animals were returned to the mother for care and nursing. Animals were killed by plunging the whole animal or the amputated head into liquid nitrogen. After decapitation, blood was collected from the severed neck vessels in chilled heparinized capillary tubes. Brain was sampled at two intervals after injection. The 30 min interval was chosen so that a comparison could be made with the study by HIMWICH and HIMWICH (1955) of the blood–brain barrier to glutamate in newborn rats, despite the different routes of injection (subcutaneous vs. intraperitoneal). Brain was also sampled 3 h after injection of glutamate because at this time lesions in the retina and brain are reported to be far advanced (OLNEY, 1969).

Immediately after injection, one or two mice from each litter appeared seriously ill and exhibited cyanosis. Some of these mice survived with stimulation, others died. The mortality rate in glutamate-injected mice was twice that of controls. All animals which exhibited any signs of distress were excluded from the study. The mice which had been returned to their mothers for intervals of 1–3 h after injection appeared healthy at these times, and the clinical behaviour of the control and glutamate-injected mice was indistinguishable.

Preparation of blood and tissue extracts and methods of assay for metabolites. Prior to dissection all tissues were kept at −80°C. Dissections were performed in a cryostat at −35°C. As much as possible of the brain anterior to the cerebellum was removed. The entire sample was powdered and portions were weighed at −20°C. Tissue extracts were prepared according to the procedure of LOWRY, PASSONNEAU, HASSELBERGER and SCHULZ, (1964) and were stored at −80°C until the time of assay.

For the determination of the concentration of blood glutamate, samples of heparinized blood were

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added to a 10- to 20-fold vol. of 0·5 M-perchloric acid and centrifuged at 4°C. The supernatant fluid was removed and stored at −20°C until the time of assay.

All metabolites were measured fluorometrically by specific enzymic methods. ATP, P-creatine and glucose were assayed by the methods of LOWRY *et al.*, (1964). Lactate and glycogen were measured by the methods of GATFIELD, LOWRY, SCHULZ and PASSONNEAU (1966). The method of assay for glutamate in blood and brain was that of YOUNG and LOWRY (1966), with slight modifications (1·6 mM-NAD^+ was used instead of acetyl-NAD^+, and the concentration of glutamic dehydrogenase in glycerol was 50 μg/ml of reagent.) In the determination of blood concentrations of glutamate, the addition of portions of the perchloric acid extracts of blood in the volume required for a satisfactory assay in control samples (50–80 μl/ml of reagent) did not produce a change in the pH of the reagent buffer (0·1 M-tris-HCl, pH 8·73) or affect the complete recovery of glutamate standards. Therefore, no preliminary neutralization of the extracts was made. γ-Aminobutyric acid was measured by the method of HIRSCH and ROBINS (1962).

Calculation of the cerebral energy reserve as high-energy phosphate (~P). The energy reserve of the brain which would be available in the event of total ischaemia was calculated, following the example of LOWRY *et al.* (1964), as one equivalent of high-energy phosphate for each mole of P-creatine, two for each mole of ATP and glucose and 2·9 equivalents for each glucose equivalent of glycogen. (ADP was not measured so that the small contribution which it would make to this reserve could not be included.)

Reagents (*enzymes*). Phosphorylase-*a* (α-1,4-glucan:orthophosphate glucosyltransferase; EC 2.4.1.1) was purchased from Sigma Chemical Co. (St. Louis, Mo.); ox heart lactate dehydrogenase (L-lactate:NAD oxidoreductase; EC 1.1.1.27) was from Worthington Biochemical Corp. (Freehold, N.J.); and Gabase (a partially purified mixture of 4-aminobutyrate:2-oxoglutarate aminotransferase EC 2.6.1.19, and succinate semialdehyde:NAD (P) oxidoreductase EC 1.2.1.16 from *Pseudomonas fluorescens*) was obtained from International Mines and Chemical Corp. (Wasco, Calif.) All other enzymes were obtained from Boehringer and Sons through CalBiochem (Berkeley, Calif.).

RESULTS

Blood levels of glutamate were measured from 30 min to 3 h after injection of 2 g (11·8 mmol) of monosodium L-glutamate per kg body wt subcutaneously (Fig. 1). In control animals glutamate levels in blood sampled from 30 min to 2 h did not change with time, and results were pooled. At 30 min after injection of glutamate the concentration of glutamate in blood was 100-fold higher than the control value (12·2 mM ± 1·07 *vs.* 0·127 mM ± 0·008). In older animals (at 5 days of postnatal age) injected subcutaneously with one-half of this dose, peak levels 40-fold greater than the control value were reached as early as 5 min after injection. As plotted in Fig. 1, it cannot be clearly appreciated that

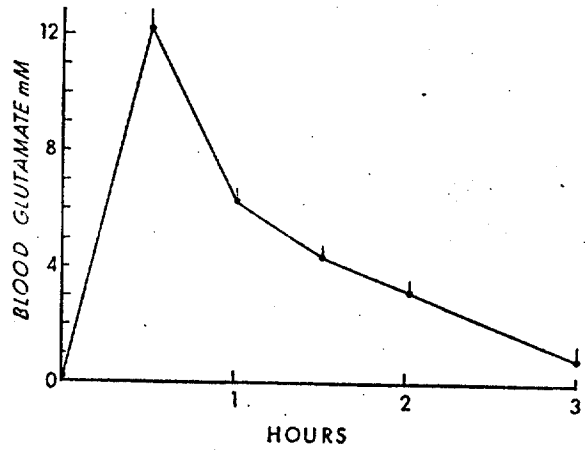

FIG. 1.—Levels of glutamate in the blood of newborn mice at various intervals after the injection of monosodium L-glutamate [2 g (11·8 mmol)/kg subcutaneously]. Each point represents the average of three or four individual blood samples; vertical lines represent ± 1 S.E.M. Control values for blood glutamate at intervals up to 2 h for 13 saline-injected mice did not differ with time and the results were pooled to give a mean value of 0·127 ± 0·008 mM (indicated on the ordinate at zero time.)

even at 3 h after the glutamate injection, the blood concentrations of glutamate were still nearly seven-fold higher than the control value (0·85 ± 0·55 mM *vs.* 0·127 ± 0·008 mM for controls). From the regression curve (Fig. 1), the half-life of glutamate in the blood of newborn mice after the injection was calculated to be 48·5 min, under the conditions specified.

Levels of glutamate were measured in the brains of control and glutamate-injected animals from four litters (Table 1). In about one-half of these animals blood and brain levels of glutamate were obtained for the same animal. Despite the marked elevation of blood levels of glutamate at 30 min post-injection, there was no significant increase in brain content of glutamate at this time. This finding differs from that reported in newborn rats (HIMWICH and HIMWICH, 1955). For an equivalent dose of glutamate these investigators found that at 30 min after injection, brain glutamate had increased 35 per cent. This discrepancy may reflect a species difference, or it could be a consequence of

TABLE 1.—EFFECT OF INJECTED GLUTAMATE ON GLUTAMATE LEVELS IN BRAINS OF NEWBORN MICE

Control	+ Glutamate	*P* value
(30 min post-inj.)		
4·30 ± 0·21 (7)	4·66 ± 0·14 (9)	0·182
(3 h post-inj.)		
4·22 ± 0·03 (9)	4·62 ± 0·08 (10)	< 0·001

Four litters of mice were used in this study, two for the 30-min interval, two for the 3 h experiment. One-half of each litter served as controls. The numbers of control and gluatamate-injected mice are given in parentheses.

Values represent means ± S.E.M.

the different routes of injection (the neonatal rats were injected intraperitoneally, whereas our mice were injected subcutaneously), but there are other differences. In the rats the blood level of glutamate at 30 min after injection was 18·3 mM, whereas in our mice, the blood level of glutamate at 30 min was 12·1 mM. However, the control value of glutamate in the brains of newborn rats was also much higher (6·3 mmol/kg) than in our animals (4·3 mmol/kg), so that direct comparisons cannot be made. Nevertheless, at 3 h after injection the small increase (9 per cent) in brain levels of glutamate in our mice was statistically highly significant ($P < 0·001$). Possibly if brains had been sampled at intervals other than 30 min and 3 h, larger elevations of brain glutamate might have been seen.

Because glutamate might be metabolized so quickly that no marked increase could be expected, the related metabolites α-ketoglutarate and GABA were also measured (Table 2). No differences between

TABLE 2.—EFFECT OF INJECTED GLUTAMATE ON LEVELS OF SELECTED METABOLITES IN BRAINS OF NEWBORN MICE

Metabolite	(mmol/kg) Control (N = 16)	+Glutamate (N = 19)
ATP	2·76 ± 0·08	2·74 ± 0·02
P-Creatine	3·19 ± 0·1	3·27 ± 0·07
Glucose	1·34 ± 0·04	1·34 ± 0·04
Glycogen	2·32 ± 0·19	2·02 ± 0·07
Lactate	1·66 ± 0·07	1·79 ± 0·10
α-Ketoglutarate	0·104 ± 0·007	0·109 ± 0·002
GABA	1·01 ± 0·05	1·07 ± 0·03
~ P	18·11 ± 0·73	16·66 ± 0·32

Four litters of newborn mice were used in this study. One-half of each litter served as controls. Animals were treated as described in methods. Biochemical findings at 30 min and 3 h after injection did not differ in either the control or glutamate-injected mice and results at these two times were pooled. Glutamate levels in the brains of these same animals are shown in Table 1. ~ P was calculated as described in Methods. All values represent the means ± S.E.M. There were no statistically significant differences between control and glutamate-injected mice in the levels of any of the metabolites shown.

experimental and control animals were found for these two substances. The effects of the injection of glutamate on the major components of the energy reserve of the brain and other selected metabolites are also summarized in Table 2. The injection of glutamate had no effect on any of the metabolites measured.

DISCUSSION

We think it unlikely that the small increase in brain levels of glutamate after the injection of glutamate into newborn mice or even the larger increase reported by HIMWICH and HIMWICH (1955) in newborn rats could account for the devastating pathological effects reported in the literature. Certainly no effects of this modest increase could be seen on any of the important components of the energy reserve of the brain. It is possible, however, that the inner layers of the retina and the hypothalamus could be particularly susceptible to any increased concentration of glutamate and might exhibit significant deviations if metabolites were measured at these sites.

Another possibility is that these tissue regions (inner retina and hypothalamus) are more permeable to glutamate than other areas of the brain. In view of the negative findings on whole brain, a biochemical investigation of the specific areas involved seems clearly indicated. Moreover, since the lesions in the eye and brain may be secondary to changes elsewhere in the body as a result of glutamate excess (adrenal, liver, kidney, etc.), a biochemical study of these extracranial tissues might be rewarding in this unsolved problem.

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sentence." But to have down in print what we presently know and what we presently think of monosodium glutamate will be of great usefulness. Glutamate has been the center of a great deal of attention in recent years—and a part of that attention has been of Armed Forces origin.

In concluding my brief remarks let me express the appreciation of the Institute for your presence here today and for the contributions that, by virtue of your past and current investigations, I know you will be making.

History of the Glutamate Industry

DONALD K. TRESSLER
Scientific Director, Quartermaster Food and Container Institute for the Armed Forces, Chicago, Illinois

Background of early interest. During World War I, the supply of potassium salts, which had been imported in large quantity, was first greatly reduced and finally completely cut off. Because of this, an intensive search for other sources of potash was carried out in the United States. It had long been recognized that desugarized beet molasses and distillery slops were relatively high in potassium salts. In Germany, beet molasses, desugarized by the strontium process, had been concentrated and then destructively distilled, since the end of the 19th century. The data available indicated that about 5,000 tons of potassium cyanide, 5,000 tons of ammonium sulfate and large quantities of potassium salts were produced annually from this source. Consequently, the utilization of Steffen's waste for the production of potassium salts in the United States was proposed.

During the beet slicing campaign of 1917, the Great Western Sugar Co. had impounded the dilute Steffen filtrate produced at its Fort Collins, Colorado, factory. Following the slicing operations, this was concentrated to produce some 5,000 tons of concentrated Steffen's filtrate (62%–65% dry substance) and a specially-designed furnace, suitable for reducing this product to an ash, was constructed. This furnace utilized the combustible products in the concentrated Steffens filtrate, supplemented by small amounts of coal, for evaporating the residual water in the concentrated Steffen's filtrate. It produced an ash containing some 40% of potash.

A large quantity of Steffen's waste water was also concentrated by evaporation in the American Beet Sugar Company's Mason City, Iowa, plant to a concentration of 81.5% solids (sp. gr. 1.41),

and stored in large steel tanks. From one ton of this raw material, approximately 320 lbs. of crude potassium chloride (70% potassium chloride) could be obtained. However, before the production of potash got under way, World War I ended, and the price of potash salts dropped so low that their manufacture from this concentrated waste did not appear to be inviting.

In many beet sugar factories the disposal of Steffen's waste water had been a problem for many years. If the waste from a midwestern sugar plant was run into a river or creek, the water in the sluggish streams became foul and the fish and other aquatic life died. The citizens of Mason City, Iowa, objected very strenuously to this pollution by the American Beet Sugar Company, and passed an ordinance forbidding it. The company, therefore, impounded the waste in large ponds, which held it during the autumn and winter. However, when spring floods came, the impounded waste would break the earthen dams holding it and escape into the swollen streams. This "accidental" escape of the waste usually caused considerable argument.

Partly because of the ever-increasing difficulty of disposing of the Steffen's waste, and partly because of the very large quantity of the concentrated waste held in the large storage tanks, for which there was no practical use, Mr. James E. Larrowe, President of the Larrowe Milling Company, a large manufacturer of animal feeds, approached the Mellon Institute of Industrial Research, Pittsburgh, Pa., in 1923, asking for assistance in finding some use for the waste.

Previously, an unfortunate attempt had been made to use the concentrated waste to replace alcohol as an antifreeze in automobile radiators. In the winter of 1920, a construction engineer noted that the Steffen's waste concentrate (81.5% solids) in the large tanks did not freeze even when temperature dropped to —30° F. Shortly thereafter, certain individuals began to market as an antifreeze the concentrated waste without consideration of the corrosive properties. This business lasted only a month or two, because the users soon found that, although the concentrated waste did not freeze, it severely damaged the motors of the cars in which it was used. Mr. Larrowe put some in the radiator of his own new Packard car and nearly ruined the motor.

Larrowe's original request was for some one to determine how the concentrated waste could be modified so that it would not affect the motor gaskets and connections and retard or eliminate its corrosive action. Mr. Larrowe offered to support an Industrial Fellowship to carry out the study. When the Industrial Fellowship was offered to the writer, he pointed out that the high concentration of potassium, sodium and amine salts would be certain to be

very corrosive, and that chances of success in making concentrated Steffen's waste into a satisfactory antifreeze were very slim; however, he felt that it was highly probable that a number of valuable products could be separated from it, and agreed to accept the fellowship provided he were given a free hand to determine what useful products could be produced therefrom. With this understanding, he accepted the fellowship and started work on the problem on December 1, 1923.

The work at the Mellon Institute. During the first year's work on this fellowship, the dry matter of the concentrated Steffen's waste was determined to contain relatively large amounts of potassium and sodium salts, betaine and a compound of glutamic acid. The conditions necessary for the liberation of glutamic acid from the glutamic acid compound and a process, by which a high yield of betaine hydrochloride, glutamic acid hydrochloride and crude potassium salts could be obtained, were determined.

Maximum yields of these products obtained from the Mason City concentrated Steffen's waste were as follows:

	Grams/Kg. 1.41 sp. gr. Waste	% of Total Solids
Crude potassium chloride	211.9	26.0
Betaine hydrochloride	142.6	17.5
Crude glutamic acid hydrochloride	134.6	16.5
(Equivalent to glutamic acid of 92% purity	107.9	13.2)

Based on these observations, patents covering the acid hydrolysis and production of betaine hydrochloride and glutamic acid from concentrated Steffen's waste were obtained in the United States, Canada, Germany, and Japan.

In the second year of the fellowship, the writer consulted Professor Elmer V. McCollum, the famous vitamin chemist of Johns Hopkins University, in an effort to learn of possible uses for either glutamic acid or betaine hydrochloride. Dr. McCollum advised the Fellow that monosodium glutamate was used by the Japanese as a condiment. A sample of the Japanese product, Aji-no-moto, obtained by Professor C. A. L. Schmidt of the University of California, was analyzed and found to be nearly pure monosodium glutamate. On March 21, 1925, the Fellow wrote to S. Suzuki and Company of Tokyo, Japan, who manufacture Aji-no-moto from wheat gluten, inquiring if that company would be interested in purchasing monosodium glutamate. Mr. C. Suzuki replied indicating interest and in company with his son and Dr. K. Ikeda and Mr. Domen came to this country to negotiate for the purchase of glutamic acid (Jan. 19 to Feb. 5, 1926). On the latter day, the Larrowe-Suzuki Company was formed. The Larrowe Construction

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Company subscribed for 60% and the S. Suzuki and Company 40% of the capital required for the new company. S. Suzuki and Company agreed to buy 400,000 lbs. of glutamic acid during the first year of operation. That company agreed to buy somewhat larger amounts for each of the succeeding four years.

Plans were immediately made for the construction of a small pilot plant and then a commercial-scale plant at Rossford, Ohio. The fellowship was enlarged by employing two junior fellows, Mr. Max Donauer, a chemical engineer, and Dr. C. L. Butler, an organic chemist, and an assistant, Mr. James Kammerer. Mr. Donauer aided in the construction of the small pilot plant on the top floor of the Mellon Institute and then proceeded with the design of the commercial-scale plant.

The study of the utilization of betaine hydrochloride and other by-products was also pushed. Procedures were worked out for the production of (1) trimethylamine, (2) triethylamine oxide hydrate, (3) methyl chloride, (4) dimethylamine, and (5) dimethylaminoacetic acid from this hydrochloride. Methods were also worked out for the preparation of amine mixtures by the destructive distillation of the waste mother liquor left after the removal of the glutamic acid hydrochloride. Betaine, betaine phosphate, tartrate, citrate, acetate, malate, and sulfate were prepared and studied. The pH of betaine hydrochloride solutions of normality varying from 0.0001 to 0.5 were determined. Procedures for the preparation of pyrrolidone carboxylic hydroxyglutaric and glutaconic acids from glutamic acid were developed.

The use of monosodium glutamate in sauces and for seasoning various foods was studied.

An analysis of fourteen samples of concentrated Steffen's waste obtained from various beet sugar plants indicated a glutamic acid content varying from a minimum of 3.5% to a maximum of 11.5%.

Another important study carried out by this fellowship was the conversion of crude carbon obtained during the manufacture of glutamic acid into activated carbon suitable for decolorizing sugar and other solutions.

Shortly after the Larrowe Construction Company-S. Suzuki and Co. contract had been signed, the Chemical Construction Company was employed to make cost estimates for the construction of the proposed glutamic acid plant. Following this, Mr. Max Donauer, assisted by the engineers of A. Bentley and Sons and the Fellow, designed the plant. A. Bentley and Sons Company, of Toledo, Ohio, contracted for the construction of the plant to be erected at Rossford, Ohio, on property owned by the Larrowe Milling Company. Mr. Max Donauer, aided by the Purchasing and Engineering Departments of A. Bentley and Sons, prepared the final specifications

for the equipment required and purchased it. Mr. Albert E. Marshall, consulting engineer, was employed as consultant but had little to do with the design of the original plant.

The plant was completed and ready for a preliminary tryout on February 1, 1927. Preliminary trials, carried out during February and March, 1927, indicated certain changes were necessary before actual production operations could start. These changes involving principally the ventilation system, were made during April and May, and the plant was again tried out. An 8.5% yield (dry matter basis) of glutamic acid was obtained and the capacity of the plant appeared to be 1,000 lbs. of glutamic acid daily. This yield and capacity were lower than anticipated. Further, it was found that the plant alterations had not eliminated the corrosion problem.

Although it was highly probable that the yield of glutamic acid and the daily capacity could be increased by continued study and trials, because of the difficulties caused by corrosion, the process involving the use of hydrochloric acid for hydrolysis was dropped after only a few thousand pounds of monosodium glutamate had been produced, and studies were begun to determine methods of separating glutamic acid from the concentrate without the use of strong hydrochloric acid.

Development of the alkaline hydrolysis process. The Larrowe Fellowship at the Mellon Institute of Industrial Research was discontinued at the end of 1927, and studies were begun by the Larrowe-Suzuki Company in an endeavor to work out a process of obtaining glutamic acid from concentrated Steffen's waste which would eliminate the major corrosion difficulties.

Dr. Ikeda of S. Suzuki and Company proposed a process in which sulfuric acid was substituted for hydrochloric acid as the hydrolytic agent and included the step of precipitating glutamic acid as calcium glutamate. This process was used to produce approximately 70,000 lbs. of monosodium glutamate during 1928.

In endeavoring to get completely away from corrosion problems, the research carried out by Dr. C. L. Royal under Mr. A. E. Marshall's direction then turned toward work involving alkaline hydrolysis, and, in 1931, the process was tried out on a pilot plant scale.

According to this process, concentrated Steffen's filtrate, the desugarized molasses from beet sugar plants, is hydrolyzed with alkali then acidified with hydrochloric acid and concentrated further. After removal of mineral salts, the filtrate is adjusted carefully to the isoelectric point of glutamic acid which crystallizes out over a period of 5 to 6 days. This glutamic acid is then refined,

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neutralized with caustic soda solution and monosodium glutamate crystallized from this neutral solution.

In 1936 the Larrowe-Suzuki Company was reorganized as the Amino Products Co., and the Rossford plant was rebuilt to suit the new alkaline hydrolysis process.

International Minerals and Chemical Corporation process. In 1942, the Amino Products Company, owned principally by J. E. Larrowe and A. E. Marshall, was sold to International Minerals and Chemical Corporation. Shortly afterward, the wartime need for beet molasses caused the eastern beet sugar processors to discontinue Steffen's operations and International was faced with the problem of replacing its raw material.

Wheat gluten was available as a by-product from the production of ethyl alcohol from wheat flour and from the production of dextrose from the same source. International's research and production teams joined to develop a process for the utilization of this raw material. The process chosen involved the acid hydrolysis of wheat gluten under pressure, followed by neutralization with caustic soda. This reduced the corrosion problem which had always been characteristic of acid hydrolysis. Additional technological improvements were made to improve the yields and later the process was adapted to corn gluten.

In recent years, the Rossford plant has processed wheat and corn gluten and Steffen's waste (in the limited quantities available). Some by-product recovery has also been found feasible.

In 1946, International completed a new plant at San Jose, having an annual capacity in excess of 3,000,000 pounds of glutamate. Raw material for this plant is from the beet sugar processors located in California and on the western slope. The plant capacity has been steadily increased as a result of a strong research and development program. Significant additions to the plant have been made during the past eight years.

The Huron Milling Company process. The Huron Milling Company started laboratory work on glutamate manufacture in January 1929 under the supervision of Mr. A. J. Patten, and began commercial production in August 1934. The Huron Milling process was similar to that devised at the Mellon Institute of Industrial Research for the manufacture of glutamates from concentrated Steffen's waste (except that the Huron Milling Company used wheat gluten as the raw material) since it involved hydrolysis with hydrochloric acid, isolation of the glutamic acid as its hydrochloride with subsequent crystallizations as free glutamic acid and then as monosodium glutamate.

In recent years, the Huron Milling Company has made many improvements in its plant and process. Great strides in the direction of keeping hydrogen chloride fumes out of the air have been made but this problem has not yet been entirely solved. One of the worst sources of fumes in the past was what are called filter boxes which allowed the escape of a rather large amount of hydrogen chloride fumes into the air. These are now being replaced with enclosed continuous acid-proof filters.

One step in the operation consists in concentrating the hydrolysate under vacuum in order to recover excess hydrogen chloride. This was formerly done in several small glass-lined stills and these have now been replaced by one large continuous still which was made largely according to a new design.

Another improvement is that glazed tile has been substituted for brick in nearly all parts of the plant, particularly in the finishing end. Also, the finishing end has been streamlined and made as sanitary as possible.

The Staley process. The A. E. Staley Manufacturing Company conducted research work on the preparation of monosodium glutamate from vegetable proteins from 1931 to 1948. In the latter year that company completed work on a glutamate plant at Decatur, Illinois, for the manufacture of monosodium glutamate from corn gluten.

According to Dr. Walter G. Meyer, process development director, and Dr. Paul Shildneck, director of research, who have headed up the glutamate research program for the Staley Company, the process is not extremely complicated although it is quite difficult to operate. One of the first and principal steps in the production of glutamate is the hydrolysis operation which may be accomplished in two ways. This reaction may be carried out at atmospheric pressure, requiring 15 to 20 hours for completion, or it may be carried out at elevated temperatures and pressures in about four hours.

When this resulting hydrolysate is diluted to a certain point, it can then be neutralized by the addition of an alkali without causing precipitation of amino acids. After cooling to a predetermined temperature, this solution is then filtered in rubber-lined presses. The choice of the alkali to be used depends upon the means of waste disposal or the possible use elsewhere in the company's other operations of the inorganic salt resulting from neutralization.

After neutralization, the humin or carbonaceous material is press filtered and washed thoroughly. The washes resulting from this operation are used for dilution of the following batch. All of the amino acids present in the hydrolysate are contained in the filtrate at this point and only one, glutamic, is desired in this process. By concentrating this clear filtrate, the least soluble amino

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acids, principally tyrosine and leucine, can be precipitated without inclusion of large amounts of the salts. The amino acids which crystallize when the solution is concentrated are recovered on rotary suction filters.

This filtrate is further concentrated in order to remove inorganic salts by precipitation. A small residual amount of inorganic salts does not interfere with the crystallization of the glutamic acid. After another filtration stage, the filtrate then contains some residual inorganic salts and other amino acids. Then it is cooled and the pH adjusted to the isoelectric point of glutamic acid. The crystallization of glutamic acid from this solution requires from four to eight days, depending on the glutamic acid content in the solution and the amount which can be economically removed.

After this crystallization, the glutamic acid is separated from its mother liquor by centrifugation and is then redissolved in water for neutralization by caustic soda or conversion into the monosodium salt. The monosodium glutamate is then crystallized from its water solution by a carefully controlled process. A clean, white crystalline product is obtained and dried in a rotary dryer to free it from the last traces of moisture.

The Great Western Sugar Company's glutamate manufacturing operations. The Great Western Sugar Company started production of monosodium glutamate November 1, 1954. An annual production of 2 to 2½ million pounds of monosodium glutamate is expected.

According to Dr. E. H. Hungerford, Director of Research and Chemical Control of that company, the raw material used for the production of monosodium glutamate is the residuum from the desugarization of beet molasses. The raw material differs from that of International Minerals and Chemical Corporation in that filtrate from the barium desugarization process, as well as filtrate from the Steffen process, is used. There is no marked difference in these two raw materials except the filtrate from the barium desugarization process contains more raffinose and other non-sucrose carbohydrates.

The process is essentially the same as that used by International Minerals and Chemical Corporation, except in the manner in which hydrolysis of pyrrolidone-carboxylic acid to glutamic acid is accomplished. In the International Minerals and Chemical Corporation process this hydrolysis is accomplished by heating the concentrated Steffen filtrate after the addition of caustic soda. In the Great Western process the hydrolysis is accomplished by holding the hot alkaline filtrates as they are produced in the factory for a considerable length of time in insulated tanks.

General Mills operations. The only other monosodium glutamate manufacturer in the United States is General Mills, Inc. This company began production of this glutamate from wheat gluten in 1943 and is continuing these operations in their plant in Keokuk, Iowa. The process used by General Mills, Inc., in the manufacture of monosodium glutamate has been patented by Mr. Vern S. Waters (U. S. Patent 2,380,890, July 31, 1945). This process has been summarized by the inventor as follows:

"A process of hydrolyzing gluten for the production of glutamic acid which comprises subjecting gluten to the action of hydrochloric acid containing in excess of 25 per cent hydrogen chloride under superatmospheric pressure for a sufficient period of time to effect a substantial hydrolysis while preventing the escape of hydrogen chloride gas, and crystallizing the resultant glutamic acid hydrochloride from the hydrolyzate while said hydrolyzate contains hydrogen chloride in a concentration not less than about 25 per cent."

General Mills' method of manufacture crystallizes glutamic acid hydrochloride near the start of the processing, thus eliminating the need for handling neutralization salts through much of the processing equipment. There is no necessity of recovering corrosive hydrochloric acid which is hard to handle. In the second step of manufacture, General Mills separates glutamic acid from the other amino acids and then purifies it for conversion to monosodium glutamate.

Production statistics. Since 1943, monosodium glutamate production in the United States has increased from 3,054,445 pounds to 13,000,906 (Table 1). With the exception of 1954, there has been a steady increase in monosodium glutamate production every year. Few industries have had such a steady phenomenal growth.

Broadening uses. The production of the monosodium glutamate industry 5 years ago went largely to improve the flavor of canned soups. Today it is used to improve the flavor of hundreds of commercially prepared food products. But this phenomenal growth has not been as easy as this may sound, for glutamate manufacture involves one of the most technically difficult of manufacturing processes. The problems of inadequate research and education of the food industry have been largely overcome due to the pioneering spirit of the glutamate industry which has had the necessary courage to invest many millions of dollars in new plants, equipment, and personnel.

The industry's long-range future is bright because monosodium glutamate is a natural, valuable ingredient in practically all foods except sweets and fruits. And who knows for sure that future

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U. S. Production of Monosodium Glutamate, 1943-54	
(Pounds)	
1943	3,054,445
1944	3,742,260
1945	4,500,000
1946	6,100,000
1947	(not available)
1948	6,159,000
1949	7,643,000
1950	8,577,000
1951	10,001,000
1952	11,642,000
1953	14,738,000
1954	13,000,906

research will not turn up worthwhile applications even in these categories?

The food industry, in its highly competitive fight for consumer favor and dollar, is in the midst of developing two new giants for the American market—frozen foods and precooked frozen foods. The use of monosodium glutamate in these types of food products will help materially in fulfilling the desire to supply Americans with the best tasting, best looking food products that the food industry is striving so earnestly to produce.

Chronology of Glutamate Research and Development Events

1866-68—The chemical composition of glutamate was determined by the German chemist Ritthausen, thus laying the theoretical basis for modern manufacture.

1890 —Glutamic acid was successfully synthesized by Wolff and his colleagues.

1909 —British patent 9940 was issued to Kikunae Ikeda and S. Suzuki, Tokyo, Japan; this historic patent describes "the manufacture of a flavouring material," and contains the first printed reference to the flavor characteristics of sodium glutamate. The product of this process was the "Aji-no-moto" of commerce.

1912 —Dr. Kikunae Ikeda, at the Congress of Applied Chemistry, New York, called attention to the flavor improvement properties of *Laminaria Japonica*, a seaweed, and reported that the hydrolysate of this product when neutralized with soda yielded sodium glutamate, which possessed "a meat-like taste."

1921 —Manufacture of glutamate was initiated in China, with the added refinement of alcoholic purification; the leading Chinese product of this period was known as Ve-tsin.

1923 —Work to retrieve useful by-products from Steffen's Waste was initiated at Mellon Institute under an Industrial Fellowship sponsored by James E. Larrowe.

1927 —A plant for the manufacture of glutamic acid and other by-products was erected at Rossford, Ohio, by the Larrowe-Suzuki Company and commercial production was initiated.

1928 —Use of the alkaline hydrolysis process was introduced by Dr. K. Ikeda of S. Suzuki and Company.

1929 —Experimental work on glutamate manufacture was undertaken by A. J. Patten, Director of Research, the Huron Milling Company, Harbor Beach, Michigan.

1934 —Commercial production was begun by the Huron Milling Company at Harbor Beach, Michigan; the source was wheat gluten.

1936 —The Larrowe-Suzuki Company was organized as the Amino Products Company.

1942 —The Amino Products Company was sold to the International Minerals and Chemical Corporation.

1943 —General Mills, Inc. initiated glutamate production from wheat gluten at their plant in Keokuk, Iowa.

1946 —A plant for the production of glutamate from Steffen's Waste was erected at San Jose, California, by International Minerals and Chemical Corporation.

1948 —Commercial production of glutamate from corn gluten was begun by the A. E. Staley Manufacturing Company, using the Staley process, at Decatur, Illinois.

1954 —The Great Western Sugar Company began production of monosodium glutamate from beet waste.

1954 —Monosodium glutamate reached a total production of over 13,000,000 pounds.

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Documentation

The Work of the Mellon Institute

Annual Summary Reports of Larrowe Construction Company's Industrial Fellowship No. 1 (1924), No. 2 (1925), No. 3 (1926), Donald K. Tressler, Fellow.

Monograph of Larrowe Construction Company's Industrial Fellowships Nos. 1, 2, 3 and 4 (1927), Donald K. Tressler, Fellow.

U. S. Pat. 1,634,221. Manufacture of betaine hydrochloride, etc., Donald K. Tressler, June 28, 1927.

U. S. Pat. 1,634,222. Process of preparing betaine hydrochloride. Donald K. Tressler, June 28, 1927.

U. S. Pat. 1,840,798. Preparation of betaine hydrochloride, etc. Donald K. Tressler, Sept. 25, 1927.

Processes (personal letters or statements)

E. H. Ziegenfuss, Special Commodities Division, General Mills, Inc., Minneapolis, Minnesota.

E. H. Hangerford, Director of Research and Chemical Control, Great Western Sugar Company, Denver, Colorado.

J. V. Jenks, Huron Milling Co., Harbor Beach, Michigan.

R. W. Slator, General Production Manager, Amino Products Division, International Minerals and Chemical Corp., San Jose, California.

Walter G. Meyer, Process Development Director, A. E. Staley Mfg. Co., Decatur, Illinois.

R. L. Nagle, Supervisor of MSG Sales and Distribution, A. E. Staley Mfg. Co., Decatur, Illinois.

W. R. Eichelberger, Market Development Department, A. E. Staley Mfg. Co., Decatur, Illinois.

Production Data

Data for the years 1943-46 were furnished by the Dept. of Commerce, compiled from estimates made by the War Food Administration and the Grain Branch of PMA, U. S. Dept. of Agriculture.

Data for the years 1948-53 were furnished by the Tariff Commission, from a report *Synthetic Organic Chemicals*, U. S. Production and Sales.

Data for the year 1954 (10 months, actual; last 2 months, estimated) were compiled by Ernst & Ernst, based on figures submitted by the companies filing this statement.

General

Dan Gutleben, The painful growth of MSG, *Chemical Engineering*, **55**, 223-224, 226, 228, 230, 232, 234.

Proceedings of the Symposium, Flavor and Acceptability of Monosodium Glutamate, held March 4, 1948, sponsored jointly by the Quartermaster Food and Container Institute for the Armed Forces and Research and Development Associates, Food and Container Institute, Inc., Chicago, Illinois.

History of Glutamate Manufacture, Albert E. Marshal, President, Rumford Chemical Works, Rumford, Rhode Island.

Quality Production of Glutamate, Paul D. V. Manning, Vice President in Charge of Research, and B. F. Buchanan, Director of Technical Service, Amino Products Division, International Minerals and Chemical Corp., Chicago, Ill.

II. Military Evaluations of Glutamate

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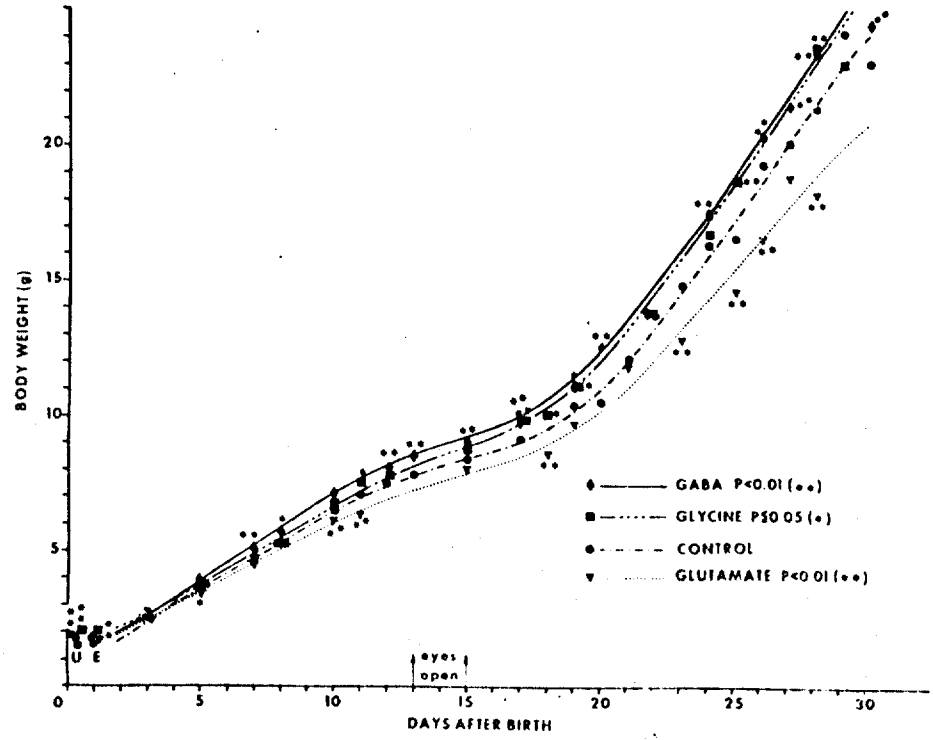
Brain weight and growth of mice fed gamma-aminobutyric acid, glycine or L-glutamic acid diet

Numerous studies in past years have demonstrated that the nutritional quality of diets has a considerable influence on the development of animals (for review see ref. 3). As part of an investigation concerning possible environmental factors which may influence the appearance of certain enzymes in the CNS, 40-day-old albino mice (Sprague Dawley ARS Ha/ICR) were maintained on pelleted Purina Rat Chow and distilled water, supplemented with 0.5% (w/w and w/v) L-glutamic acid, glycine or γ-aminobutyric acid (GABA). Mating, pregnancy and subsequent growth of the offspring all occurred while the animals had free access to one of the special diets or to the non-supplemented (control) diet and water. Each litter was equalized to 8 individuals within 24 h after birth by elimination of those young having a weight farthest removed (heavier and/or lighter) from the average weight of the litter. A careful record was kept of these animals and, incidental to the main purpose of this study, certain observations indicated that the growth rate and final appearance of the mice in the 3 experimental groups differed. Unusual differences in brain weight also became apparent.

A comparison of the growth rate of the litters indicated that, starting in the first week after birth, the glycine and GABA groups were approximately 10% heavier than the control mice (Fig. 1A, B, top two curves). GABA in the diet proved more effective in this respect than glycine. Around the same period after birth, the weight increase of the young in the group fed glutamic acid began to lag and the mice remained undersized throughout their development (Fig. 1A, B, bottom curve). It may be noted that the average weight of the young after equalization of the litters was very similar for the experimental groups (Fig. $1A_E$). The mice shown in Fig. 2 demonstrate that the differences in weight gain represented true variations in development. The mature animals, raised on GABA, were larger with respect to body size and size of the head, reflecting a better developed musculature, larger paws and a longer tail, as well as an increase in the length and width of the skull bones (Fig. 2, top). In contrast, mice fed L-glutamic acid in a normal diet were clearly undersized on attaining maturity (Fig. 2). The effect of glycine was less obvious, despite the observed increase in weight of such animals. While the addition of 0.5% L-glutamic acid to the normal diet resulted in mice which were about 20% smaller than controls fed the same diet without a supplement of the amino acids, these animals were nevertheless in all other respects quite normal. In partial contrast to previous reports[1,5] macroscopic and light microscopic examination of the brain revealed no *obvious* abnormalities and males as well as females proved to be fertile. The males raised on GABA seemed considerably more aggressive than the other groups.

The enhanced weight increase of the glycine or GABA fed animals as compared to the control group was apparently not directly attributable to a greater food intake. Food consumption of normal mice placed on the special diets was comparable (Table I). In the animals raised on such diets, only the group matured on glutamic acid appeared to eat less (Table II). To a large extent this could be due to the small size of

1A



1B

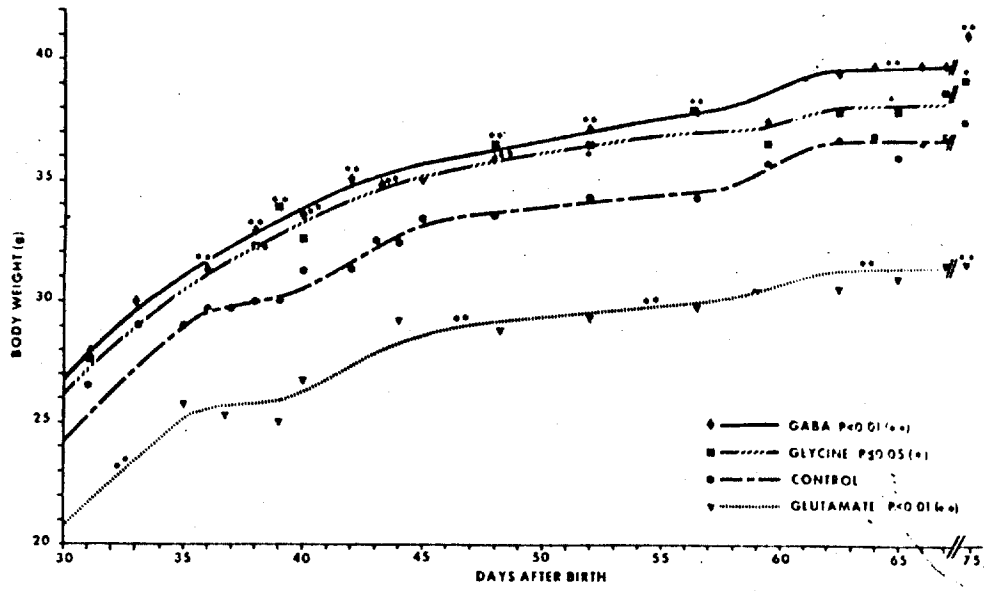


Fig. 1. Effect of supplementing a normal diet, fully adequate to sustain growth, and water with 0.5% (w/w and w/v) L-glutamic acid, glycine or GABA. Normal mice (40 days) were placed one week prior to mating on one of the special diets or on an identical diet without amino acid supplement. Record A shows growth of offspring (males and females) up to day 30, while B demonstrates growth rate of males only, up to day 75. Females were from 3 to 7 g lighter throughout this period but differences in weight gain between dietary groups were identical to the one shown (see Fig. 2). Each point on the curve represents the average weight of a minimum of 24 animals. In A, U indicates average weight of young at birth, E the weight after each litter had been equalized to 8 individuals 24 h after birth.

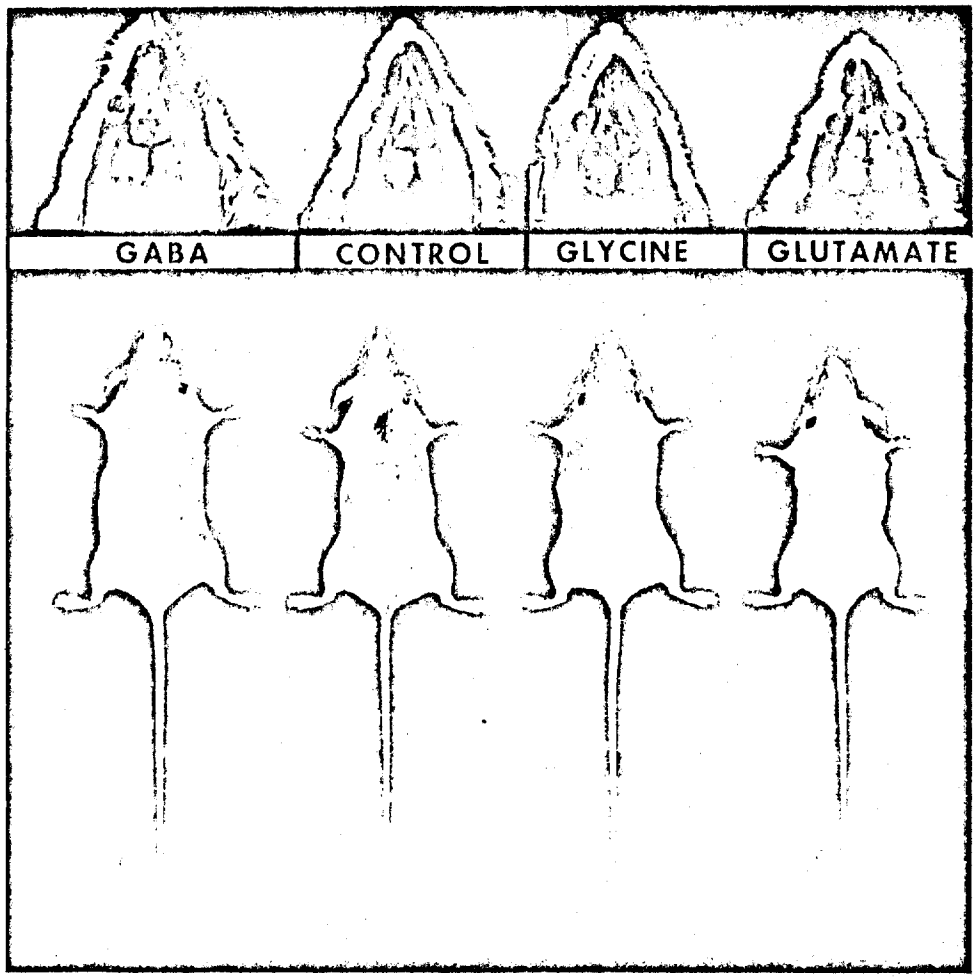


Fig. 2. Representative differences in appearance of female mice, having been raised on Purina Rat Chow and water, or on an identical diet supplemented with either L-glutamic acid, glycine or GABA. Animals were 60–65 days old and each had given birth to one litter. Note abnormally small size of female raised on glutamic acid and the sturdier appearance and larger skull size of GABA female.

TABLE I

MICE RAISED NORMALLY AND PLACED ON SPECIAL DIETS (FEMALES)

Group	*Weight (day 1)*	*Weight (gain/15 d.)*	*Food (g/day/5 mice)*	*Water (g/day/5 mice)*
Control (5)	29.56 ± 1.90 (S.D.)	0.36 ± 0.97	4.18 ± 0.39	5.71 ± 0.41
GABA diet (5)	29.54 ± 1.90	−0.82 ± 1.16	3.93 ± 0.48	5.49 ± 0.55
Glycine diet (5)	29.22 ± 1.69	1.08 ± 0.97	4.16 ± 0.59	6.33 ± 0.5**
Glutamate diet (5)	29.78 ± 1.14	−0.22 ± 0.87	3.89 ± 0.93	4.72 ± 0.56***

** 0.01 < P < 0.02.
*** P < 0.01.

TABLE II

MICE RAISED ON SPECIAL DIETS AND MAINTAINED ON SPECIAL DIETS (FEMALES)

Group	*Weight (day 1)*	*Weight (gain/15 d.)*	*Food (g/day/5 mice)*	*Water (g/day/5 mice)*
Control (5)	32.38 ± 0.56 (S.D.)	3.20 ± 0.66	5.40 ± 0.41	7.36 ± 0.67
GABA fed (5)	34.04 ± 0.85**	3.58 ± 1.05	5.68 ± 0.40	8.14 ± 0.76**
Glycine fed (5)	32.30 ± 0.48	2.38 ± 0.61	5.10 ± 0.45	7.66 ± 0.83
Glutamate fed (5)	27.08 ± 0.58**	0.80 ± 0.26**	4.30 ± 0.42**	4.97 ± 1.04**

** $P < 0.01$.

the glutamate raised mice. A greater variability in water consumption was noted for the 3 experimental groups. Since the pH of the water was not adjusted in order to eliminate a possible effect of sodium or other ions on brain growth, the smaller water intake of the glutamic acid group (Tables I and II) may have been due to the increased acidity of the water. The effect of water intake *per se* on development is not known but the possibility that water consumption beyond a certain minimum requirement can influence size appears somewhat remote. All the young were weaned at day 20 and were noted to consume water and solid food soon after opening of the eyes (day 13–15). Variations in lactation by the mothers could therefore account for some of the initial differences in growth between the 3 experimental groups but it appears unlikely that such differences were maintained and even enhanced throughout development, when the offspring had free access to food and water[3].

Another and unexpected effect of the special diets was their influence on brain weight (Table III). Weighings in past years of well over 500 mature mouse brains never revealed a marked difference between the weights of male and female brains, despite considerable variations in body weight. This was corroborated in the present experiments (Table III). However, it soon became apparent that of the mice fed GABA or glycine, the adult female brain was usually heavier when compared to the control females. Furthermore, a less pronounced but rather consistent difference in brain weight was noted between females and males of the dietary groups (especially glycine, Table III), even though the females were from 3 to 7 g lighter. In this respect L-glutamic acid was almost as effective as GABA or glycine. Thus, whereas females raised on GABA or glycine had an average brain weight significantly larger than normal, the brains of females in the L-glutamic acid group were within the normal weight range, despite their smaller skull size (Fig. 2). The average brain weights of the GABA and glycine males were only slightly greater than those of the controls, and the brain weight of the glutamate males was more in accordance with their smaller size (Table III).

A third observation, related specifically to the GABA diet, concerned the number of animals born per litter as well as their weight at birth (Fig. 3). GABA

TABLE III

EFFECT OF SPECIAL DIETS ON BRAIN WEIGHT

Values represent average brain weight ± standard deviation (g wet weight). Cerebellum and brain stem were removed. Numbers in parentheses refer to animals per group. Significant differences, indicated by asterisks, were obtained by comparing control group with each of the special groups: * $p < 0.01$; ** $p < 0.01$ (Fisher's t distribution). Difference between average brain weight of glycine females (italics) as compared to that of the males was significant at the less than one per cent level. No significant differences between the four groups were noted when the combined weight of cerebellum plus brain stem was compared.

	Female			*Male*		
Control	0.345	± 0.014 (S.D.)	(15)	0.345	± 0.019	(17)
GABA diet	0.364**	± 0.017	(20)	0.357	± 0.020	(21)
Glycine diet	*0.366***	± 0.014	(21)	0.352	± 0.015	(17)
Glutamate diet	0.339	± 0.023	(18)	0.329*	± 0.016	(22)

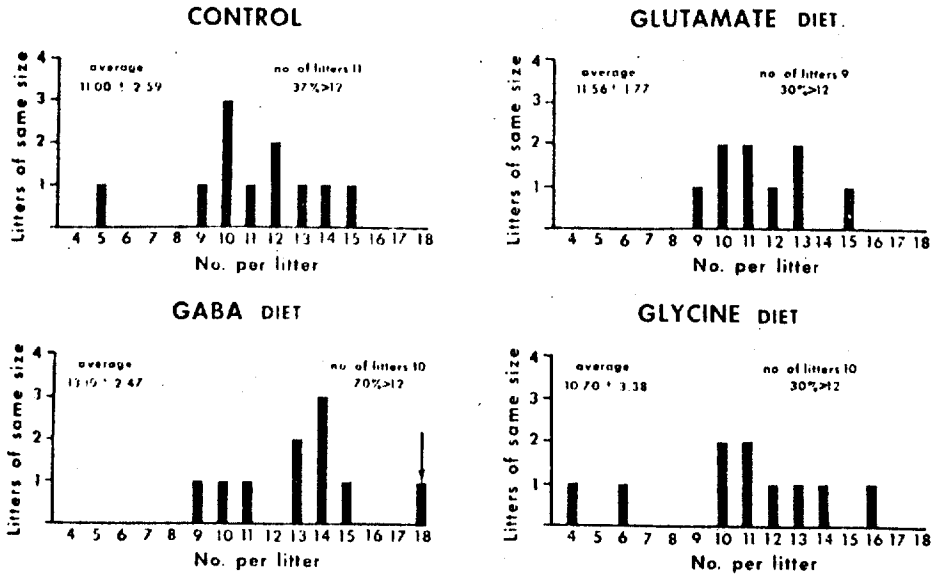


Fig. 3. The influence of diet on litter size. GABA litters numbered on the average 13 young and most of the litters (70%) were of this size or larger. In contrast, the litters of the groups fed the glycine, L-glutamic acid or control diet averaged 11 young and only 30–37% of the litters were larger than that size.

litters numbered on the average 13 young and most of the litters (70%) were of this size or larger. In contrast, the litters of the groups fed the glycine, L-glutamic acid or control diet averaged 11 young and only 30–37% of the litters were larger than that size. Despite a litter of as many as 18 pups (Fig. 3, arrow), the average weight and size of the young at birth in the GABA group was comparable to the control litters

numbering only 10 individuals. Clearly then, GABA added to a normal diet appeared to have a beneficial effect on the *in utero* development of the embryos.

The present study suggests that further investigations may be warranted on the effect of supplementing diets, fully adequate for normal growth, with low concentrations of certain amino acids. Previous studies, using diets augmented with 7–10% glycine or glutamate, indicated that these amino acids were by themselves either detrimental to growth or without effect[3].

As to the mechanism responsible for the effect of chronic administration of glycine, L-glutamate or GABA, one can only hazard a guess at this time. It is possible that the action of glycine is related to an enhanced incorporation into proteins. However, this leaves unexplained the effect of glutamate or GABA. In the case of GABA, it is known that this amino acid is either metabolized in the liver or excreted as such in the urine[6,8]. In fact, GABA reputedly only affects the physiology of neurons and this immediately raises the possibility that this compound may influence the activity of the hypothalamus during development, thereby affecting pituitary hormone metabolism. The differential effect of the amino acid on female brain weight as well as the increased litter size and the abnormal aggressiveness of the GABA males, strongly suggest hormonal involvement. Moreover, both GABA and glycine depress neuronal activity whereas glutamate has an excitatory action[2], which could explain the two opposite effects of these amino acids on growth. The slightly greater effectiveness of GABA over glycine in stimulating growth would then be compatible with observations[4] that the inhibitory action of glycine is more marked on neurons in spinal cord while that of GABA appears most pronounced in higher CNS structures. Preliminary results indicate that each special diet causes a characteristic change in amino acid metabolism of the CNS. The exact nature of these changes is at present under investigation[7].

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THE ROLE OF GLUTAMIC ACID IN COGNITIVE BEHAVIORS [1]

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Despite recent judgements to the contrary, considerable evidence exists suggesting that glutamic acid does play a significant role in cognitive behavior. Its effects upon intelligence are evident both in normal and in retarded persons. Variations in results are believed to be related to (a) types of Ss employed, (b) the condition under which the drug is administered, (c) the method of assessing change in cognitive behavior, (d) method of drug administration, and (e) type of glutamic acid employed. Relevant physiological literature is reviewed.

Basic biochemical research pointing to a central role in neural functioning for glutamic acid (Weil-Malherbe, 1936) has been followed by a continuing series of biological studies further implicating this substance in metabolic and neural processes. In 1944, Zimmerman and Ross reported positive effects of glutamic acid administration upon the learning behavior of the white rat. Later behavioral studies reported that glutamic acid had positive effects upon animal learning; upon intelligence and personality in retarded, psychoneurotic, and normal persons; and that it suppressed abnormal EEG phenomena and controlled epilepsy. Glutamine (a metabolite of glutamic acid) was also reported to have positive effects upon retardate intelligence.

It was quickly recognized, however, that many of the early, positive reports of the effects of glutamic acid upon retardate intelligence were derived from methodologically weak studies. Consequently, experimentally oriented psychologists became active with, presumably, better controlled studies. This research, however, generally failed to find positive effects of glutamic acid upon retardate intelligence. In 1960, Astin and Ross reviewed the literature on the effect of glutamic acid upon retardate intelligence and concluded that no beneficial effect of glutamic acid administration upon retardate intelligence had been demonstrated. They regarded the optimistic conclusions of the early reports as attributable to a particular defect in experimental design, specifically, a failure to employ control groups.

Astin and Ross' paper made no claim to being comprehensive and, in fact, it was not; we have located a total of 25 additional research papers (representing 50% of the available literature) on the effect of glutamic acid administration upon mental retardation published before 1960, the year of Astin and Ross' review. An examination of these papers suggests that their inclusion would have, of necessity, altered Astin and Ross' conclusions, since a complete compilation of glutamic acid studies reveals that as many "positive" as "negative" studies employed control groups. However, we do not fault Astin and Ross primarily because of the incompleteness of their review. We would dispute their conclusions even if the discussion were limited to the material they cited. We take issue with Astin and Ross primarily because of salient aspects of the research with glutamic acid which they did not consider, but which, had they done so, might have led them to a different conclusion. Specifically, we wish to focus upon the following methodological problems: (*a*) the characteristics of subject samples used in the studies reporting positive versus negative results, (*b*) the manner of administration of glutamic acid in the studies reporting positive versus negative results, (*c*) salient questions

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TABLE 1

THIRTY-THREE STUDIES CLASSIFIED IN TERMS OF RESULTS AND USE OF CONTROLS

	Positive results	Negative results
Control	Albert, Hoch, & Waelsch (1946, 1951) Foale (1952) Head (1955) Kurland & Gilgash (1953) Zimmerman, Burgemeister, & Putnam (1948)	Beigins (1954) Ellson, Fuller, & Ernston (1950) Ernsting (1949) Kantor & Boyes (1951) Kerr & Szurek (1950) Loeb & Tuddenham (1950) Lombard, Gilbert, & Donofrio (1955) McCulloch (1950) Milliken & Standen (1951) Oldfelt (1952) Quinn & Durling (1950b) Zabarenko & Chambers (1952) Zublin & Lutz (1953)
	(f = 6)	(f = 13)
No control	de la Fuente Muniz, Zuniga, & Yanowski (1950) Delay, Pichot, Puech, & Perse (1951) Harney (1950) Hoven (1951) Kane (1953) Levine (1949) Müller (1953a, 1954) Quinn & Durling (1950a) Schwöbel (1950, 1952) Zimmerman & Burgemeister (1950) Zimmerman, Burgemeister, & Putnam (1949a, 1949b)	
	(f = 14)	(f = 0)

relating to placebo effects, (d) the manner in which intellectual change was assessed, and last, (e) the nature of the patients' environment while they were being medicated with glutamic acid.

GLUTAMIC ACID AND RETARDATE INTELLIGENCE

Controls

In their review of glutamic acid and retardate intelligence, Astin and Ross concluded that "positive effects tend to be reported in studies not employing a control group"; and that "the few positive studies employing controls contain methodological flaws . . . [p. 433]." The suggestion that a lack of controlled observations and the presence of methodological errors were responsible for the reports of beneficial effects of glutamic acid is not original with Astin and Ross, but is rather a note which sounded through the earliest reviews of this work (Anonymous, 1951; Arbitman, 1952; Hirai, 1951). It is not a claim, however, which stands close scrutiny, particularly in the light of the more recent literature.

Astin and Ross examined the studies listed in Table 1. These studies were classified into those yielding positive versus negative results; and those which employed a control group versus those which did not. The computed chi-square, with a correction for continuity, was "significant at the .001 level ($\chi^2 = 12.99$), indicating that positive results tend to be related to a lack of controls [p. 429]."

Astin and Ross' table, however, can be corrected in several ways. First, there is every indication that Astin and Ross meant to exclude the extensive literature on the effect of glutamic acid upon the intelligence of normal subjects and nonretarded psychiatric subjects, and to limit their review to "studies with mentally deficient subjects [p. 429]," yet five articles which did not employ retarded subjects were included in their Table 1 (Delay, Pichot, Puech, & Perse, 1951; Kantor & Boyes, 1951; Müller, 1953a, 1954; Schwöbel, 1950). Second, there are a considerable number of articles (at least 25) which were published prior to Astin and Ross' review, but which were not cited by them. Third, Astin and Ross were inconsistent in their classification of studies which did or did not have a control group ("liberally defined as those [studies] in which a similarly diagnosed group was studied in the absence of glutamic acid medication.") An article by Kerr and Szurek (1950) was cited as having employed a control group when, in fact, it did not, and a study by Schwöbel (1952) was cited as not having a control group when, in fact, it did. Further, Astin and Ross listed Albert, Hoch, and Waelsch (1946) and Zimmerman, Burgemeister, and Putnam (1948) as having employed control groups; these studies employed each subject as his own control. Judging from their discussion of these studies, Astin and Ross regarded this method of control as satisfying their definition. However, Harney (1950) used the same method, but was listed as "no control." Finally, in at least one instance, a study was listed in the table twice (Zimmerman et al., 1949a, is a preliminary report of the same data in

TABLE 2

GLUTAMIC ACID STUDIES WITH RETARDED SUBJECTS, CLASSIFIED IN TERMS OF RESULTS, AND PRESENCE OR ABSENCE OF CONTROL GROUPS

	Positive results	Negative results
Controls	Albert, Hoch, & Waelsch (1946) Albert, Hoch, & Waelsch (1951) Contini-Poli (1950) Desclaux, Benoit, & Aussagel (1953) DuPlessis (1953) Foale (1952) Fortunato & Canella (1951) Harney (1950) Jaeger-Lee, Gilbert, Washington, & Williams (1953) Koch (1954) Kurland & Gilgash (1953) Schwöbel (1952) Zimmerman & Burgemeister (1959a) Zimmerman, Burgemeister, & Putnam (1948)[b]	Bergius (1954) Ellson, Fuller, & Urmston (1950) Ernsting (1949) Harbauer & DeBoor (1952) Head (1955)[a] Hellstrom & Melin (1952) Leedham (1955) Loeb & Tuddenham (1950) Lombard, Gilbert, & Donofrio (1955) McCulloch (1950) Milliken & Standen (1951) Oldfelt (1952) Pallister & Stevens (1957) Quinn & Durling (1950b) Zabarenko & Chambers (1952) Zublin & Lutz (1953)
No controls	Bergamini (1956) Borak & Borak (1957) Clapp (1949) de la Fuente Muniz, Zuniga, & Yanowski (1950) de Moragos (1949) Dragunova (1955) Goldstein (1954) Hoven (1951) Kane (1953) Lafon, Faure, & Bascou (1952) Levine (1949) Pabst & Wurst (1952) Quinn & Durling (1950a) Tschakert (1953) Zimmerman & Burgemeister (1950) Zimmerman & Burgemeister (1959b) Zimmerman, Burgemeister, & Putnam (1949b)[c]	Donnadieu & Achalle (1951) Göllnitz (1952) Kerr & Szurek (1950) Shchembelev (1959) Vasquez & Farago (1957) Zimmerman, Burgemeister, & Putnam (1949b)[c,d]

[a] The difference in IQ scores between the experimental and control group was reported as significant by Head, but was found to be not significant upon reanalysis of the data by Astin and Ross (1960).
[b] Completed report of an earlier paper (Zimmerman, Burgemeister, & Putnam, 1947).
[c] Positive results obtained with a nonmongol sample; negative results with a mongol sample.
[d] Completed report of an earlier paper (Zimmerman, Burgemeister, & Putnam, 1949a).

Zimmerman et al., 1949b). In line with the above discussion, the table presented by Astin and Ross has been corrected (see Table 2). As may be seen, there are now almost as many "positive results, control" ($N = 14$) as "negative results, control" ($N = 16$). The chi-square (corrected for continuity) of 2.93 is not statistically significant. Thus it would seem that the simple presence or absence of a control group does not differentiate the studies which obtained positive results from those which did not. The question of what differentiates studies reporting positive results from studies reporting negative results, then, remains to be answered.

Moreover, we believe that because it is based on certain false assumptions, no classification of studies, such as in Table 1 or 2, can ever result in a proper appreciation of the literature. For example, after alleging a relationship between positive results and the absence of control groups, Astin and Ross (1960) proceeded to dismiss the results of the "no control" studies without further comment. Presumably, their assumption was that in the absence of a control group, positive results may be attributable to placebo effects of glutamic acid; to environmental stimulation that might be attendant to a retarded subject's being included in a glutamic acid study; to natural increments in IQ as a function of growth and experience; to regression effects; or to chance. In fact, Astin and Ross did not utilize the available evidence to investigate whether the results of the "no control" studies were attributable to these variables; they simply regarded the absence of a control group as adequate grounds for dismissal of a study. Yet it can be demonstrated, by inference, at least, that it is most unlikely that the IQ increments observed in the "no control" studies which reported positive results were due to factors other than glutamic acid. Of the 30 glutamic acid studies with mental retardates using control groups, only 4 studies reported significant rises in intelligence in the control groups (Contini-Poli, 1950; DuPlessis, 1953; Ellson, Fuller, & Urmston, 1950; Quinn & Durling, 1950b). The mean rises in IQ of the control groups in these four studies were, respectively, 1.52, 2.15, 1.9, and 4.5 IQ points.[2] In contrast, considerably greater IQ gains are reported in the studies which did not employ control groups, but which reported significant test gains in subjects fed glutamic acid: six such studies reported mean IQ gains of 5 to 10 IQ points (Clapp, 1949; de la Fuente Muniz, 1950; Goldstein, 1954; Zimmerman & Burgemeister, 1950, 1959b; Zimmerman, Burgemeister, & Putnam, 1949b); two re-

[2] Astin and Ross (1960) incorrectly cited Zabarenko and Chambers (1952) and Ernsting (1949) as reporting significant rises in IQ in control groups.

ported gains of 10-20 points (Kane, 1953; Levine, 1949), and only one reported a mean gain of less than five points (Quinn & Durling, 1950a). The much greater size of the IQ gains following glutamic acid therapy compared to the IQ gains of control groups suggest that the gains in the experimental groups are not attributable to environmental factors other than glutamic acid. Consequently, any summary dismissal of a study on the grounds that a "no treatment, control" group was not employed does not seem to be justifiable.

A case in point is a paper by Kane (1953), which was one of those dismissed without discussion by Astin and Ross on the grounds that it failed to include a "no [glutamic acid] treatment" control group. Kane reported that glutamic acid was "of decided value in cases in which the triad: emotional immaturity, mental retardation, and brain insult" appeared; and that it might be of some value to the psychotic and epileptic, but that it was of no value in other cases of retardation. Kane's sample consisted of 106 "immature, brain-injured, retarded" children who gained an average of 17 IQ points after 3 months of treatment with glutamic acid. She reported no change as a result of glutamic acid treatment in a number of other samples whose members manifested none, one, or two characteristics of the triad. It seems most unlikely that Kane's results could have been due simply to placebo effects since no studies in the literature reported effects anywhere near so great following glutamic acid treatment, let alone placebo; and none have reported such dramatic effects on so large a number of persons. The Kane study might well have served to initiate well-controlled, methodologically sophisticated studies to investigate the validity of her claim, but in fact it did not. No later study has utilized a sample similar to that employed by Kane.

It is possible that studies reporting positive results from glutamic-acid therapy may capitalize on regression effects resulting from an initial selection of subjects with low IQs. Regression effects are usually controlled by using a matched control group. However, Ellson, Fuller, and Urmston (1950) noted that in at least one instance (Zimmerman, Burgemeister, & Putnam, 1947) a control group was chosen in such a manner that a regression effect magnified the difference between a glutamic acid and a control group. Upon reanalysis of the data (Ellson, Fuller, & Urmston, 1950), the difference in favor of the glutamic acid group was found to be lessened, although still present.

The problem of regression effects is left unresolved in studies which select subjects on the basis of IQ and do not use a control group. Studies which obtained positive results after glutamic acid treatment but which did not employ a control group have, therefore, been closely examined to ascertain the extent to which regression effects may have contributed to their positive results. Such an examination has suggested, however, that regression effects probably did not play a major role in the "positive results, no control" studies, for the following reasons: First, we have already shown that in the 30 studies in which control groups were employed, and in which placebo effects, practice effects, and any possible regression effects were presumably working in the same direction to produce elevated scores, only four control groups showed significant rises upon retest; and, in three of the four cases, the rises were less than 2.15 points. In contrast, the rises in the "no control, glutamic acid" studies were, with one exception (Quinn & Durling, 1950a), greater than five points. Second, although it is sometimes difficult to be certain from the written reports, it appears that most studies did not select their subjects on the basis of IQ except insofar as they had been catagorized as retardates. The usual procedure seems to have been to select subjects on some basis other than IQ, for example, institutionalized retardates, noninstitutionalized retardates, mongoloids, familial retardates, etc., and then determine the pre- and post-treatment IQs. This selection procedure does not induce regression effects. Only two "positive results, no control" studies appear to have chosen subjects from the lower end of a test distribution (Goldstein, 1954; Quinn & Durling, 1950a). Consequently, prudence would suggest that these two studies be eliminated from further consideration in the ensuing discussions. It should be noted that with the omission of the two studies, the value of the chi-square based on the con-

tingencies in Table 2 changes from 2.93 to 2.16, remaining not statistically significant; thus, the conclusion remains tenable that the simple presence or absence of a control group does not differentiate the studies which obtained positive results from those that did not.

Subject Samples

The studies reporting positive results tended to use noninstitutionalized retardates who were either living in their parents' homes or attending private boarding schools, whereas the studies reporting negative results tended to employ institutionalized subjects.

Of the positive studies listed in Table 2 (excepting Goldstein, 1954; Quinn & Durling, 1950a) six studies employed institutionalized subjects (Desclaux, Benoit, & Aussagel, 1953; Foale, 1952; Hoven, 1951; Kurland & Gilgash, 1953; Schwöbel, 1952; Tschakert, 1953). In four positive studies, the composition of the subject sample cannot be inferred with certainty (Bergamini, 1956; Borak & Borak, 1957; Clapp, 1949; de Moragos, 1949), and in one study (Fortunato & Canella, 1951) the sample was evenly mixed. In each one of the remaining 18 positive studies, the subjects were noninstitutionalized retardates who were living in their parents' homes or attending private boarding schools.

Of the *negative* studies listed in Table 2, three employed samples composed exclusively or almost exclusively of noninstitutionalized subjects (Head, 1955; Kerr & Szurek, 1950; Vasquez & Farago, 1957); in five studies, the composition of the samples cannot be inferred with certainty (Donnadieu & Achalle, 1951; Ernsting, 1949; Harbauer & DeBoor, 1952; Leedham, 1955; Shchlembelev, 1959); and in one study, it was evenly mixed (the mongol sample of Zimmerman, Burgemeister, and Putnam, 1949b). The remaining 13 studies employed samples composed exclusively or almost exclusively of institutionalized subjects.

Summarizing, of the positive studies in which the composition of the sample can be ascertained, 18 employed noninstitutionalized, and 6 employed institutionalized retardates; while of the negative studies, 3 employed noninstitutionalized retardates and 13 employed institutionalized retardates ($\chi^2 = 10.03$, $p < .005$).

This tendency of studies reporting positive findings to employ outpatients rather than inpatients had been noted earlier (Oldfelt, 1952); however, no investigation of the phenomenon has been reported. Several explanations suggest themselves. First, the parents of the outpatients, aware that something special is being done for their child (since it is they who administer the drug at home) may watch their child closely for some slight sign of positive change, which is then, as Quinn and Durling (1950b) put it, "capitalized on and implemented by [the] doting parents." This would seem to be a definite danger when, as in the early Zimmerman studies (Zimmerman et al., 1947, 1948), the patients and parents knew the nature of the drug. The danger, of course, disappears entirely in cases where glutamic acid can be shown to have a more positive effect than a placebo. Second, the outpatient retardates, living at home, are more likely to be in a favorable situation than institutionalized retardates—they have access to more individualized care, more personal attention, and a more even environment, all of which may create the kind of positive atmosphere that might make them more responsive to therapeutic intervention. Third, the fact that the noninstitutionalized retardates have the resources to maintain themselves outside of an institution suggests better adjustment, greater maturity, or a lesser degree of disability than institutionalized retardates and, consequently, a greater amenability to therapeutic intervention. Finally, there may be a debilitating and therapeutically inimical effect of institutionalization upon certain classes of retarded persons.

The question of why certain investigators chose to work with outpatients while others worked with inpatients may relate to experimental versus clinical orientations of the investigators. In some cases, at least, the use of institutionalized patients was determined by a desire for greater experimental rigor, specifically, a wish to keep the environmental conditions similar for all subjects (Ellson, Fuller, & Urmston, 1950; Oldfelt, 1952; Zabarenko & Chambers, 1952). No recognition was evident, in these instances, that this procedure might result in less thera-

peutically amenable patient samples. On the other hand, some clinically oriented investigators seem to have employed noninstitutionalized subjects out of a wish to work with the more promising varieties of retarded patients, at the same time displaying a relative lack of concern over the problem of environmental control (Zimmerman & Burgemeister, 1950).

Individual Differences

Another difference between the positive and negative studies which may relate to a clinical versus experimental orientation involves attitudes towards individual differences. The clinician who usually deals with individuals is quite naturally concerned with constitutional phenomena governing individual differences. The experimental psychologist, on the other hand, has been typically mainly interested in the effects of externally manipulated variables on a sample of subjects.

In this context, it is pertinent that the positive studies often compared the responses to glutamic acid of various diagnostic categories of retardates: for example, mongols versus nonmongols, primary versus secondary retardates, severe versus mild retardation, etc., (Bergamini, 1956; Borak & Borak, 1957; Clapp, 1949; de la Fuente Muniz et al., 1950; de Moragos, 1949; Kane, 1953; Koch, 1954; Lafon et al., 1952; Zimmerman et al., 1948, 1949b, 1950, 1959a, 1959b). When differences were found, it was usually concluded that glutamic acid may affect some varieties of retardation more than others (Borak & Borak, 1957; Kane, 1953; Lafon, Faure, & Bascou, 1952; Zimmerman et al., 1959a; 1959b). Thus, concern with individual differences tends to be present in the methodological approach of the positive studies. On the other hand, concern with differential responses to glutamic acid as a function of type of mental retardation is present in only a few of the negative studies (Gollnitz, 1952; Vasquez & Farago, 1957; Zabarenko & Chambers, 1952; Zimmerman, Burgemeister, & Putnam, 1949b). If glutamic acid is therapeutically effective with only a segment of the retarded population, this fact would be disguised by the methodological approach employed in the majority of the "negative" studies.

Glutamic Acid Administration: The Problem of Dosage Level

In administering a drug to patients over a long period of time, clinicians often follow a standard procedure in determining dosage level: the initial dose of the drug is small, and is gradually increased up to the point where the patient shows a toxic response to the drug. The dosage level is then reduced to below the level of toxicity. In this manner, a maximal drug response is obtained from all patients. This method of drug administration may be termed an "individualized" method, that is, the level of medication a patient receives is determined by his own reaction to the drug. The method may be modified in that the dose may not be increased to toxicity but may be varied depending upon the patient's response to lower than toxic levels. Throughout their numerous publications, Zimmerman and his colleagues have stressed the necessity of adhering to the individualized method of administering glutamic acid if positive results are to be obtained. They have been echoed in their plea by Contini-Poli (1950), de la Fuente Muniz et al. (1950), and others.

However, the individual or clinical approach to drug administration greatly complicates matters for scientists who are attempting to evaluate the efficacy of drugs. Astin and Ross (1960) argued against the clinical method on the grounds that if the dosage is increased to toxicity, it becomes apparent who is in the placebo and who is in the experimental group. Also, if the members of an experimental sample are on various dosages, it can be argued that individual differences in response to the drug are attributable to the differences in dose level. It seems more "scientific," then, to give all subjects the same amount of the drug, even though the internal response to the drug is allowed to vary across individuals. The procedure of giving all subjects the same dosage level of a drug ignores the pharmacological facts in favor of a superficially clean-cut experimental design.

Studies which administer the same dose to all subjects are presented with a dilemma: They may administer a small dose to all sub-

jects, whereupon they risk not obtaining the sought-after behavioral response. Or, they may give all subjects a very high dose and try to maximize a positive response, while ignoring the problem of toxic effects. Zabarenko and Chambers (1952), for example, chose the latter course and gave all subjects 40 grams—a massive dose, considering half their subjects were children 5–11 years of age. While they reported that "in 10,000 patient days of medication, there were at most half a dozen instances of gastric disturbance," there appears to have been definite toxic effects apparent on intelligence testing, since, in a later report on the same data (Chambers & Zabarenko, 1956), they stated that after glutamic-acid therapy there was a "lessening in ability to inhibit responses, which led to impulsive errors." "Impulsivity," it must be noted, is one of the behavioral effects of an overdose of glutamic acid. Since Zabarenko and Chambers (1952) reported no positive effect of glutamic acid on intelligence testing, one wonders if their method of administration of glutamic acid was to blame. Similar observations may be made of the study of Ellson, Fuller, and Urmston (1950) in which subjects (all of whom were children) were maintained on fairly high dosage levels (30 grams). Ellson et al. reported no positive effect of glutamic acid upon intelligence, and at the same time reported that the glutamic acid group evidenced significantly more impulsive, error-prone behaviors on one of the cognitive tests.

Comparison of the positive and negative studies indicates a statistically significant tendency for the positive studies to vary the dosage level for individuals, while the negative studies tend to administer the same dosage level to all subjects ($\chi^2 = 7.11$, $p < .01$). Specifically, of the positive studies listed in Table 2 (once more excepting Goldstein, 1954; and Quinn & Durling, 1950a), 9 studies administered a fixed dose to all subjects (Albert, Hoch, & Waelsch, 1946; Bergamini, 1956; Desclaux et al., 1953; DuPlessis, 1953; Foale, 1952; Kurland & Gilgash, 1953; Levine, 1949; Pabst & Wurst, 1952; Tschakert, 1953). The remaining 20 used the individualized method of drug administration. Of the negative studies, 16 gave all subjects the same dose, while only 6 studies used an individualized method of drug administration (Bergius, 1954; Hellstrom & Melin, 1952; Kerr & Szurek, 1950; Milliken & Standen, 1951; Vasquez & Farago, 1957; Zimmerman et al., 1949b).

Glutamic Acid versus Glutamate Salts

The early positive studies of glutamic acid treatment of retardates used the free form of the natural glutamic acid. For reasons not entirely clear, but which probably had to do with convenience of administration, the sodium salt of glutamic acid, monosodium glutamate, later came into common use. The assumption of course was that glutamic acid and monosodium glutamate were equivalent in their physiological effect. The available evidence now suggests that this assumption is not a valid one. Pond and Pond (1951), for example, reported that the salts of glutamic acid *increased*, while the free acid *decreased* epileptic activity both clinically and in the EEG. In addition, no study which used monosodium glutamate reported positive effects of the drug on the intellectual functioning of retardates (Ellson, Fuller, & Urmston, 1950; Loeb & Tuddenheim, 1950; Milliken & Standen, 1951; Pallister & Stevens, 1957).

Only one paper reported an experimental comparison of the effects of the free acid and any one of its salts upon mental functioning. Albert, Hoch, and Waelsch (1951) found significant positive effects of the free, natural form of glutamic acid upon intellectual functioning of retardates. These investigators then selected 10 mental defectives who had responded to the free, natural acid with significant IQ rises and gave these patients a placebo, until their scores had returned to pretest level. At this point, the retardates were administered potassium glutamate for 3 to 4 months without their evidencing any signs of positive or negative intellectual change.

The Problem of Intellectual Assessment

Zimmerman and his colleagues (1948, 1949a, 1949b, 1950) originally held that intellectual ability was increased by glutamic acid in a way that was assessable with psychometric devices. Albert, Hoch, and Waelsch

(1946, 1951) held that intellectual ability as such was not increased by glutamic acid but that certain factors were enhanced which enabled a person to effectuate what intellectual ability was already present. These factors were concentration, attention, motivation, and persistence—what Wechsler (1944) has termed the "nonintellective" or "temperamental" components of intelligence. It follows from the position of Albert et al. that glutamic acid could effect cognitive behavior in a way which would be clinically apparent, but which would not necessarily be reflected in increased IQ scores. Not until quite late did Zimmerman (1959a, 1959b) accept the view of Albert et al. Finally, there were a number of writers who held that no aspect of intellectual functioning was affected by glutamic acid. Thus, the fact that a division existed among the glutamic acid adherents as to whether a clinical or a psychometric method should be employed to evaluate intellectual changes was lost in the larger issue of whether there was an effect of glutamic acid at all.

The evidence for or against any of these positions is difficult to evaluate, primarily because of the difficulty in ascertaining the scope of the clinical procedures, if any, which were employed in the various studies. It is not uncommon to find the comment "no clinical changes were noted" without any description of the procedures used to observe clinical phenomena. The studies reporting positive psychometric results, however, do tend to report associated clinical changes. For example, of the 31 positive studies listed in Table 2, 25 instituted clinical assessment procedures (all except Desclaux et al., 1953; DuPlessis, 1953; Harney, 1950; Jaeger-Lee, 1953; Kane, 1953; Zimmerman et al., 1949b), and all 25 reported some kind of clinically observable intellectual changes, generally in the direction of increases in mental activity, alertness, spontaneity, and motivation. On the other hand, if glutamic acid affects clinical aspects of cognitive behaviors independent of psychometric change, there should be studies which report clinical changes in the absence of psychometric changes. Several such studies, in fact, exist (Bergius, 1954; Göllnitz, 1952; Harbauer & DeBoor, 1952). However, it cannot be denied that reports of positive clinical change may be systematically biased in those instances in which the judge is aware of which subjects are receiving the active medication.

Thus, an aspect of the psychometric assessment procedure which deserves mention involves the tester's or clinician's knowledge of whether he is examining a control or a glutamic acid patient. Astin and Ross stressed the desirability of keeping the tester "blind" in order to preserve his objectivity. It was partially on the basis of more frequent "blind testing" that they based their judgment of the superior methodology of the negative studies; blind testing has been employed by only four positive studies (Albert, Hoch, & Waelsch, 1951; Contini-Poli, 1950; Zimmerman & Burgemeister, 1959a, 1959b), but by nine negative studies (Bergius, 1954; Ellson, Fuller, & Urmston, 1950; Harbauer & DeBoor, 1952; Hellstrom & Melin, 1952; Lombard, Gilbert, & Donofrio, 1955; McCulloch, 1950; Milliken & Standen, 1951; Pallister & Stevens, 1957; Zabarenko & Chambers, 1952).

Intellectual Stimulation and Glutamic Acid

Glutamic acid studies have followed a general pattern: subjects were first evaluated intellectually, then given glutamic acid and reevaluated; the degree of intellectual change over the course of treatment was usually compared with that of a "no treatment" group. The environmental stimulation intervening between the beginning and end of glutamic acid or placebo treatment was held constant for both groups. This has usually meant that, with the exception of the brief periods each day during which the experimental or control medication was taken, the subjects were not subjected to any special training experiences, that is, they continued with their daily routines as before.

The difficulty with this method is that it does not conform to the general theoretical view of the origins of abilities upon which estimates of intelligence are based. Abilities are generally thought to be acquired as a function of experience, reward, etc. (Ferguson, 1954; 1956). It is meaningless to give an intelligence test which requires demonstra-

tion of particular abilities and kinds of information if the testee has never been exposed to situations which permit him to acquire these skills and information. Similarly, it is meaningless to assess the effects of a drug on intelligence with tests of specific skills and information when the subject has had no opportunity during the course of the drug administration to acquire new information or skills. At least one investigator (Clapp, 1949) has made the clinical observation that glutamic acid seems to have little effect unless it is embedded in a program of formal training.

The literature does provide several examples of studies in which children who were attending special classes for retardates were given glutamic acid therapy; such a class may be thought of as providing an intellectually enriched environment. It is especially suggestive that two of the most successful glutamic acid studies employed students who were enrolled in classroom situations: Levine (1949) reported a mean rise of 13 IQ points for retarded pupils fed glutamic acid who were enrolled in special classes for the deaf. Contini-Poli (1950) reported a mean rise of 15 IQ points for slow school children fed glutamic acid who were enrolled in special classes, as opposed to a 2-point rise for control pupils on a placebo. Other investigators have also reported statistically significant IQ rises or significant improvements on cognitive tasks after glutamic acid treatment was given to children who were receiving training in special schools (DuPlessis, 1953; Harney, 1950; Koch, 1954; Schwöbel, 1952). On the other hand, at least four studies of retarded children in special classes observed no effect of glutamic acid treatment upon cognition (Harbauer & DeBoor, 1952; Lombard et al., 1955; Oldfelt, 1952; Zublin & Lutz, 1953). However, Oldfelt's (1952) results should be regarded with caution since half of the subjects were not given their postexperimental psychometric evaluations until 3 months after treatment had ended. If Oldfelt's study is set aside, a total of seven studies which have administered glutamic acid in a context of formal training have reported positive results (Clapp, 1949; Contini-Poli, 1950; DuPlessis, 1953; Harney, 1950; Koch, 1954; Levine, 1949; Schwöbel, 1952) as opposed to three studies which reported negative results (Harbauer & DeBoor, 1952; Lombard et al., 1955; Zublin & Lutz, 1953). Thus, it seems that embedding glutamic acid in a program of classroom training improves the chance of obtaining an increment in IQ.

Effect of Glutamic Acid upon Retardate Intelligence: A Summary

Several important differences discriminate the positive and negative glutamic acid studies. The positive studies tended (*a*) to employ outpatient as opposed to inpatient retardates; (*b*) to pay more attention to individual differences among retardates in response to the effects of glutamic acid; (*c*) to employ varying dosage levels of glutamic acid for different individuals rather than giving all subjects the same amount of the drug; (*d*) to medicate patients with the free form rather than with the salt of glutamic acid; and (*e*) to utilize clinical assessments in addition to psychometrics, rather than relying solely on the latter to determine intellectual functioning. About an equal number of positive and negative studies employed control groups and used placebos. There are two areas in which the negative studies, taken as a group, may be said to evidence greater experimental rigor than the positive studies: first, more negative than positive studies employed "blind" psychological testing. Second, (a point not mentioned earlier) more negative than positive studies attempted to equate glutamic acid and the placebo for taste.[3]

In those cases in which control groups were employed, the reported procedures do not seem to differentiate in any major way between the positive and negative glutamic acid studies. For example, no instances were found in which the experimental and control groups were not (*a*) chosen from the same parent population, (*b*) of equivalent chronological

[3] It should be noted that of the studies reporting attempts to equate the placebo and glutamic acid for taste, Loeb and Tuddenham (1950) and Oldfield (1952) reported that they were unable to do so. Ellson et al. (1950) reported only that the two tastes were "similar." It is unclear what degree of success the other studies enjoyed in this regard.

age, (c) kept under (apparently) equivalent environmental conditions. However, there were three studies in which the several retardate groups under investigation were not of equivalent intelligence levels on initial testing (McCulloch, 1950; Zabarenko & Chambers, 1952; Zimmerman, Burgemeister, & Putnam, 1947). In the first two studies, no effect of glutamic acid was reported; in the last, there was a regression effect, but, as discussed previously, the superiority of the glutamic acid group remained even after the regression effect had been taken into account. In Oldfelt's (1952) negative study, half of the subjects did not receive their final psychometric evaluation until 3 months after treatment had ended; thus, it seems possible that any beneficial effect of treatment upon cognition might have been lost in the interim between end of treatment and final evaluation.

It appears then that the effectiveness of glutamic acid treatment in mental retardation which has come to be considered a closed question with an answer in the negative might well be reopened. The application of both methodological rigor and clinical sophistication is required in any psychological research venture; it is clear that the two have not been combined in any optimum balance in the history of the investigation of the effects of glutamic acid upon mental retardation.

Results of Glutamic-Acid Administration on the Intellectual Processes of Normal Persons

While it is generally unrecognized, a large number of well-designed studies have reported positive effects of glutamic acid upon the cognitive functioning of normals. In fact, the efficacy of glutamic acid in improving the cognitive capacity of normal subjects is apparently more pronounced than its effect with retardates.

The first study to report positive effects of glutamic acid treatment upon normals was that of Milliken and Standen (1951), who administered monosodium glutamate to a sample of boys under 10 years of age and a sample of boys over 10 years of age. An individualized method of drug administration was employed (the boys received from 20–36 grams per day). By any technical standard, the study was well designed: control groups, matched for age, received placebos matched for appearance and taste with glutamic acid; treatment was continued for 3 months, after which the experimental and placebo medication was switched. The study was double-blind. No significant differences between placebo and glutamic administrations were found in the younger sample, but, in the older sample, the glutamic acid group was superior to the control group on the Arithmetic ($p < .05$) and Digit Symbol ($p < .01$) subtests of the Wechsler-Bellevue intelligence test. After the medication was switched, the current glutamic acid group showed superior performance to the current control group on the Comprehension, Block Design, and Digit Symbol subtests ($p < .025$).

Subsequently, Müller (1955), using six groups of normal adults, reported that a group of subjects fed 6 grams daily of the free, natural form of glutamic acid showed significantly greater resistance to fatigue and increased learning rates on simple tasks when compared to five other groups of subjects fed either lesser amounts of the same substance, or glutamic acid in the protein-bound form. Working with monozygotic twins, Müller (1957) fed half his subjects glutamic acid supplement and gave their twins another amino acid; he observed that the glutamic-acid-fed twins showed superior performance, and when the medication of the two groups was switched, the glutamic acid group again showed superior performance. Kergl (1952) reported superior intelligence-test performance in 57 normal adults and children fed glutamic acid as compared with 10 normals fed a placebo. Koch (1954), employing children of normal intelligence, also found superior performance on intelligence tests in a group fed glutamic acid compared to a placebo-fed control group. Schwöbel and Tamm (1952) gave normal adults glutamic acid and reported improved performance on simple cognitive tasks, decreases in reaction time, improved motivation, and increased energy levels; however, they employed no control group.

Other positive studies have compared motivational and personality traits of groups of normal subjects fed glutamic acid, with groups of normals fed placebos matched for

taste and appearance with glutamic acid. Müller (1953a) reported that glutamic acid led to "increased drive, and heightened ability to utilize one's potential. Changes in self-concept occur so that the person feels more competent, and acts more competently." Studying convicted criminals of normal IQ, Müller (1953b) reported that the Rorschachs of the glutamic acid groups became less constricted than those of the placebo group. Kronicke, and Zinits and Kuhr, in unpublished studies cited by Mehl (1956), both reported positive effects of glutamic acid upon the motivational and drive states of high-level normals (Kronicke employed university professors and graduate students; Zinits and Kuhr used university students).

On the other hand, there have also been reports that glutamic acid had no effect on the performance of normal children or adults (Kane, 1953; Head, 1955; Mehl, 1956). Interestingly, neither Head nor Mehl employed the individualized method of drug administration.

Thus, most of the studies with normals support the view that glutamic acid has an influence on mental functioning. The German writers generally have expressed the opinion that glutamic acid achieves its effects by increasing drive, and by positively affecting other personality characteristics which, if they are not intellectual traits in the narrow sense of the term, are certainly related to intellectual performance (Kronicke, and Zinits & Kuhr, cited by Mehl, 1956; Müller, 1953a, 1953b, 1957; Schwöbel & Tamm, 1952). Alternatively, glutamic acid may increase capacity to deal with simple or speed-related cognitive tasks (Müller, 1955; Schwöbel & Tamm, 1952).

Significance of Glutamic Acid in Physiological Functioning

The voluminous biological literature on glutamic acid has focused heavily upon such theoretical issues as the role of glutamic acid in carbohydrate metabolism, in neural functioning, and in the removal of waste products. However, the psychiatric and psychological literature on the role of glutamic acid in intellectual functioning rarely makes reference to the biological literature. The mistaken impression is given that the only rationale for expecting glutamic acid to affect mental functioning is the purely empirical one that such an effect has been claimed in the past. Consequently, it seems appropriate at this point to discuss the physiological effects of glutamic acid which may be relevant to intellectual functioning.

1. One of the most comprehensively presented theories in regard to the behavioral effects of glutamic acid was Weil-Malherbe's (1950) proposal that the behavioral effects of glutamic acid were adrenergic; that is, that glutamic acid stimulated the release of adrenaline, thus stimulating increased mental and physical activity. However, Strecker (1957), reviewing the biochemical and physiological literature since Weil-Malherbe's paper, concluded that a number of mechanisms must necessarily be involved in promoting the varied physiological effects of glutamic acid, although he did not deny that adrenergic mechanisms were among them.

2. A generally accepted role of glutamic acid in the brain is the removal of intracellular ammonia; a convicing and well-documented statement of this thesis was presented by Weil-Malherbe (1950). Glutamic acid may exercise its effect upon mental functioning through its role in the removal of toxic waste, such as ammonia, from the brain.

3. There is evidence that in emergency conditions when glucose is in short supply, such as hypoglycaemic coma, glutamic acid substitutes for glucose in supporting respiration in brain tissue (Cravioto, Massieu, & Izquierdo, 1951; Dawson, 1949; Himwich & Sullivan, 1956; Himwich & Peterson, 1958; Waelsch, 1951; Waelsch, Schwerin, & Bessman, 1949). Consequently, it seems possible that glutamic acid may support the respiration of brain tissues in chronic conditions in which brain functioning has been impaired by reduced glucose supply.

4. Glutamic acid plays a central role in a complex pattern of protein and carbohydrate metabolism in nervous tissue generally and in the brain in particular; some excellent reviews of the physiological literature testify to this point (Waelsch, 1951; Tower, 1959). In fact, there are few metabolites in carbohydrate and protein metabolism

which are not related by some process to glutamic acid. Consequently, glutamic acid would be implicated in conditions in which disturbances of cognitive functioning are traceable to disturbances in the major metabolic processes supplying nervous tissue.

5. In 1943, Nachmansohn and his colleagues (Nachmansohn, Cox, Coates, & Machado, 1943; Nachmansohn & Machado, 1943) established the mechanism of the acetycholine cycle, a biochemical process which provides energy for neural transmission; and later Nachmansohn, John, and Waelsch (1943) accorded glutamic acid a major role in the cycle. Subsequently, glutamic acid became centrally implicated in many aspects of neural metabolism and functioning (Ames, 1956; Coxon & Peters, 1950; Davies & Krebs, 1952; Krnjević, Randić, & Straughn, 1964; Strecker, 1957; Terner, Eggleston, & Krebs, 1950; Tower, 1955, 1957; Way & Sutherland, 1963). If, as seems probable, disturbances in glutamic acid metabolism may lead to disturbances of cortical neural transmission, then these disturbances might also lead to disruption of cognitive functioning.

6. It is now generally assumed that the major locus of action of glutamic acid is in the brain. It is known that glutamic acid passes the blood-brain barrier and enters the brain (Lajtha, Berl, & Waelsch, 1959; Roberts, Flexner, & Flexner, 1959; Waelsch, 1958). However, the brain level of glutamic acid remains constant for a given individual no matter how much glutamic acid is introduced into the bloodstream (Friedberg & Greenberg, 1947; Schwerin, Bessman, & Waelsch, 1950). On the other hand, glutamine supplementation results in increases of the brain levels of glutamine (Waelsch, 1951; Weil-Malherbe, 1950). Consequently, it has been suggested that glutamic acid may effect behavioral change through the effect upon the brain of its metabolite, glutamine (Waelsch, 1951). Subsequent research has indicated that glutamine serves a major role in the maintenance of cerebral tissues (Strecker, 1957); indeed, there is indication that glutamine is essential for the growth and maintenance of almost all living cells (Meister, 1956).

Consequently, some writers (Rogers & Pelton, 1957) came to believe that any facilitory effects of glutamic acid upon mental functioning may rest in its ability to produce glutamine. Rogers and Pelton (1957) found that glutamine increased the intelligence test scores of retardates significantly more than did a placebo, and Beley, Caustier, and Olievenstein (1964) also found significant differences between the effects of glutamine and a placebo on the test behavior of mentally retarded children. Farina, Collinet, and Collinet-Marchal (1961) reported IQ improvement following glutamine administration in children of normal intelligence, but these authors did not employ a control group.

7. Glutamic acid is metabolized, in part, into an amine, gamma-aminobutyric acid (GABA). It has recently been suggested that GABA plays a major role at postsynaptic junctions by coordinating and regulating electrical activity, and by aiding the depolarized nerve to recover and fire once more (Roberts, Wein, & Simonsen, 1964). As such, GABA inhibits neural fatigue and, conversely, enables the neural fibers to be receptive to continuous stimulation. The ability to resist neural fatigue might well underlie the increased attention, persistence, and ability to perform simple repetitive tasks reported to follow the administration of glutamic acid (Müller, 1955; Schwöbel & Tamm, 1952). In this connection, it is interesting to note that GABA is a monoamine which is metabolized by the enzyme monoamine oxydase (MAO). MAO has recently been reported to be inversely related to speed of performance of simple repetitive tasks in normal adults (Klaiber, Broverman, & Kobyashi, 1965).

Conclusion

A considerable amount of evidence indicates a role for glutamic acid in cogntive functioning. The sheer weight of this evidence is surprising; indeed, there is more sound experimental confirmation of the positive effects of glutamic acid than appears to exist for the most of the psychotropic drugs now in common therapeutic use. The earlier judgments that glutamic acid has little behavioral effect may be considered to be based upon an incomplete or unsound analysis.

We have tried to discover and analyze the problems which have hampered research. The major problem seems to have been a lack of rapport among behavioral scientists, clinicians, and biological scientists in an area in which communication would seem imperative if a competent research effort is to be maintained.

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Preliminary Communication

THE EFFECT OF GLUTAMIC ACID ON THE COMA OF HEPATIC FAILURE

DESPITE much recent research the underlying biochemical disturbances in hepatic coma remain obscure. In an earlier communication [1] the rôle of disordered amino-acid metabolism in the pathogenesis of this syndrome was emphasised, particularly with reference to glutamic-acid metabolism in the brain and the possible presence of methionine sulphoxide, a glutamic-acid antimetabolite, in the plasma and cerebrospinal fluid of certain cases of hepatic coma. Glutamic acid ($COOH.CH_2.CH_2CH.NH_2COOH$) is the only amino-acid oxidised by the brain [2]; it is deaminated to α-ketoglutaric acid, which enters the Krebs cycle and therefore associates glutamic acid with carbohydrate metabolism. It binds ammonia to form glutamine, which is then metabolised without liberating ammonia. Waelsch [3] believed that the equilibrium between glutamic acid and glutamine in the brain might act as one of the metabolic buffers of the tricarboxylic-acid cycle and also ensure an adequate supply of α-ketoglutarate. However, according to Weil-Malherbe [4] the primary rôle of glutamic acid in cerebral metabolism is based on the three enzymic reactions deamination, transamination, and amidation, which are all part of an integrated system for the removal of intracellular ammonia. Glutamic acid is also essential for the synthesis of acetylcholine [4] and for cation transport in the brain,[5] kidney,[6] and probably in other tissues.

In view of the important rôle of glutamic acid in both cerebral and hepatic metabolism and its action in

1. Walshe, J. M. *Quart. J. Med.* 1951, **20**, 421.
2. Quastel, J. H., Wheatley, A. H. M. *Biochem. J.* 1932, **26**, 725; Krebs, H. A., *Ibid*, 1935, **29**, 1620, 1951; Weil-Malherbe, H. *Ibid*, 1936, **30**, 665.
3. Waelsch, H. *Lancet*, 1949, ii. 1.
4. Weil-Malherbe, H. *Physiol. Rev.* 1950, **30**, 549.
5. Terner, C., Eggleston, L. V., Krebs, H. A. *Biochem. J.* 1950, **47**, 139.
6. Stern, J. M., Eggleston, L. V., Hems, R., Krebs, H. A. *Ibid*, 1949, **44**, 410.

removing excess ammonia, which may be one of the toxic factors producing hepatic coma, it was decided to try the effect of large doses of the amino-acid intravenously in hepatic coma. Glutamic acid was prepared for intravenous use, as the sodium salt, by the method of Mayer-Gross and Walker.[7] To date it has been used in five episodes of coma in three patients with subacute or chronic liver damage. It has not yet been possible to try this therapy in any patient with acute virus necrosis of the liver.

CASE-RECORDS

Case 1.—A man, aged 45, with long-standing cirrhosis of the liver associated with ulcerative colitis, had hepatosplenomegaly, ascites, and œsophageal varices. Laboratory tests showed strongly positive serum flocculation reactions, a low serum-albumin level and a slightly raised serum-bilirubin level. He was admitted to hospital in February, 1952, after a "flare-up" of his colitis. There was little change in his condition until the morning of March 9, 1952, when he was found to be in deep coma. There was a strong fetor hepatis and the plantar responses were doubtfully extensor. Intravenous glucose therapy was started together with B vitamins. Next day coma was deeper, the patient was hiccuping almost continuously, his plantar responses were definitely extensor, and he had a transient ankle clonus. By that evening his condition appeared to be deteriorating, and it was decided to give intravenous glutamic acid; 23 g. of the sodium salt was given at 6 P.M. The immediate effect was flushing, salivation, and increased depth of respiration. Some four hours later the patient regained consciousness, next day he was mentally normal, and he has remained so to the time of writing fourteen months later.

Case 2.—A man, aged 51, was admitted to hospital after a hæmorrhage from large œsophageal varices. As the laboratory tests suggested that liver damage was slight, he was considered a suitable case for surgery, and on March 25, 1952, a side-to-side portacaval shunt was constructed. Four days after operation he became confused and was found to have increased tendon-jerks and bilateral ankle clonus; there was a strong fetor hepatis. Next day, March 30, a high-protein milk drip by intragastric tube was started. By April 2 he was in deep coma and, besides his other abnormal reflex signs, he had developed bilateral extensor responses. Fluid balance was maintained with intravenous glucose and electrolyte solutions, and on this day 23 g. of sodium glutamate was added to the intravenous drip. This led to a lessening in the depth of coma, and he became noisy and restless. On April 4 a further 23 g. of glutamate was given, and during the day he regained consciousness, although his abnormal reflex signs persisted for a further twenty-four hours. He progressed well until April 22, when he became mentally confused, and examination again revealed a prolonged ankle clonus. Next day he was in light coma, 10 g. of glutamic acid was given by gastric drip, and by the following morning his mental state was almost normal. Oral glutamic acid was given until April 28, when it was discontinued. On the morning of April 29 he was confused and noisy, and all the abnormal neurological signs noted earlier had returned. Intravenous glucose was again started, but by evening he was in light coma; 23 g. of sodium glutamate was therefore added to the drip, and next morning he was sufficiently improved to coöperate and to take food by mouth; treatment with oral glutamic acid was started again, and he was given 10 g. daily for three weeks before the dose was reduced over the subsequent two weeks. No further episode of coma occurred until he died at home in coma some eight months later.

Case 3.—A married woman, aged 61, was admitted to hospital for investigation with a history of recurrent attacks of jaundice over the previous twenty months; these had been associated with loss of weight and a gradual deterioration in her health. Laboratory tests showed strongly positive serum flocculation reactions, a low serum-albumin level and a high serum-globulin level. A firm liver was palpable just below the right costal margin, and there was a large spider nævus on the left shoulder. On April 11, 1953, three weeks after admission to hospital, she became confused and did not recognise her relations. She remained able to take milk and glucose by mouth, and B vitamins were given intramuscularly. By April 14 there was much fluid retention and oliguria, for which she was treated with the ion-exchange resin 'Katonium' (in the usual 75% ammonium form); she was also given oral glutamic acid 20 g. daily. Urine flow was re-established by April 17, but coma was deeper, and there were extensor plantar responses and ankle clonus. Treatment with intravenous glucose and electrolytes was started, and all other therapy was stopped. Next day, the eighth day in coma, her condition was deteriorating, and in the evening 23 g. of sodium glutamate was added to the intravenous drip. During the night the coma became lighter, and by the following morning she was just able to recognise her relations and to answer simple questions. That afternoon a further 23 g. of glutamate was given, and by 8 P.M. she was fully conscious and demanding food. Treatment with oral glutamic acid was started, but she became confused and aggressive about thirty-six hours after emerging from coma; the dosage of the glutamic acid was therefore raised to 30 g. daily, which coincided with a change to a normal mental state, and her tendon-jerks and plantar responses returned to normal. At the time of writing (May 19) this improvement has been maintained.

DISCUSSION

The failure of oral glutamic acid to terminate the episode of coma in case 3 may well have been due to the simultaneous administration of the ion-exchange resin katonium in the 75% ammonium form. Possibly this neutralised the ammonia-binding power of the glutamic acid before it could influence cerebral metabolism. It has certainly been shown that ammonium ion-exchange resins and ammonium chloride can produce a syndrome closely mimicking hepatic coma in patients with cirrhosis of the liver.[8] Apart from this one failure glutamic acid appeared to exert a beneficial effect in five episodes of coma, on four occasions after intravenous use and once on oral administration; on one occasion also the withdrawal of glutamic-acid therapy appeared to precipitate coma. This evidence strongly supports the thesis that glutamic acid influences favourably the coma that may develop in patients with subacute and chronic liver disease. Whether it will have any effect on the course of acute virus necrosis of the liver remains to be seen, but in this condition it cannot act as more than a supportive therapy until such time as the virus invasion can be dealt with by the natural defence mechanisms. The exact means whereby glutamic acid exerts its effect in restoring consciousness to patients in hepatic coma is not clear; it is not even certain if this action is on cerebral or hepatic metabolism, although it seems more likely that the beneficial effect is on the brain rather than the liver. The most probable explanation is that glutamic acid acts by its ammonia-binding power, but its action on the Krebs cycle may also be important in view of the findings of Amatuzio and Nesbitt,[9] that the blood-pyruvate level is considerably raised in hepatic coma, and the work of Seligson et al.,[10] who have found that in blood-pyruvate and blood-α-ketoglutarate levels are raised in patients with liver injury. Whatever the mechanism of its action, intravenous glutamic acid appears worthy of further trial in the treatment of hepatic coma. In this connection the following method of administration has proved satisfactory: 20 g. of glutamic acid is neutralised with sodium hydroxide to give 23 g. of sodium glutamate in 80 ml. of water[7]; this solution is added to 500 ml. of 5% glucose and given intravenously over three or four hours. Too rapid administration leads to salivation, flushing, and vomiting and should therefore be avoided.

Not more than 23 g. of sodium glutamate has been given intravenously on any one day, because this contains 130 m.eq. of sodium and, if used in larger doses, might lead to undesirable retention of water. For oral use

7. Mayer-Gross, W., Walker, J. W. *Ibid*, p. 92.

8. Gabuzda, G. J. jun., Phillips, G. B., Davidson, C. S. *New Engl. J. Med.* 1952, **246**, 124. Phillips, G. B., Schwartz, R., Gabuzda, G. J. jun., Davidson, C. S. *Ibid*, **247**, 239.
9. Amatuzio, D. S., Nesbitt, S. *J. clin. Invest.* 1950, **29**, 1486.
10. Seligson, D., McCormick, G. J., Sborov, V. *Ibid*, 1952, **31**, 661.

0·5 g. tablets of the free amino-acid have been used and appear to be well tolerated by these patients when given in divided doses to a total of 20 g. daily.

SUMMARY

The effect of glutamic acid on the coma of hepatic failure has been investigated.

Five episodes of coma in three patients with subacute or chronic liver injury were treated with glutamic acid.

On each occasion a return of consciousness closely followed the administration of glutamic acid, which therefore appears worthy of further trial in the treatment of hepatic coma.

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72. *Effects of Prolonged Monosodium Glutamate and Other High-Salt Diets on Arterial Pressure and Learning Ability in Rats.* L. R. WEISS, J. F. REILLY, J. WILLIAMS, and S. KROP, Food and Drug Administration, Washington, D.C.

A study was undertaken to compare and estimate the toxicologic significance of certain high-salt diets in rats and to test pharmacologically for changes in learning ability, motor activity, blood pressure, and minimal convulsive thresholds. Diets containing monosodium glutamate (MSG) and monopotassium glutamate (MKG), each 10%, or sodium chloride (NaCl) and potassium chloride (KCl), each equivalent to the cation in the glutamate salt, were given to groups of male weanling rats for approximately 34 wk. Observations on growth rates and food intakes were made weekly. Pharmacologic measurements were made after 24 wk on these diets. After 12 and 24 wk, animals receiving MSG showed reductions in weight gain as a group significantly different from controls, while groups receiving the other substances had smaller changes in this respect. Food consumption was similar in all groups. After 24 wk, indirect arterial systolic pressures, as measured in unanesthesized rats by a tail-cuff (plethysmographic) technique, were significantly elevated in MSG rats, as were those in NaCl rats, though to a lesser extent; in contrast, MKG and KCl rats were normotensive. Motor activity was unchanged but amphetamine-induced stimulation of motor activity was reduced in MKG and KCl rats as compared with controls, MSG and NaCl rats. Tests of learning deficits in an avoidance-escape situation indicated that animals receiving MSG failed to develop avoidance lever-pressing behavior. This effect was also seen in some NaCl rats. In contrast, MKG, and especially KCl, animals showed a high rate of avoidance responding suggesting an enhancement of avoidance learning by potassium salts in rats; these results point to the possibility of pharmacologic interactions between drugs and high-salt diets.

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Variation of Glutamate Metabolism with the Site of Injection*

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Previous reports from this laboratory (1, 2) suggested a pathway of glutamate-2-C^{14} catabolism in rats other than direct conversion to succinate via α-ketoglutarate. This pathway was not observed in rats poisoned with fluoroacetate (2).

These conclusions were based on the observation (1, 2) that, after supposed intraperitoneal adminstration of DL-glutamic acid-2-C^{14} to normal rats, a considerable percentage of the total radioactivity was found in the noncarboxyl portion of aspartic acid, alanine, and serine, and in carbon atoms 3 and 4 of glutamic acid. In contrast, rats poisoned with fluoroacetate synthesized aspartate, alanine, and serine essentially carboxyl-labeled and glutamate labeled in positions 1 and 2.

All attempts to repeat the experiments (1, 2) involving "intraperitoneal" administration of DL-glutamic acid-2-C^{14} to normal rats have been unsuccessful. Therefore, the possibility that the previous "intraperitoneal" injections (1, 2) had actually been injections into some part of the gastrointestinal tract was tested with results described herein.

EXPERIMENTAL

Male albino rats (of Wistar origin) from our own colony were used. DL-Glutamic acid-2-C^{14}, obtained from Isotopes Specialties Company (0.4 mc. per mmole) or from Volk Radiochemical Company (0.7 mc. per mmole), was dissolved in 1 to 2 ml. of 0.9 per cent sodium chloride and administered to rats by intraperitoneal injection, by stomach tube, or by injection into the cecum. The animals which received the isotopic material by injection into the cecum were anesthetized with ether before the opening of the abdominal wall. After the abdominal wall had been closed, these rats were placed in a glass metabolism chamber, as were the other animals.

The rats were killed 4 hours after the administration of DL-glutamic acid-2-C^{14}, and the "carcass" and liver amino acids were isolated, degraded, and assayed for radioactivity by methods previously described (2, 3). "Carcass" refers to the entire animal, except liver, including the washed gastrointestinal tract.

RESULTS AND DISCUSSION

The results after intraperitoneal injections are presented in Table I, and the data obtained from the rats given DL-glutamic acid-2-C^{14} by stomach tube or by injection into the cecum are presented in Table II below.

Conversion of glutamic acid-2-C^{14} to α-ketoglutarate and hence to succinate should yield aspartic acid labeled in the carboxyl carbons and glutamic acid labeled in carbon atoms 1 and 2. The data in Table I show that most of the glutamate metabolized after intraperitoneal injection was converted to succinate via α-ketoglutarate. Variations in animal size or nutritional state or in the dosage of glutamate (Table I) did not affect the results. Thus, we have been unable to confirm our previous observations (1, 2) that a significant portion of the total radioactivity was present in the noncarboxyl carbons of aspartic acid and in carbon atoms 3 and 4 of glutamic acid. The data presented in Table I are actually in close agreement with those previously obtained after "intraperitoneal" administration of DL-glutamic acid-2-C^{14} to rats poisoned with fluoroacetate (2). Therefore, our previous claim (2) that fluoroacetate poisoning drastically alters glutamate metabolism is not valid.

In contrast to the data presented in Table I, the results obtained from the injections into the cecum (Rats 54 and 62, Table II) duplicate those previously reported (1, 2) for "intraperitoneal" injections. Therefore, it appears that the "intraperitoneal" injections (1, 2) were actually intragastrointestinal. The labeling patterns observed in Rats 54 and 62 are almost identical to those observed after intraperitoneal injection of acetate-2-C^{14} (3). Thus, it must be concluded that in the cecum or during absorption from the cecum, carbon 2 of glutamate is converted to the methyl carbon of acetate. Whatever the mechanism, this conversion occurs rapidly and almost quantitatively; little or no glutamate is converted directly to α-ketoglutarate. The very slight preponderance of carbon 2 of isolated glutamate over carbon 3 supports this concept.

The data presented do not suggest a mechanism for acetate formation from glutamate. Wachsman and Barker (4) and Munch-Petersen and Barker (5) have reported that *Clostridium tetanomorphum* converts carbon 2 of glutamate to the methyl carbon of acetate, with mesaconate and citramalate as intermediates. A similar sequence of metabolism in cecum bacteria would explain our results. However, reductive deamination of glutamate-2-C^{14} to glutarate-2,4-C^{14} via the Stickland reaction (6) with subsequent formation of acetate-2-C^{14} is another feasible mechanism. Work in this laboratory (7) has shown that carbon atom 3 of glutarate is metabolized via the carboxyl carbon of acetate. Although the mechanism is unknown, carbons 2 and 4 of glutarate could be precursors of the methyl of acetate.

The results found after feeding DL-glutamic acid-2-C^{14} by stomach tube (Rats 49 and 52, Table II) are intermediate between the data from the intraperitoneally-injected and from the cecum-injected animals. Apparently a portion of the glutamate fed was absorbed and metabolized via α-ketoglutarate and succinate,

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TABLE I

Labeling patterns in carcass glutamic and aspartic acids after administration of DL-glutamic acid-2-C^{14} by intraperitoneal injection

	Rat 25*	Rat 26†	Rat 27‡	Rat 39§	Rat 41¶
Rat weight (gm.)	151	240	136	125	125
Dose injected (μc.)	25.1	26.8	25.0	51.3	44.5
Per cent injected, exhaled as $C^{14}O_2$	26.3	32.3		30.8	25.7

C^{14} in carcass amino acids

	mμc/mM	per cent of total	mμc/mM	per cent of total	mμc/mM	per cent of total	mμc/mM	per cent of total	mμc/mM	per cent of total
Glutamic acid										
Total	30.2		26.6		56.3		76.3		56.1	
Carbon 1, COOH		19.8		20.2		17.2		20.2		17.5
Carbon 2, $CHNH_2$		77.8		66.0		77.6		74.7		78.4
Carbon 3, CH_2		1.7		0.8		1.5		1.0		0.9
Carbon 4, CH_2		1.0		0.2		0.9		0.3		0.2
Carbon 5, COOH		0.7		1.6		0.6		1.5		0.3
tic acid										
al	10.8		9.0		17.3		26.9		18.0	
arbon 1 + 4, COOH		95.3		94.0		89.6		95.5		94.1

* Rat 25 was given 1 gm. of glucose by stomach tube 30 minutes before injection of DL-glutamic acid-2-C^{14}.

† Rat 26 weighed about twice as much (240 gm.) as did the other animals.

‡ Rat 27 was an exact duplicate of previous (1, 2) normal rats.

§ Rat 39 was given 12 mg. of carrier glutamic acid with about 12 mg. of the labeled compound.

¶ Rat 41 was fasted for 44 hours before the administration of DL-glutamic acid-2-C^{14}.

TABLE II

Labeling in carcass amino acids after administration of DL-glutamic acid-2-C^{14} by stomach tube and cecum injection

	Rat 49*	Rat 52*	Rat 54†	Rat 62†
Rat weight (gm.)	135	110	145	136
Dose injected, (μc.)		37.8	42.3	33.0
Per cent injected, exhaled as $C^{14}O_2$		32.0	18.7	28.5

C^{14} in carcass amino acids

	mμc/mM	per cent of total	mμc/mM	per cent of total	mμc/mM	per cent of total	mμc/mM	per cent of total
Glutamic acid								
Total	9.1		29.5		54.5		75.0	
Carbon 1, COOH		16.4		19.7		9.9		10.9
Carbon 2, $CHNH_2$		60.4		55.9		23.1		27.5
Carbon 3, CH_2		7.7		4.7		21.8		19.6
Carbon 4, CH_2		12.1		9.6		38.7		33.9
Carbon 5, COOH		3.3		1.0		3.6		2.0
Aspartic acid								
Total	3.4		14.2		30.9		40.8	
Carbon 1 + 4 COOH		73.0		82.4		31.4		35.0
Alanine								
Total			11.3	11.2		14.4		
Carbon 1, COOH				88.5		17.9		22.4
Carbon 2, $CHNH_2$				5.3		40.5		36.8
Carbon 3, CH_3				4.4		40.7		31.3

* DL-Glutamic acid-2-C^{14} given by stomach tube.

† DL-Glutamic acid-2-C^{14} injected into the cecum.

and a significant amount was metabolized in a manner whereby carbon 2 was converted to the methyl carbon of acetate.

Glutamic and aspartic acids were isolated from the liver protein of Rats 39, 54, and 62. The labeling patterns in these amino acids were in every case similar to those observed in the corresponding carcass amino acids.

SUMMARY

The labeling patterns of tissue glutamic acid, aspartic acid, and alanine have been determined after administration of DL-glutamic acid-2-C^{14} to rats by intraperitoneal injection, by stomach tube, and by injection into the cecum. Conversion to succinate via α-ketoglutarate was the primary route of catabolism when glutamate was injected intraperitoneally. When glutamic acid was injected into the cecum, carbon atom 2 was converted to the methyl carbon of acetate. Glutamic acid administered by stomach tube was partially converted to succinate via α-ketoglutarate and was partially metabolized in a manner whereby carbon 2 was converted to the methyl carbon of acetate.

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THE EFFECTS OF GLUTAMIC ACID UPON OPERANT CONDITIONING IN RATS*

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A. INTRODUCTION

In a recent article, Vogel, Broverman, Klaiber, and Draguns (13) demonstrated that, despite previous judgments to the contrary, ample evidence exists to indicate that glutamic acid plays a significant role in the cognitive behavior of both normal and retarded persons. Since the gultamic acid literature in the areas of human and rat behavior is methodologically and theoretically interdependent, it seems worthwhile to re-examine the basis for previous judgments that dietary supplements of glutamic acid do not affect the learning abilities of rats.

The majority of studies which have investigated the effects of glutamic acid supplementation upon rat learning have employed mazes. Hughes and Zubek (3), Phillips (7), and Zimmerman and Ross (15) reported a beneficial effect of glutamic acid supplementation on maze learning, although Phillips was unable to obtain significant results with a second group of animals, with the use of a modified design. On the other hand, there have been many reports of no beneficial effect of glutamic acid upon maze learning (2, 4, 5, 6, 8, 9, 10, 12). These negative reports include two unsuccessful attempts by Stellar and McElroy (12) and Hughes, Cooper, and Zubek (4) to replicate the earlier positive findings of Zimmerman and Ross (15) and of Hughes and Zubek (3), respectively.

The failure to establish a positive effect of glutamic acid supplementation on maze learning may be interpretable in terms of early work by Weil-Malherbe (14), and recent work by Broverman, Klaiber, Kobayashi, and Vogel (1). Broverman *et al.* (1) have reported that adrenergic states, such as are induced by epinephrine and norepinephrine, facilitate performances of

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simple, overlearned, perceptual-motor tasks (i.e., bar pressing), but impair performance of perceptual-restructuring tasks (i.e., maze learning). Weil-Malherbe (14) argued that the beneficial results of glutamic acid therapy which had been obtained with mentally retarded persons were attributable to its adrenergic effects: i.e., the stimulation of epinephrine release by glutamic acid. Weil-Malherbe's theory would account for the heightened levels of spontaneous behavioral and mental activity so commonly noted in both retarded and normal recipients of glutamic acid supplementation (13).

Thus, the generally negative findings in regard to the effect of dietary supplements of glutamic acid upon maze performance may be attributable to the fact that maze learning is not a task which is facilitated by adrenergia; in fact, it is normally impaired by an enhanced adrenergic state (1). In this view, the appropriate tasks to employ in order to ascertain if glutamic acid facilitates learning would be simple, perceptual-motor tasks, such as the operant conditioning of bar pressing, or the turning of an activity wheel, both of which would place a premium on the heightened spontaneous activity characteristic of adrenergic states (1). No study of the effect of glutamic acid upon operant conditioning has been reported in the literature. In the only study reported of the effects of glutamic acid upon activity-wheel behavior, Phillips (7) found animals fed glutamic acid to be significantly more active than a group of control rats.

The purpose of this study, then, is to ascertain the effect of different levels of glutamic acid supplementation upon the operant conditioning of bar-pressing behavior in rats, and upon the spontaneous activity levels of rats. The effects upon these same behaviors of vitamin B_6, an enzyme active in the metabolism of glutamic acid, will also be studied. The additional vitamin B_6 should have the effect of speeding the metabolism of glutamic acid. However, it is unclear whether the behavioral effect would be to increase activity levels by enhancing the speed and efficiency of glutamic acid metabolism, or to decrease activity levels through overly rapid depletion and clearance of glutamic acid.

B. Method

Thirty-day-old Sprague-Dawley male rats were divided into six groups of 10 rats each. The groups were each given daily one of the dietary supplements in the following list; in addition, all groups received a standard diet of Purina Rat Chow: (*a*) no additional supplement (control group); (*b*) 50 mg of L (+) glutamic acid; (*c*) 200 mg of L (+) glutamic acid; (*d*) 400 mg of L (+) glutamic acid; (*e*) 200 mg of L (+) glutamic acid plus 1/100 mg vitamin B_6; and (*f*) 1/100 mg vitamin B_6.

All rats were supplied Purina Rat Chow *ad libidum,* from 9 p.m. through 9 a.m., when the food was removed. Each day, prior to 9 p.m., the various experimental diets were fed to the rats. The experimental drugs were mixed with applesauce in order to disguise the bitter taste of glutamic acid. The control group was also fed applesauce, but with no B_6 or glutamic acid supplement. There were no significant differences in weight among the various groups of animals at either the beginning or the end of the experiment.

The animals were administered the experimental tasks after puberty had been passed, at 90 days of age, after having been on their respective diets for 60 days. On each day of testing, each rat to be tested was given only the applesauce mixed with his supplemental diet and was not fed the normal basal diet. Thus, when tested, all rats were 23 hours hungry except for the ingestion of applesauce and the supplemental diet. All rats were tested at 10 p.m., when they were placed in the Grason-Stadler testing chamber for the first time.

Each rat was initially reinforced 50 percent of the time whenever the lever was pressed in the Grason-Stadler testing chamber. After being reinforced in this manner for 25 reinforcements, the rats were then reinforced according to a fixed ratio schedule of four to one until 125 pellets were dispensed. The total amount of food pellets was precalculated to approximate 50 percent of a rat's normal diet. Thus, when extinction was initiated, all rats had been reinforced an equal number of times. Learning was measured in terms of both number of responses, and the amount of time until the criteria of mastery and extinction were reached. Mastery was operationally defined as the first significant concentration of responses; e.g., 50 responses in 10 minutes. Extinction was operationally defined as no response for one-half hour.

Approximately two days after testing in the Grason-Stadler chamber, each rat was placed in an activity wheel for a period of one-half hour in order to ascertain directly the animal's spontaneous level of activity when placed in a novel situation. The total revolutions for each rat were measured by an automatic counter.

C. Results

Nonparametric tests have been employed throughout, since in several instances the distribution of data appeared to depart from normality.

1. *Activity Wheel*

A Kruskal-Wallis one-way analysis of variance applied to the activity-wheel data for all groups (see Table 1) reveals a significant difference ($p < .05$) among the various groups. Mann-Whitney U tests between pairs of groups

TABLE 1
ACTIVITY-WHEEL AND BAR-PRESSING PERFORMANCE

Group	N	Number of activity-wheel revolutions		Time required (minutes) to attain bar-pressing mastery		Number of responses required to attain bar-pressing mastery		Time required (minutes) to extinguish bar-pressing response		Number of responses required to extinguish bar-pressing response	
		M	SD	M	SD	M	SD	M	SD	M	SD
Control	10	38.7	24.6	65.6	37.3	74.2	13.2	98.8	46.5	269.1	76.8
50 mg GA	10	26.0	17.8	69.8	29.6	72.6	27.8	70.5	24.1	149.0	37.9
200 mg GA	10	51.4	29.0	54.8	29.5	57.0	24.8	43.7	23.1	134.9	67.4
400 mg GA	10	33.6	11.1	71.4	31.8	80.5	31.2	46.5	24.5	135.0	33.8
B_6 200 mg GA	10	39.2	12.3	91.4	48.0	72.0	26.6	55.7	20.8	168.9	62.9
B_6	10	25.5	8.4	73.2	22.2	91.3	34.1	41.9	29.5	147.2	87.6

reveal that at the $p < .05$ level of confidence, the 200 mg glutamic acid and the 200 mg glutamic acid-B_6 groups evidence more revolutions than either the 50 mg glutamic acid group, or the B_6 group. There are no other significant differences between groups.

2. *Mastery of Bar-Pressing Responses*

The means and standard deviations for both the number of responses and the time required to reach the criterion of mastery are presented in Table 1. A Kruskal-Wallis one-way analysis of variance reveals a significant difference ($p < .01$) among the groups in regard to number of responses to mastery. Further analysis with Mann-Whitney U tests between pairs of groups reveals that at the $p < .05$ level of confidence, the 200 mg glutamic acid group reaches the criterion of learning in fewer responses than either the B_6 or the control groups, and the 50 mg glutamic acid group reaches the criterion of learning in fewer responses than the B_6 group. A Kruskal-Wallis one-way analysis of variance reveals no significant difference among the various groups on the basis of time until mastery.

3. *Extinction of Bar-Pressing Responses*

The means and standard deviations for the number of responses until extinction, and for time elapsed until the criterion of extinction is reached, are reported in Table 1. A Kruskal-Wallis one-way analysis of variance, applied to the data for number of responses to reach the criterion for extinction, reveals a significant difference ($p < .001$) among the groups. Mann-Whitney U tests between pairs of groups show the control group to have emitted a significantly ($p < .01$) greater number of responses during the extinction trials than any other group. There were no other significant differences between groups.

In regard to the time required to reach the criterion of extinction, a Kruskal-Wallis one-way analysis of variance reveals a significant difference ($p < .05$) among the groups. Mann-Whitney U tests between pairs of groups indicate that the control group required significantly more time ($p < .05$) to extinguish than each of the other groups, with the exception of the 50 mg glutamic acid group. The 50 mg glutamic acid group required significantly more time to reach the criterion for extinction ($p < .05$) than the 200 mg glutamic acid group, the 400 mg glutamic acid group, and the B_6 group.

4. *Relationship Between Activity-Wheel and Bar-Pressing Performance*

Correlations were obtained for the group of 60 animals between revolutions of the activity wheel and the criteria for mastery and extinction of the

bar-pressing response. Animals with a large number of revolutions in the activity wheel attained the criteria for mastery of the bar-pressing response in significantly less time ($rho = -.42$, $p < .01$), and with a smaller number of bar-pressing responses ($rho = -.25$, $p < .07$) than did animals with a small number of revolutions. There were no relationships between activity-wheel performance and the criteria for extinction of the bar-pressing response.

D. Discussion

1. *Mastery of the Bar-Pressing Task and Performance on the Activity Wheel*

It is apparent from Table 1 that, as contrasted with the other groups, the 200 mg glutamic acid group displayed consistent superiority on the performance measures, especially in regard to mastery of the bar-pressing task and mastery of the activity wheel. The significant correlations between activity-wheel behavior and the criteria for mastery of bar pressing suggest that, as we had anticipated, both tasks are affected by a common factor, presumably the organism's level of activation. Consequently, performance on both tasks should be sensitive to the administration of glutamic acid, a sympathetic stimulant which increases activation. However, previous workers generally have made the assumption of a linear relationship between the amount of glutamic acid administered and specific criteria of learning. Our data suggest that the assumption is unwarranted. It appears that a curvilinear relationship exists between the optimum level of glutamic acid supplementation and its effectiveness in promoting simple, perceptual-motor rat behaviors.

The animals in the 400 mg glutamic group were hyperemotional, taking fright easily, huddling frequently, and attacking and biting with minimal provocation. These behaviors are reminiscent of hyperemotional traits associated with toxic levels of glutamic acid supplementation in human subjects. Such results would be expected on the basis of Weil-Malherbe's (14) formulation that glutamic acid was an adrenergic stimulant. Since these animals were relatively inactive in the activity wheel and were relatively slow to attain the criteria for mastery of the bar-pressing task, it would appear that too-high levels of glutamic acid intake result in overactivation to the point that adaptive behavioral patterns become disorganized.

Thus, the data from both the 200 mg and 400 mg glutamic acid groups are consistent with Weil-Malherbe's (14) theory that glutamic acid is a sympathetic stimulant, and are also consistent with the argument that, within limits, sympathetic stimulation is beneficial to performance on simple perceptual-motor tasks (1).

Table 1 also indicates that the rats fed the supplementary diet of 200 mg glutamic acid plus vitamin B_6 exhibited behavioral performances inferior to the 200 mg glutamic acid group. Vitamin B_6 is an essential component in the metabolism of glutamic acid. The increased intake of vitamin B_6 above normal levels, while glutamic acid intake is held constant, could result in the depletion of glutamic acid levels, which would result in a lowered level of sympathetic stimulation and thus produce a debilitating effect in regard to activity-wheel performance and mastery of the bar-pressing task. The results from the present study are similar to those of Phillips (7) who found that rats fed diets supplemented with 200 mg of glutamic acid displayed increased activity when compared to control animals, whereas rats fed diets supplemented with both 200 mg of glutamic acid and vitamin B_6 exhibited activity levels identical to the control animals. Supranormal levels of vitamin B_6 intake, while glutamic acid intake is held constant, might result in behavioral impairment for any one of several reasons, since numerous key physiological processes may be adversely affected by glutamic acid depletion. These processes include (*a*) the removal of intracellular ammonia; (*b*) protein and carbohydrate metabolism in nervous tissue generally and in the brain in particular; (*c*) the maintenance of the acetylcholine cycle; and (*d*) production of glutamine and GABA, both of which play key roles in cerebral tissue (13).

2. *Extinction of the Bar-Pressing Response*

The control group required significantly more responses to extinguish the bar-pressing response than any of the other groups, and required significantly more time to extinguish than any other group with the exception of the 50 mg glutamic acid group.

These findings might imply that the control group was evidencing greater habit strength on the bar-pressing task. This explanation seems unlikely. While for many years it was thought that the greater the number of reinforced acquisition trials, the slower the extinction, recent work suggests the relationship is much more complex. Sperling (11), in a review of the relationships between number of acquisition trials and resistance to extinction, concluded that resistance to extinction was a decreasing function of amount of training if the training reward was large, but an increasing function of amount of training if the training reward was small.

Sperling's conclusions may be applicable to our data, even though the amount of training reward for the various groups was held constant. The control groups received no dietary supplement; consequently, this group may

have manifested a stronger hunger drive than the other groups, since, in the 24 hours preceding training, the control group received the least nourishment of any group. If this hypothesis is tenable, then the control group would have received a smaller food reward in the Skinner box, relative to the animals' hunger needs. The control group animals would, therefore, be expected, in terms of Sperling's formulation, to make more extinction responses relative to the other groups, as was indeed the case..

E. Summary

Previous studies of the effect of glutamic acid on rat learning ability generally employed mazes. However, glutamic acid, as a sympathetic stimulant, should not be expected to improve performance on perceptual-restructuring tasks (e.g., mazes), but should be expected to improve performance on simple perceptual-motor tasks, such as operant conditioning of bar pressing, and activity-wheel performance. The findings indicate a curvilinear relationship between mastery of these tasks and amount of glutamic acid ingestion. Moderate levels of glutamic acid most quickly lead to learning; high levels lead to overactivation and behavioral disorganization.

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QUANTITATIVE METHODS FOR MEASURING THE HISTOCHEMICAL DISTRIBUTION OF ALANINE, GLUTAMATE AND GLUTAMINE IN BRAIN*

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THE most abundant and most metabolically active free amino acids in brain are glutamic acid, glutamine, aspartic acid, alanine and γ-aminobutyric acid. This is the record of methods of sufficient sensitivity for measuring glutamate, glutamine and alanine at the histochemical level. A companion paper (FLEMING and LOWRY, 1966) describes comparable procedures for aspartate as well as *N*-acetyl aspartate. HIRSCH and ROBINS (1962) described a method of measuring γ-aminobutyrate for histochemical purposes.

Enzymic methods for measuring alanine, glutamine and glutamate, similar in principle to those presented here, have been described by a number of authors (PFLEIDERER, GREIN and WIELAND, 1955; ALBERS, KOVAL, MCKHANN and RICKS, 1961; SOWERBY and OTTAWAY 1961; HOLZER and SOELING, 1962. MEISS, PEYSER and MILLER, 1964; GRAHAM, WERMAN and APRISON, 1965). All but the last of these are based on spectrophotometric measurement and require from 5 to 50 $\times$ 10^{-9} moles of material. The method of GRAHAM *et al.* (1965) for glutamate is based on fluorometric measurement of NADH produced in the reaction and is capable of measuring as little as 2×10^{-10} moles of material. The proposed methods require no more than 10^{-11} moles of material. Since the methods may be useful for studies with larger samples, both macro and micro procedures are described.

The use of the micromethods is illustrated by a record of (a) the distribution of alanine in the several layers of Ammon's horn and cerebellum and (b) the distribution of glutamine and glutamate in three layers of Ammon's horn.

ANALYTICAL METHODS

Materials. Amino acids were obtained from the Mann Research Laboratories (L-alanine), the California Corporation for Biochemical Research (L-glutamine), and Accent International (crystalline monosodium glutamate). ADP, α-oxoglutarate, NAD^+ and NADH were obtained from the Sigma Chemical Company. Glutamate dehydrogenase (ammonia free), rabbit skeletal muscle lactate dehydrogenase, $NADP^+$ and NADPH were obtained from Boehringer and Sons, Mannheim, Germany through the California Corporation for Biochemical Research. Acetyl NAD^+ was supplied by the Pabst Laboratories.

NH_4-free water was prepared by making it 1 mM in NaOH, boiling for 30 min, and neutralizing with HCl.

Glutamate-pyruvate transminase. Frozen ox heart (350 g) was passed through a meat grinder and homogenized in a Waring Blender for 30 sec in 2 volumes of 0·05 M-maleic acid buffer, pH 6·0 (0·05

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M-maleic acid, 0·075 N-NaOH), that had been heated to 90° (giving the mixture a temperature of about 60°). After further heating for 20 min at 65° the preparation was filtered through cheese cloth and chilled to 0°. (This initial step was suggested by Dr. SIDNEY F. VELICK.) The filtrate was adjusted to pH 5·0 with 4 M-acetic acid and centrifuged (all centrifugations were for 20 min at 20,000 *g*). The supernatant fluid (30 per cent of the original activity) was brought to pH 6·0 with 2 N-NaOH followed by solid $(NH_4)_2SO_4$ to give a concentration of 2·8 M. The precipitate (13 ml) was taken up in 2 or 3 volumes of 0·1 M-phosphate buffer, pH 6·7, reprecipitated once at an $(NH_4)_2SO_4$ concentration of 2·5 M, and again at a concentration of 1·9 M. The dissolved precipitate was brought to a concentration of 1·3 M, the precipitate was discarded, saturated $(NH_4)_2SO_4$ was added to make the concentration 1·5 M, and 2 days at 4° were allowed for precipitation. The collected precipitate represented a yield at this stage of 14 per cent; the presence of NADH oxidase made it unsuitable for the analytical purpose. The preparation was dialysed overnight against 150 volumes of 0·01 M-acetate buffer, pH 5·7, diluted to a protein concentration of 1 per cent with the same acetate buffer, and an equal volume of $Ca_3(PO_4)_2$ gel (1 mg per ml) was added. After 30 min the supernatant fluid (which contained 15 per cent of the NADH oxidase and 85 per cent of the transaminase) was removed, freeze-dried, and redissolved in a small volume of H_2O. This preparation had an activity of 1300 moles per kg protein per hr at 25° and pH 8 with 120 mM-alanine and 12 mM-α-oxoglutarate. The yield was 12 per cent. No loss of activity was observed after several months at −20°. (A transaminase preparation is now available from Boehringer and Sons, but it has not been tested for the present purpose.)

Glutaminase. (The preparation incorporates elements of the procedures of OTEY, BERNBAUM and GREENSTEIN (1945), KLINGMAN and HANDLER (1957) and SAYRE and ROBERTS (1958).) The cortex of two previously frozen hog kidneys (174 g) was homogenized at 0° in 4 volumes of 0·12 M-potassium phosphate buffer, pH 8·1, which contained 15 mM-sodium borate and 1 mM-EDTA. To the supernatant fluid obtained by centrifuging 30 min at 80,000 *g* was added 0·4 volume of a 3·7 M-phosphate buffer (3·4 M-K_2HPO_4:0·34 M-KH_2PO_4). The precipitate (10,000 *g* for 30 min) was dissolved in about 2 volumes of a 0·1 M-phosphate buffer (0·1 M-potassium phosphate, pH 8·1, containing 12 mM-sodium borate and 0·8 mM-EDTA) to give a protein concentration of 3·5 per cent, and the 3·7 M-buffer was added to give a total P_i concentration of 0·7 M. Inert protein was removed by centrifuging, and the activity again precipitated by increasing the P_i concentration to 1·0 M. The precipitate was dissolved in the 0·1 M phosphate buffer, the protein concentration adjusted to 4·5 per cent, and the P_i concentration raised to 0·9 M with the 3·7 M-buffer. The resulting precipitate was suspended in 1·5 volumes of 2·9 M-phosphate buffer (2·6 M-K_2HPO_4:0·26 M-KH_2PO_4) containing 15 mM-sodium borate. The yield was 8 per cent, the purification 16-fold, and the activity 130 moles per kg protein per hr measured at 38°, pH 8·1, with 125 mM-glutamine. Activity was stable for 6 months at 4° but was seriously reduced by freezing.

Preparation of tissue for analysis. For macro analyses of glutamate and glutamine, because of the high levels of these substances, whole homogenates of brain could be analysed satisfactorily without removal of proteins. For measuring alanine at macro levels extracts were prepared by homogenizing at 0–4° with 10 volumes of 0·3 M-$HClO_4$. The extracts were promptly neutralized with a slight calculated excess of 2 M-$KHCO_3$.

After most of the CO_2 had come off, the supernatant fluid was removed from the $KClO_4$ precipitate and brought to a pH of about 8 by the addition of 1/10 volume of 1 M-tris base.

Analyses at the histochemical level were made on samples dissected from frozen-dried tissue sections (LOWRY, 1953). At this level extracts were not prepared. Instead the tissue enzymes were destroyed by heating the samples either with alkali (alanine) or acid (glutamate and glutamine) as described below.

A Farrand Fluorometer Model A was used throughout with Corning filter No. 5840 as primary, and Nos. 3387 and 4308 as secondaries.

Measurement of alanine. Principle. The analytical steps are those proposed by PFLEIDERER *et al.* (1955):

$$\text{alanine} + \alpha\text{-oxoglutarate} \rightarrow \text{pyruvate} + \text{glutamate}, \quad (1)$$

$$\text{pyruvate} + \text{NADH} \rightarrow \text{lactate} + \text{NAD}^+. \quad (2)$$

In the case of larger samples the NAD^+ is measured by the fluorescence produced in strong alkali (KAPLAN, COTOWICK and BARNES, 1951; LOWRY, ROBERTS and KAPPHAHN, 1957). With small samples the NAD^+ is measured by enzymic cycling (LOWRY, PASSONNEAU, SCHULZ and ROCK, 1961*a*).

Macro method. Into tubes of 7 mm-bore are placed 50 μl of 0·1 M-tris-HCl buffer, pH 8·0, containing 0·2 mM-α-oxoglutarate, and 35 μg per ml of glutamate–pyruvate transaminase of the purity described above. This enzyme concentration is sufficient to give a calculated velocity of 45 m-moles per litre per hr with saturating levels of the substrates. A second set (Set 2) of tubes containing 50 μl of reagent without transaminase are simultaneously prepared. To the first set of tubes are added 3 μl volumes of water, standards (0·03 to 0·13 mM-alanine), or brain extracts. (The brain extracts are equivalent to 0·1 to 0·2 mg of tissue.) To provide uniformity of treatment the tubes are kept in a

rack on ice until all samples have been prepared. Both sets of tubes are incubated for 45 min at 50°, and the reaction is arrested by heating in a boiling water bath for 1·5 min. The samples are chilled once more on ice, and 3 μl of water or extracts are added to Set 2.

All samples are now incubated 15 min at 25° with 5 μl of 0·1 M-tris-HCl buffer, pH 8·0, containing 0·1 mM-NADH, and 1 μg per ml of rabbit skeletal muscle lactate dehydrogenase. Residual NADH is destroyed with 25 μl of 0·2 N-HCl and a portion of 75 μl is added to 1 ml of 6 N-NaOH with immediate mixing. After heating 10 min at 60° the fluorescence is read. (This final procedure for measuring NAD^+ avoids a dilution step recommended previously (LOWRY *et al.* (1957).) In consequence, the fluorescent product remains in strong alkali in which it is quite light sensitive. Therefore the samples should be protected from light before reading, and the strength of the exciting light in the fluorometer should be kept to a minimum (LOWRY and PASSONNEAU, 1963).

The fluorescence produced from standard solutions is proportional to alanine present (Fig. 1). In tissues, however, for accuracy it is necessary to correct for a tissue blank in which transaminase is

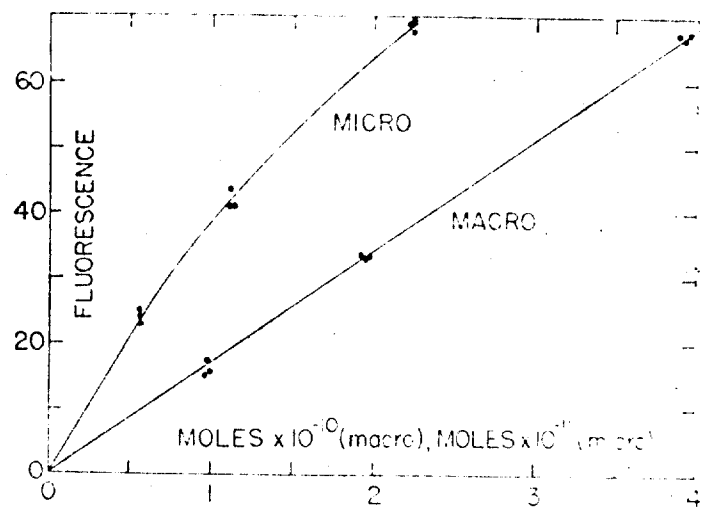

FIG. 1.—Relationship between net fluorescence and amount of L-alanine as measured by the macro and micro ('histochemical') procedures. Fluorescence units represent arbitrary galvanometer readings. Triplicates are recorded individually.

omitted (Set 2). This blank is due in part to preformed pyruvate, in part to other substances which react more slowly with NADH and lactate dehydrogenase (LOWRY, PASSONNEAU, HASSELBERGER and SCHULZ, 1964), and in part to native fluorescence of tissue extracts. The correction averages about 0·2 m-mole per kg and is minimized by using no more lactate dehydrogenase than necessary. With the preparation of transaminase available there was also a small tissue blank when lactate dehydrogenase was omitted. This blank would be eliminated with more highly purified transaminase.

Determination of alanine in histochemical samples. Samples of frozen-dried tissue weighing 1 or 2 μg are placed in tubes of 1·8 mm bore, and 1 μl of 0·02 N-NaOH is added. Blanks (1 μl of 0·02 N-NaOH) and standards (1 μl of 0·02 N-NaOH containing 0·005 to 0·02 mM-alanine) are simultaneously prepared. Samples are covered with aluminum foil to prevent evaporation and are heated in an oven for 30 min at 70°.

After chilling, to each tube is added 1 μl of 0·2 M-tris-HCl buffer, pH 8·0, containing 0·4 mM-α-oxoglutarate, and glutamate–pyruvate transaminase equivalent to 70 μg per ml of the above preparation. The tubes are incubated 45 min at 50° in a water bath, and the reaction is arrested by heating in a boiling-water bath for 1·5 min. After cooling, 0·75 μl of 0·1 M-tris-HCl buffer, pH 8·0, containing 0·04 mM-NADH and 3·5 μg of lactate dehydrogenase per ml is added. This is allowed to react for at least 15 min at 25°.

Residual NADH is destroyed with 2·5 μl of 0·2 N-HCl and vigorous buzzing. A 1·5 μl portion is added to 100 μl of NAD^+ cycling reagent (LOWRY and PASSONNEAU, 1963), and the analysis completed as described in that publication.

The yield of product in the cycling step is not strictly proportional to NAD^+ present; consequently the final fluorescence is not proportional to alanine (Fig. 1). Calculations are therefore made from a standard curve. In the method given for histochemical analyses no provision has been made for tissue

blanks of the kind described in the macro procedure. Such blanks could be included if the problem warranted. Judging from studies with whole brain, analyses are unlikely to be more than 25 per cent too high as the result of this omission.

Measurement of L-*glutamate and* L-*glutamine. Principle.* In the macro procedures, *glutamate* reduces acetyl NAD^+ with the aid of glutamate dehydrogenase:

$$\text{Acetyl NAD}^+ + \text{glutamate} \rightleftharpoons \text{Acetyl NADH} + \alpha\text{-oxoglutarate} + NH_3. \quad (1)$$

Acetyl NAD^+ is used to take advantage of the favourable equilibrium (KAPLAN, CHIOTTI and STOLZENBACH, 1956; HOLZER and SOELING, 1962). The acetyl NADH produced is determined in part of the sample by its fluorescence.

Glutamine is measured in the rest of the sample by converting it to glutamate with glutaminase after first destroying acetyl NADH with acid.

$$\text{Glutamine} \rightarrow \text{glutamate} + NH_3. \quad (2)$$

Step 1 is now repeated and the acetyl NADH is determined as before.

In the histochemical procedures, $NADP^+$ is substituted for acetyl NAD^+ and the NADPH generated is measured by enzymic cycling (LOWRY *et al.*, 1961*a*). Even though the equilibrium is less favourable than with acetyl NAD^+, it is possible to achieve near equilibrium with high coenzyme levels, and the advantages of the $NADP^+$ cycle for measuring small amounts of pyridine nucleotide outweigh the disadvantage. Also, the equilibrium of reaction (1) and of its $NADP^+$ counterpart is favourably influenced by lowering the initial glutamate concentration, since two reactants produce three products. At the histochemical level, glutamate concentration is lowered, almost of necessity; consequently nearly quantitative conversion to α-oxoglutarate can be achieved even with $NADP^+$.

Glutamate (macro). Brain tissue is homogenized in 100 volumes of 0·002 N-HCl (or in 10 volumes of 0·02 N-HCl and diluted 10-fold). Portions of 20 μl are placed in tubes of 3 mm bore, heated for 2 min in a 100° water bath, and placed in an ice bath. Blanks and standards consist of 20 μl of 0·002 N-HCl or of 0·05 mM-glutamate in 0·002 N-HCl. If glutamine is also to be measured, a second set of tissue samples (Set 2) is needed to provide proper blanks (see below), and the standards are made to contain both glutamate and glutamine (0·15 mM). (Glutamine standards should be prepared freshly from stock solutions in water.) To each sample is added 20 μl of 0·1 M-tris–HCl buffer, pH 8·4, containing 0·04 per cent bovine plasma albumin, 0·04 mM-ADP, 2·4 mM-acetyl NAD^+ and 0·03 per cent glutamate dehydrogenase. After 30 min at 25°, a 10 μl sample is diluted in 1 ml of 0·05 M-tris buffer, pH 8·4, and the fluorescence measured.

Over the range of concentration described, fluorescence is proportional to glutamate (Fig. 2) although the yield of α-oxoglutarate is only about 95 per cent of theory owing to the position of equilibrium under the analytical conditions. The large excess of acetyl NAD^+ is necessary to achieve this near-complete reaction. It is desirable to select a suitable commercial sample of this nucleotide since some preparations have been seriously contaminated with a yellow fluorescence impurity which would give a very troublesome reagent blank.

Glutamine (macro). To the 30 μl of the above sample, remaining after removing the portion for glutamate estimation, are added 9 μl of 0·25 N-HCl to destroy the acetyl NADH formed by glutamate. After buzzing, the samples are heated* for 10 min at 38°, chilled in ice water and treated with 9 μl of 1·0 M-tris base which contains 0·25 M-K_2HPO_4, 6 mM-sodium borate, sufficient 2 N-HCl to bring the pH to 9·5, and 0·4 mg per ml of glutaminase of the activity of the preparation described above. The mixture is incubated 30 min at 38° and heated 2 min in a boiling water bath. The samples are again chilled in ice water, and each is mixed with 9 μl of 0·05 M-tris–HCl buffer, pH 8·4, containing 1·4 mM-ADP and 0·6 mg glutamate dehydrogenase per ml. In Set 2 (see above) the glutamate dehydrogenase is omitted as a control on possible side reactions. After incubation of both sets for 30 min at 25°, 50 μl portions are diluted in 1 ml of 0·05 M-tris buffer, pH 8·4, and the fluorescence is measured.

Glutamine content is calculated from the net fluorescence obtained by subtracting the values for Set 2 from the regular set. Fluorescence is proportional to glutamine concentration (Fig. 2). Accuracy is increased if calculation is made on the basis of standards containing both glutamate and glutamine in the approximate proportion found in brain. The reason for this is that the small percentage of glutamate which is not oxidized in the first stage is added to the glutamate formed from glutamine in the second stage. If this is not taken into account the glutamine values will be somewhat too high.

Glutamate in histochemical samples. Dissected samples of frozen-dried tissue weighing 1 to 2 μg,

* The incubation at 38° is demanded because of the interesting fact that acetyl NADH is more difficult to destroy with acid than NADH. The half-time for destruction in 0·02 N-HCl is approximately 4 min at 25° and 1 min at 38°. This is 30- or 40-fold slower than in the case of NADH and 50- or 60-fold slower than in the case of NADPH (LOWRY, PASSONNEAU and ROCK, 1961*b*).

in reaction tubes of 2–2·5 mm bore, are treated with 5 μl of 0·002 N-HCl for 30 min at room temperature. Blanks of 0·002 N-HCl and standards (5 μl of 0·005 to 0·015 mM-sodium glutamate and glutamine freshly prepared in 0·002 N-HCl) are included. After chilling the rack of tubes in ice water, to each sample is added 5 μl of 0·10 M-tris HCl buffer, pH 8·4, containing 0·04 per cent bovine plasma albumin, 0·2 mM-ADP, 2·0 mM-$NADP^+$ and 0·01 per cent glutamate dehydrogenase. After incubation for 30 min at 25°, the reaction is arrested in ice water, and a 2 μl portion from each tube is transferred to a second set of similar reaction tubes. To destroy the excess $NADP^+$ 8 μl of 0·05 N-NaOH are added, and the samples are first mixed gently, then buzzed vigorously, and finally heated for 10 min at 60°. The object is to make sure that no minute droplets of sample escape contact with alkali; there is a 100-fold excess of $NADP^+$ over the NADH to be measured, and a minute fraction of this $NADP^+$, if not destroyed, would produce a disastrous blank. As a further precaution the buzzing and heating procedure is repeated once. After this treatment, the samples are chilled in ice, and

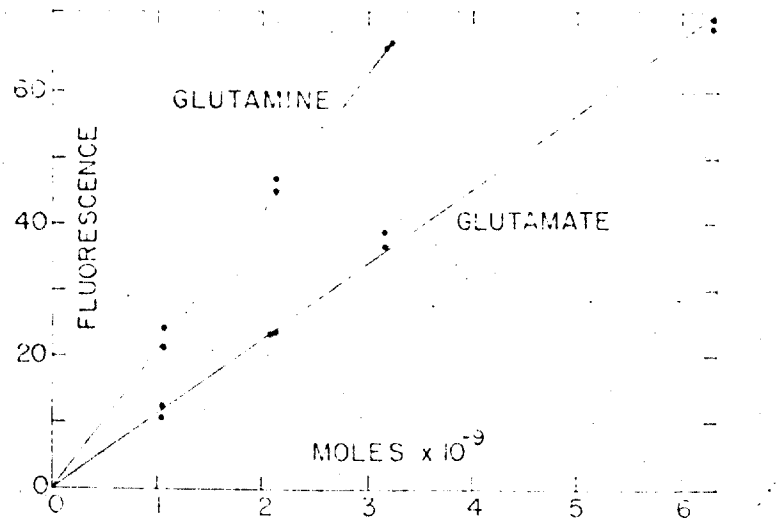

FIG. 2.—Relationship between net fluorescence and amounts of glutamine or glutamate, as measured by the 'macro' procedures. Fluroescence units are arbitrary galvanometer readings. Because of the use of different sized samples and instrumental settings for glutamate and for glutamine there is no direct relationship between the two curves. Duplicate measurements are shown at each point.

5 μl portions are analysed for NADPH formed by enzymic cycling by using 100 μl of the $NADP^+$ cycling mixture and a 30 min incubation at 38° (LOWRY and PASSONNEAU, 1963). If necessary, after stopping the cycle by heating at 100°, the tubes can be capped with parafilm and stored overnight at 4° without loss of 6-*P*-gluconate formed. Since the equilibrium conditions are not as favourable in this case as in the macro procedure, fluorescence is not quite proportional to glutamate (Fig. 3), and a standard curve may be needed for calculation. Because of the less favourable equilibrium constant it is particularly important to take pains to avoid contamination of reagents by ammonia.

Glutamine in histochemical samples. To the 8 μl remaining from the step with glutamate dehydrogenase above, 2·3 μl of 0·25 N-HCl are added, and the samples gently buzzed. At this stage samples capped with parafilm can be stored overnight at 4° without harm. To each sample are added 2·3 μl of 1·0 M-tris–HCl buffer, pH 9·5, containing 0·25 M-K_2HPO_4, 6 mM-sodium borate, and glutaminase sufficient to provide activity equivalent to 0·2 mg per ml of the preparation described above. After 30 min at 38° the reaction is stopped by heating for 2 min in a boiling-water bath.

The rack of tubes is placed in ice, and to each is added 2·3 μl of 0·05 M-tris buffer, pH 8·4, containing 0·7 mM-ADP and 0·04 per cent glutamate dehydrogenase. After 30 min at 25°, 6 μl of 0·5 N-NaOH are added and the analysis is completed as described for glutamate except that 2·5 μl rather than 5 μl of the final sample is cycled.

The concentration of glutamine in the sample is calculated from a curve obtained with glutamine standards (Fig. 3) which preferably contain glutamate in the approximate proportion found in brain (see macro method). In this micromethod, an extra set with glutamate dehydrogenase omitted is not required since NAD^+, unlike acetyl NAD^+, has not been found to give a blank with tissue extracts exposed to crude glutaminase.

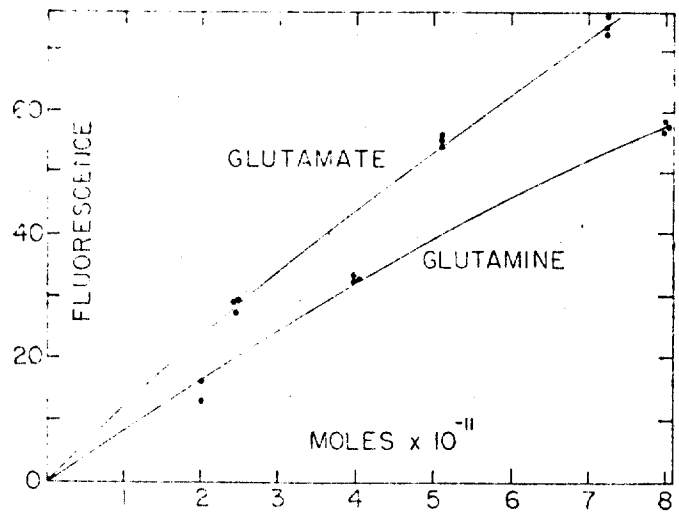

FIG. 3.—Relationship between fluorescence and amounts of glutamate and glutamine, by the 'histochemical' procedures. Fluorescence units represent arbitrary galvanometer readings. Owing to the use of different sized samples there is no direct relationship between glutamate and glutamine curves. Triplicate measurements are recorded for all but one point.

RESULTS

Alanine. Analyses of brain with the macro procedure gave the following values: Rabbit whole brain anterior to the colliculi was homogenized in $HClO_4$ within a few minutes of cutting off the blood supply. This gave values in three cases of 1·00, 1·09, 1·16 m-moles per kg wet weight respectively.

Mouse forebrains, taken from mouse heads frozen rapidly in Freon 12 at −150° and treated so as to avoid thawing (LOWRY *et al.*, 1964), gave values of 1·35 and 1·25 m-moles per kg when the heads were frozen immediately and 1·09 and 1·33 m-moles per kg when the heads were allowed to stand for 10 min at 37° before freezing. This does not agree with data on dog brain obtained by TEWS, CARTER, ROA and STONE (1963) who found values for alanine of 0·14 and 0·62 m-moles per kg, respectively, for brain frozen *in situ* and brain removed for 20 min before fixing. The present data, however, are in agreement with results of THORN, SCHOLL, PFLEIDERER and MUELDENER (1958) for rabbit, both as to level and as to lack of change with ischaemia.

Measurements of alanine in the various layers of Ammon's horn and cerebellum (Table 1) showed very little difference among the layers. It should be noted that the values have been calculated on a lipid-free dry weight basis, which is more or less equivalent to 10 times the wet weight basis (LOWRY, ROBERTS, LEINER, WU, FARR and ALBERS, 1954). It should also be reiterated that the absolute values may be as much as 25 per cent too high owing to absence of a correction for a tissue blank (see methods section).

Glutamate and glutamine. Analyses of whole brain of rabbit with the proposed methods gave average values of 9·5 and 4·2 m-moles per kg for glutamate and glutamine, respectively. A series of fifteen mouse brains was analysed after the heads

had been quick-frozen, and the brains powdered and freeze-dried. The freezing took place after the mice had been decapitated for different periods up to 10 min. There was no significant trend during this interval of ischaemia, and respective values of 45·2 ± 0·9 and 25·3 ± 1·3 m-moles per kg dry weight (± standard errors) were observed for glutamate and glutamine. These correspond to fresh weight values of 9·0 and 5·0, respectively. The lack of significant early post mortem change agrees with the data of others (e.g. TEWS *et al.*, 1963).

TABLE 1.—ALANINE CONTENT OF AMMON'S HORN AND CEREBELLUM IN RABBIT

Layer	R58	R63
Ammon's Horn		
Alveus	15·8 ± 0·2	11·8 ± 0·5
Oriens	13·6 ± 0·3	13·1 ± 0·2
Pyramidalis	12·6 ± 0·2	14·6 ± 0·6
Radiata	14·5 ± 0·4	13·5 ± 0·5
Lacunosum	17·0 ± 1·1	14·2 ± 0·6
Molecularis	14·4 ± 0·5	13·4 ± 0·5
Cerebellum		
Molecular	13·9 ± 0·5	14·8 ± 0·4
Granular	11·8 ± 0·4	13·2 ± 0·5
White	13·4 ± 0·4	12·2 ± 0·2

The values are recorded as m-moles per kg lipid-free dry weight. Each represents the mean of six determinations ± the standard error except that there were only five determinations for oriens and molecularis of animal R63. The values for lipid used in the calculation are taken from STROMINGER and LOWRY (1954). The frozen-dried sections for R58 had been stored for several months at −20°; the sections for R63 had been prepared only a few days before analysis.

These two compounds were measured in three discrete layers of Ammon's horn (Table 2). As in the case of alanine the data have been calculated on a lipid-free dry weight basis, and the values may be divided by ten for comparison with values given for whole brain. Alveus, the layer of fibres, contained much less glutamate and slightly less glutamine than the other two layers. The ratio of glutamate to glutamine in alveus is about half that in oriens and radiata. Glutamate levels presumably reflect the balance among the various enzymes which can form or degrade this amino acid. Of these, glutamate dehydrogenase is considerably more abundant in alveus than in either oriens or radiata (STROMINGER and LOWRY, 1955) and, therefore, might be a factor in this case. There is 20 per cent less glutamate-oxalacetate transaminase in alveus than in oriens (LOWRY, 1964) and 30 per cent less glutamate–pyruvate transaminase in alveus than in either oriens or radiata (unpublished data), and white matter in general is known to contain virtually zero glutamate decarboxylase (ROBERTS, 1956; LOWE, ROBINS and EYERMAN, 1958).

Although only two animals are involved, the lower glutamate levels in oriens and radiata of the anaesthetized rabbit may be significant in view of the finding of DAWSON (1953) that thiopental lowers glutamate but not glutamine in rat brain. The finding that glutamate was *not* lower in the fibre layer of Ammon's horn of the anaesthetized animal would appear to be worth further study.

TABLE 2.—GLUTAMINE AND GLUTAMATE CONTENT OF AMMON'S HORN IN RABBIT

Layer	R63	R67
Alveus		
Glutamate	41·9 ± 1·6	44·7 ± 1·8
Glutamine	29·7 ± 2·1	42·2 ± 2·3
Ratio	1·4	1·1
Oriens		
Glutamate	141 ± 2·0	115 ± 2·0
Glutamine	48·6 ± 1·8	54·3 ± 0·9
Ratio	2·9	2·1
Radiata		
Glutamate	140 ± 2·0	106 ± 2·0
Glutamine	47·0 ± 4·0	43·6 ± 3·8
Ratio	3·0	2·4

The values are recorded as m-moles per kg lipid-free dry weight. Each represents the mean ± S.E. for six determinations except there were only four determinations for oriens and radiata of animal R67. Both animals were decapitated before removing the brain. R67 only had been anaesthetized with phenobarbitone.

SUMMARY

Enzymic methods are described for measuring alanine, glutamate and glutamine in samples of tissue as small as 1 μg dry weight. With these methods alanine was found to be rather uniformly distributed among six histologically discrete layers of Ammon's horn and three layers of cerebellum (rabbit). In contrast, glutamate is only one-third as concentrated in the fibre layer (alveus) of Ammon's horn as in layers of dendrites or mixed dendrites and non-myelinated axons (radiata and oriens). Glutamine was only a little lower in alveus than in radiata or oriens.

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Olney and Sharpe administered to mice, presumably by subcutaneous injection, 0.5 g of monosodium glutamate per kilogram of body weight. They gave, by the same route, 2.7 g per kilogram of body weight to one newborn monkey. The monkey is characterized as being similar to a premature infant, but it is not clear whether the monkey was, in terms of development, itself premature. During a 3-hour period when the monkey was observed, there was no manifestation of a central nervous system disturbance. The animal was then anesthetized with Sernylan, and the brain and some other organs were removed.

There is no indication of previous observations on monkeys of similar age, no controls to indicate observation of animals handled in identical fashion but without the use of monosodium glutamate, and no quantitative relation to the dose of monosodium glutamate that could be expected to be given to the human infant.

Unless the above questions can be answered, and information can be provided about the effect of monosodium glutamate on passage through the gastrointestinal tract, the report of Olney and Sharpe has no meaning. The fears engendered among mothers with infants in arms cannot be calculated.

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