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Effect of Protein Hydrolysate Administration on Liver Composition

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SUMMARY

Intravenous administration of a 10% protein hydrolysate to adult female rats at a level of 1 gm protein/kg body weight/hour over 12 hours was followed by significant increases in the average content of RNA per cell in the liver. Similar but larger increases in RNA per cell were obtained after 44 or 48 hours intraperitoneal administration at the same level, accompanied this time by a significant increase in mitotic activity. Investigation of the time course of these changes showed that RNA per cell increased sharply during the first 24 hours of treatment and again between the 38th and 42nd hours, but that mitotic activity did not increase until about 48 hours. The protein content per cell showed the same pattern of increase as RNA, but the changes were less clear-cut.

INTRODUCTION

The regeneration of the liver fragment remaining after partial hepatectomy has been shown by Leong *et al.* (20) to be initiated by some factor in the circulating blood, but the nature, origin, and mode of action of this factor are still unknown. It is possible that the liver fragment is stimulated to grow because it has to cope with the duties normally discharged by the entire organ, as was thought by the earlier investigators (27, 32). There is some evidence that it readjusts to this situation. For example, during the first day following partial hepatectomy, the rate of urea formation is greatly increased (30). If it is the increased work load which causes the liver fragment in partially hepatectomised animals to grow, it should be possible to produce growth of the liver in an otherwise normal animal by increasing the "chemical load" with which it has to cope. In fact, it has been shown by previous workers that the liver mass is sensitive to dietary influences and that, in general, the feeding of a high protein diet to experimental animals causes the liver to increase in size (2, 18). Moreover, the refeeding of a high protein diet to animals previously starved or maintained on low protein or protein-free diets was sufficient to cause liver cell hypertrophy and an increased mitotic activity (19).

The object of the experiments reported here was to quantitatively investigate the changes in chemical composition and mitotic activity which result from the administration to rats of a protein hydrolysate in amounts roughly comparable to their normal dietary intake. In order to standardize the conditions of the experiments, it was decided to administer the protein hydrolysate either intravenously or intraperitoneally so that each animal received the same amount of hydrolysate per unit of body weight. In addition, a liver biopsy was performed on each animal at the beginning of each experiment, so that by comparing the composition of the liver before and after treatment in both test and control animals, a more precise indication of changes due to treatment would be obtained.

MATERIALS AND METHODS

Animals. Female albino rats (180 ± 20 gm) from the departmental colony were used in all experiments. They were maintained on a conventional diet of Rat-Cubes and were given water and food *ad libitum* throughout the course of the experiments. Liver biopsies were taken by delivering, under ether anesthesia, the median and left lateral lobes of the liver through a midline abdominal incision and excising the left radicle of the median lobe. This fragment represents about 10% of the total liver substance. The abdominal wound was closed with interrupted sutures.

Protein Hydrolysate Administration. The protein hydrolysate used was Aminosol Vitrum 10% (Vitrum, Stockholm, Sweden). It consists of amino acids and low molecular weight peptides obtained by enzymatic hydrolysis of casein, and it also contains 160 mM Na^+ and 0.5 mM K^+. This preparation has a biologic value of about 90; i.e., the fraction of the infused nitrogen retained in the body for maintenance and synthesis of body protein is 90%, and intravenous infusion of this preparation does not produce hyperpeptidemia in the human subject (22). In the experiment described in Table 1, Aminosol was infused intravenously at the rate of 1 gm protein/kg body weight/hour over 12 hours, and intraperitoneally at the rate of 4 gm protein/kg body weight at 4-hour intervals (Tables 2, 3). Controls received equal volumes of either saline or an isocaloric amount of glucose.

Tissue Preparations and Analytic Methods. At the end of the experiment, each animal was killed with Nembutal or by cervical dislocation. A small piece of liver tissue was fixed in Bouin's fluid for histologic examination, and about 0.5 gm was accurately weighed out and preserved at −75°C for analysis. Nucleic acids were extracted by a modification of the method of Fleck and Munro (11). The tissue was homogenized in 19 volumes of ice-cold distilled water in a blender, and to 5-ml samples (containing 125 mg wet weight tissue) was added 0.5 volume of cold 0.6 N $HClO_4$ to precipitate the proteins and nucleic acids. After standing 10 minutes, the precipitate was

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separated by centrifugation and washed twice with 0.2 N $HClO_4$. The $HClO_4$ was then poured off, 4 ml of 0.3 N KOH were added, and digestion was carried out at 37°C for 1 hour. The tube and contents were cooled in ice, 5 ml of 0.6 N $HClO_4$ were added, and after standing 10 minutes in the cold, the mixture was centrifuged. This precipitate was washed twice with 5 ml of 0.2 N $HClO_4$; the supernatant and washings were added to a cylinder and made up to 100 ml and a final concentration of 0.1 N $HClO_4$ (RNA fraction). The precipitate (DNA fraction) was dissolved in 5 ml of 0.3 N KOH and made up to 50 ml and a final concentration of 0.1 N KOH. RNA was estimated by measuring the O.D. at 260 mμ, and the DNA, by the method of Cerriotti (7). The results of these estimations are expressed in terms of ribonucleic acid phosphorus (RNA-P) and deoxyribonucleic acid phosphorus (DNA-P). Protein concentration was measured by the method of Lowry *et al.* (21) on the aqueous liver homogenate.

Histologic Methods. The mitotic index was estimated as described by Alston and Thomson (3).

Statistical Methods. Student's "t" test and analysis of variance were performed according to the methods described by Snedecor (29).

RESULTS

Table 1 shows the effect of administration of protein hydrolysate over a period of 12 hours on the composition of the liver. Since the DNA content per cell in rat liver is approximately constant (31), the DNA concentration per 1000 mg of tissue is inversely proportional to the mean cell mass, and the ratios of the other tissue components to DNA are directly proportional to the amounts of these components per cell. The figures shown in Table 1 for the treated animals and for the controls, which received glucose instead of protein hydrolysate, are comparable with those reported by other workers. Comparison for each animal of the liver biopsy taken at the beginning of the experiments with that at sacrifice makes it possible to determine what changes, if any, have taken place during treatment. In the control animals, there was only an insignificant increase in DNA tissue concentration, indicating no change in cell mass. There was also an insignificant fall in the protein/DNA ratio, i.e., in protein per cell; the RNA/DNA ratio, i.e., RNA per cell, showed no change. In the treated animals, the cell mass showed no change; there was a slight increase in protein per cell ($P < 0.05$) and a more substantial increase in RNA per cell ($P < 0.001$). In other words, in the control animals the liver cells showed little, if any, change in mass or protein content but maintained their RNA content; in the treated animals, the liver cells maintained their mass and increased their content of protein and, more especially, of RNA.

Tables 2 and 3 present the results of experiments testing the effect of more prolonged treatment with the protein hydrolysate (for 44 and 48 hours respectively) on liver composition and mitotic frequency. As controls for these experiments, animals were injected with sterile physiologic saline, the volume administered being equal to that of the protein hydrolysate given to the test animals. At sacrifice, the livers of the animals which had received protein hydrolysate had pale, fleshy consistency, dome-shaped lobes, and marked rounding of the edges, changes which were reminiscent of regeneration. No such changes were found in the control series. The apparent changes in the cell mass and protein content per cell of both control and test animals in both experiments are not significant, but a very pronounced increase occurred in the RNA per cell in the test animals, both after 44 and 48 hours of treatment with the protein hydrolysate. The increase was slightly greater after 44 hours ($P < 0.01$) than after 48 hours ($P < 0.05$). A smaller but still significant increase in RNA per cell was also obtained in the control group of the 44-hour experiment ($P < 0.02$). The corresponding increase in the control group of the 48-hour experiment was not significant. Analysis of variance between test and controls in the two experiments with respect to RNA per cell showed that the increase was significantly greater after protein hydrolysate than after saline ($P < 0.001$). Finally, a significant increase in mitotic frequency was produced both after 44 hours of protein hydrolysate treatment ($P < 0.001$) and after 48 hours ($P < 0.05$). Although a slight increase occurred in the controls of the 44-hour ex-

Table 1

Treatment	Number of animals		μg DNA-P/ gm wet wt.	μg RNA-P/ μg DNA-P	μg protein/ μg DNA-P
Protein hydrolysate	6	Biopsy before treatment	254 ± 26	3.20 ± 0.17	594 ± 61
		Liver after treatment	247 ± 16	3.74 ± 0.19	640 ± 62
		% change	−1.0 ± 4.2[a]	16.6 ± 1.2[b]	7.7 ± 2.9[c]
Glucose control	5	Biopsy before treatment	228 ± 10	3.43 ± 0.28	551 ± 50
		Liver after treatment	257 ± 21	3.51 ± 0.24	508 ± 37
		% change	12.8 ± 6.6	2.3 ± 1.5	−7.7 ± 4.3

Effect on liver composition of 12 hours intravenous administration of protein hydrolysate. DNA-P, deoxyribonucleic acid phosphorus; RNA-P, ribonucleic acid phosphorus.

[a] Values are means for the group ± S.E.

[b] Change significantly different from zero ($P < 0.001$).

[c] Change significantly different from zero ($P < 0.05$).

Table 2

Treatment	Number of animals		μg DNA-P/ gm wet wt.	Mean[a] cell mass mg wet wt./μg DNA-P	μg RNA-P/ μg DNA-P	μg protein/ μg DNA-P	Increase[b] in mitoses per 100,000 nuclei
Protein hydrolysate	5	Biopsy before treatment	204 ± 13[c]	4.99 ± 0.69	2.73 ± 0.21	649 ± 36	
		Liver after treatment	171 ± 5	5.88 ± 0.35	4.08 ± 0.23	731 ± 82	
		% change	−15.2 ± 5.3[d]	+19.6 ± 7.8	+51.6 ± 9.2[e]	+11.8 ± 8.2	442 ± 12[g]
Saline control	5	Biopsy before treatment	221 ± 11	4.58 ± 0.27	2.19 ± 0.10	485 ± 45	
		Liver after treatment	205 ± 7	4.88 ± 0.17	2.55 ± 0.05	487 ± 24	
		% change	−5.4 ± 7.5	+8.4 ± 8.14	+17.0 ± 3.8[f]	+2.2 ± 5.6	60 ± 83

Effect on liver composition of 44 hours intravenous administration of protein hydrolysate. DNA-P, deoxyribonucleic acid phosphorus; RNA-P, ribonucleic acid phosphorus.

[a] Calculated on the assumption that the DNA content per cell remains constant.
[b] Calculated from the difference between biopsy taken before treatment and liver after treatment.
[c] Values are means for the group ± S.E.
[d] Change significantly different from zero $P < 0.05$.
[e] Change significantly different from zero $P < 0.01$.
[f] Change significantly different from zero $P < 0.02$.
[g] Change significantly different from zero $P < 0.001$.

Table 3

Treatment	Number of animals		μg DNA-P/ gm wet wt.	Mean[a] cell mass mg wet wt./μg DNA-P	μg RNA-P/ μg DNA-P	μg protein/ μg DNA-P	Increase[b] in mitoses per 100,000 nuclei
Protein hydrolysate	4	Biopsy before treatment	250 ± 11[c]	4.02 ± 0.16	2.85 ± 0.06	405 ± 20	
		Liver after treatment	221 ± 14	4.59 ± 0.33	3.93 ± 0.03	476 ± 34	
		% change	−11.8 ± 4.3	14.1 ± 7.0	38.5 ± 10.7[d]	18.3 ± 8.5	284 ± 84[d]
Saline control	4	Biopsy before treatment	251 ± 19	4.06 ± 0.31	2.99 ± 0.31	435 ± 70	
		Liver after treatment	252 ± 25	4.01 ± 0.20	3.16 ± 0.26	440 ± 32	
		% change	1.0 ± 3.4	−0.75 ± 3.2	6.8 ± 3.9	2.0 ± 4.1	4 ± 6

Effect on liver composition of 48 hours intravenous administration of protein hydrolysate. DNA-P, deoxyribonucleic acid phosphorus; RNA-P, ribonucleic acid phosphorus.

[a] Calculated on the assumption that the DNA content per cell remains constant.
[b] Calculated from the difference between biopsy taken before treatment and liver after treatment.
[c] Values are means for the group ± S.E.
[d] Change significantly different from zero $P < 0.05$.

periment, the change was not significant. Again, analysis of variance for the two experiments showed that the increase in the test animals was significantly greater than that found in the controls ($P < 0.01$).

Charts 1–3 show the time course of the chemical and mitotic frequency changes produced in the liver by administration of protein hydrolysate over a period of 60 hours. Chart 1 indicates that during the first 23 hours there was a 20% increase in RNA per cell ($P < 0.01$). From 23 to 39 hours there was no further change, but between 39 and 42 hours there was a further sharp increase ($P < 0.01$) followed by a decline ($P < 0.05$) between 42 and 60 hours. This rather complicated pattern was followed by the protein per cell, but the changes here were on a smaller scale. The initial increase in protein per cell (in the first 23 hours) was not significant and the second increase (between 39 and 42 hours) was of borderline significance ($P < 0.05$); but the final decline (between 42 and 60 hours) was highly significant ($P < 0.01$). Finally, the mitotic index showed no significant change until 48 hours ($P < 0.02$), after which it showed an abrupt increase until 60 hours ($P < 0.01$).

DISCUSSION

Clearly, before any conclusions could be drawn from the results, the design and control of the present experiments had to be considered. Leduc (19) in her paper conceded that, ideally, the mitotic changes should be studied in individual animals but pointed out that repeated liver biopsies would *per se* induce mitotic changes in the remaining portions of the

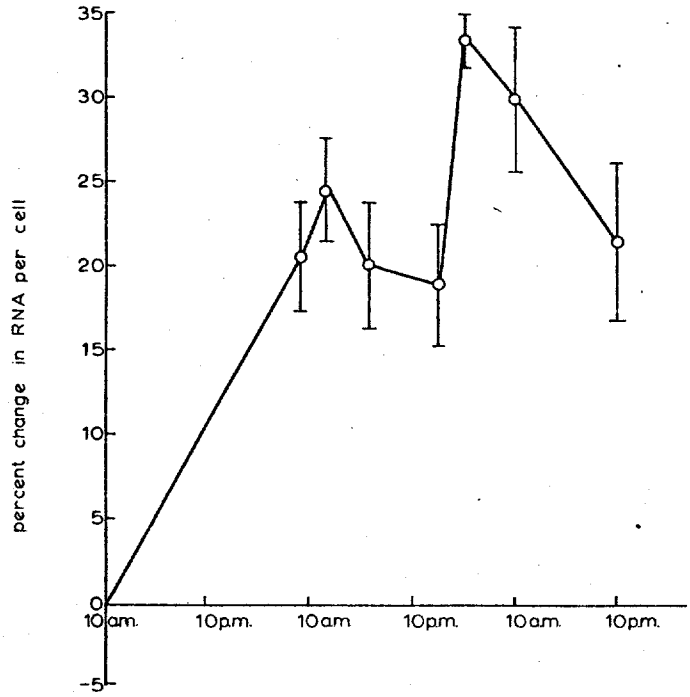

Chart 1. The effect of intraperitoneal administration of the protein hydrolysate (13.7 gm kg body weight/day) on the RNA per cell in liver. Each point is the mean for 5 or 6 animals, with the exception of the 23-hour point which represents 4 animals and the 39-hour point which represents 7 animals; the *vertical bars* represent S.E.

livers of the animals treated in this way. However, it seemed to us that, provided the treated animals were compared with saline or glucose controls, the advantages gained by studying the chemical and mitotic changes occurring in individual animals greatly outweighed this disadvantage. In this way, we had hoped to avoid some of the biologic variations inevitable in experiments of this kind. In fact, there is evidence that the removal of even less than 10% of the liver is sufficient to produce some regenerative response in the remainder (33). In particular, Halliburton and Thomson, who used the same biopsy procedure (removing approximately 10% of the liver), found that it increased the RNA per cell by about 20% in 48 hours (unpublished results). In the present experiments, the control groups of animals showed no significant increase in RNA, protein, or cell mass in the 12- or 48-hour experiments, but in the 44-hour experiment there was a 17% increase in RNA. It does seem likely, therefore, that the biopsy procedure by itself may produce some slight regenerative response, but it is evidently small in comparison to the dramatic effects produced by the protein hydrolysate. The possibility also had to be considered that the growth response evoked in the livers of the animals treated with protein hydrolysate might be a response to injury due to a toxic effect on the liver by the hydrolysate. However, careful histologic examination of the liver sections from all the experiments failed to reveal any evidence of liver cell necrosis at any time up to 60 hours of treatment.

Although the liver to body weight ratio was not recorded in

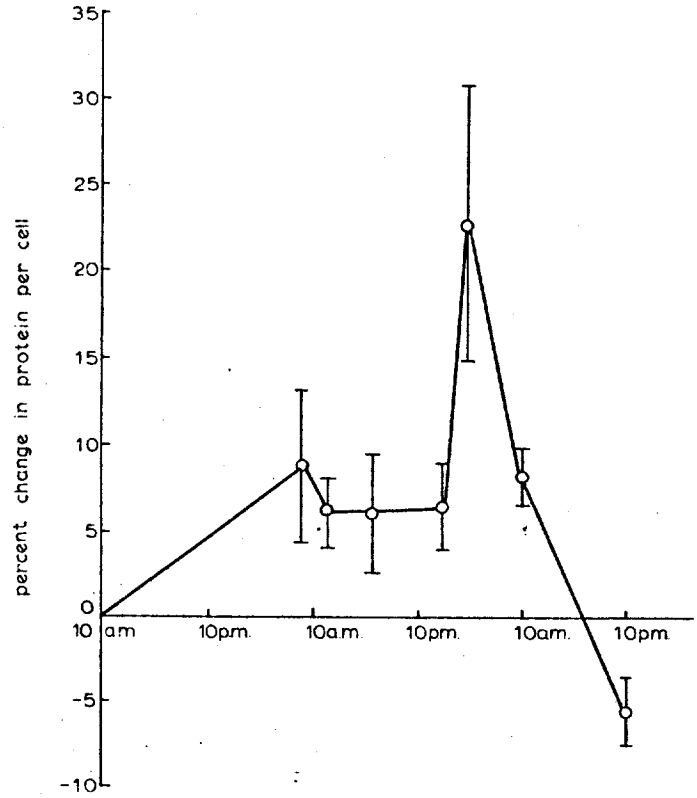

Chart 2. The effect of intraperitoneal administration of the protein hydrolysate (13.7 gm/kg body weight/day) on the protein per cell in liver. Each point is the mean for 5 animals, with the exception of the 23-hour point which represents 4 animals and the 39-hour point which represents 7 animals; the *vertical bars* represent S.E.

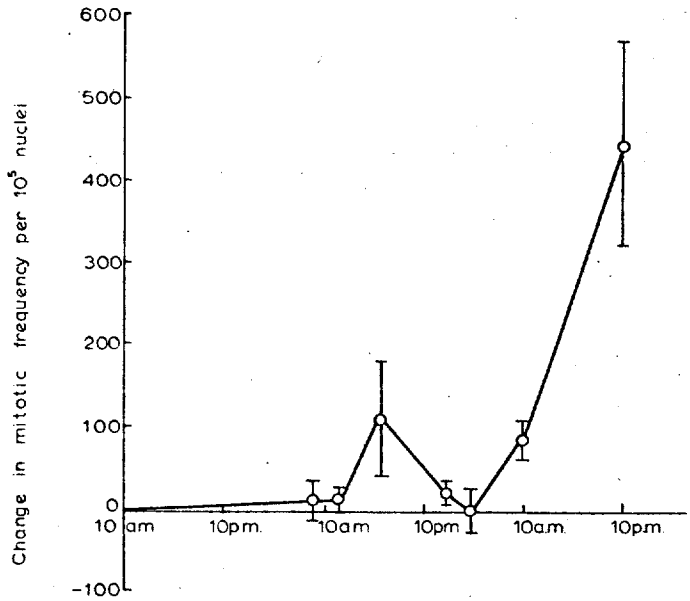

Chart 3. The effect of intraperitoneal administration of the protein hydrolysate (13.7 gm/kg body weight/day) on the mitotic frequency in liver. Each point is the mean for 5 animals, with the exception of the 39-hour point which represents 7 animals; the *vertical bars* represent S.E.

these experiments, the livers of the animals treated with the protein hydrolysate had visibly increased in mass compared to the controls, in accordance with the results reported by other workers who studied the response of liver mass to various levels of protein in the diet (2, 18). Even though mean cell mass in the liver would seem to have increased slightly after 44 and 48 hours of treatment in the test series, the changes are not significant. Leduc (19) found that liver cell size in the mouse actually diminished slightly on transferring the animals from a low to a high protein diet for the first 24 hours and increased only very gradually thereafter. In general, liver cell size was affected to a greater degree by the content of glycogen and fat, and, on feeding a high protein diet, a considerably less amount of these storage products accumulated. The increase in liver RNA in animals transferred from a low to a high protein diet is already well known (2, 18, 19). Allison *et al.* (2) found a curvilinear increase in liver weight, RNA/DNA, and protein/DNA in rats upon increased intakes of casein. This increase with respect to RNA and protein flattened out at an intake of about 4 to 5 gm of casein nitrogen per kg per day. Assuming that the increases in liver RNA and protein in our experiments were entirely due to the administered casein hydrolysate, then an increase in intake of protein nitrogen by 2.2 gm pr kg per day has increased the RNA/DNA of liver from 3 to about 4 over 48 hours. These findings are in accordance with those of Allison *et al.* (2) at the lower levels of intake. However, although in our experiments there was no significant increase in protein/DNA after 48 hours, these workers recorded an appreciable increase in this index. It may be that the differences are due to the fact that Allison *et al.* studied the compositional changes after the animals had been on the diet for 28 days.

The relationship between increased protein administration and the increase in mitotic index produced in the liver was studied in the mouse, in some detail, by Leduc (19). With the level of protein administration maintained in our experiments, a significant increase in mitotic rate was not observed until 44 to 48 hours after the beginning of treatment. Leduc, however, observed increases in mitotic rate as early as 12 to 18 hours after transition from a low to a high protein diet. She demonstrated that the actual timing and degree of mitotic activity depended on the previous length of time on the low protein diet, and the amount of protein refed in the high protein diet; in general, the higher the protein content of the diet refed, the sooner and greater the peak of ensuing mitotic activity.

The time course of changes in RNA, protein, and mitotic activity in the liver of rats treated with the protein hydrolysate yielded an interesting and complex pattern. The RNA/DNA ratio showed two distinct increases, the first after 23 hours and the second at 39–42 hours, followed by a decline thereafter. The protein/DNA, on the other hand, showed a significant increase only at 39–42 hours. The decline in both RNA and protein per cell is perhaps due to the synthesis of DNA associated with the onset of mitotic activity at 42 to 48 hours. The irregular increase in RNA per cell could be related to the known diurnal variation in RNA synthetic activity (13).

It is evident that there is a parallelism between the sequence of events obtained by stimulating liver growth with a high protein diet or protein hydrolysate administration and that observed in the liver fragment regenerating after partial hepatectomy. However, there are several important differences between the two phenomena. In the first place, the development of the changes following protein hydrolysate administration are slower in onset and smaller in degree. For example, the cells in regenerating liver increase markedly in size and by 24 hours are 63% above normal (15), whereas, in our experiments, there was no significant increase in cell size after 60 hours of treatment. Again, the increase in RNA per cell reaches a maximum in regenerating liver at 24 hours when it may be 60% (28). After protein hydrolysate the liver cell RNA did not reach a maximum until after 42 hours of treatment, and the actual increase was only 34%. In rats of the body weight range used, the mitotic peak of activity would be expected to occur between 45–49 hours after partial hepatectomy (16); but, after protein hydrolysate treatment, it was still rising after 60 hours, and the highest value attained was of the order of 0.5% of the cells in contrast to the 3.0% of the cells in regenerating liver at its peak (5). On the other hand, it is also known that the regenerative response following partial hepatectomy varies with several factors, e.g., with the diet and age of the animal; rapidly growing young rats restore tissue mass and cell population more rapidly than adults (5).

Another outstanding difference between the two situations is tissue specificity. During hepatic regeneration, no other tissue participates in the growth process to the same extent as liver, whereas, in the process following high protein administration, there is an increase also in kidney weight (23, 25), accompanied by an outburst of mitotic activity (17) and an increase in the protein content of the liver, kidney and, to a lesser extent, the carcass (1). However, Paschkis *et al.* (26) found that during liver regeneration there is a significant elevation of the mitotic index in the cornea, an increased compensatory enlargement of the residual kidney after unilateral nephrectomy, and an increased epiphyseal width of the proximal tibia in hypophysectomized rats. Therefore, the organ-specific nature of the *stimulus* to regenerative growth may be relative rather than absolute.

A still more serious objection to the analogy between the two processes is that liver regeneration occurs even in animals which have been starved (6) or maintained on a protein-free diet (12). In starved rats, the rate of mass restoration is retarded, but the rate of cellular regrowth proceeds apparently at an unimpeded rate (6). Indeed, Gurd *et al.* (12) found that the rate of appearance of new liver protein was greater in the protein-starved than in the casein-fed series. These authors found also that, in both protein-fed and protein-starved animals, the liver protein reached a plateau value after 8 days; this suggests that an equilibrium was reached between the liver and body tissues when a certain level of regeneration was attained. It seemed that the demands of other body tissues for minimal subsistence quantities of the available endogenous protein ultimately counterbalanced the emergency needs of the liver to replace its own deficiency. That a dynamic equilibrium between the liver and the body tissues exists is also suggested by the data of Harrison and Long (14) who demonstrated

that complete regeneration of liver protein occurs only after all of the body proteins lost have been replaced.

It seems possible that the dynamic equilibrium between the body tissues and the liver may be mediated by the blood amino acids, and that not only the equilibrium size of the liver but also its capacity to regenerate may be partly conditioned by the amount of work it has to perform in "processing" the endogenous amino acids from the tissues. However, in opposition to this idea is the lack of any change in the level of free amino acids in the circulating blood after partial hepatectomy (10). However, Drabkin *et al.* (8) have shown that, in the condition of experimental nephrosis in rats, there is mobilization of amino acids mainly from muscle to the liver, where they are channeled into the production of plasma proteins, and that these changes are accompanied by an increase in liver size, RNA, and DNA (24). It is also interesting that in nephrotic rats no consistent change could be observed in the concentration of free amino acids in plasma, but that there was a consistent reduction of free amino acids in muscle (9). The authors assumed that the increased rate of removal of amino acids from the blood to liver and their increased rate of incorporation into plasma proteins in liver may make plasma changes difficult to demonstrate. It could be that a similar situation prevails in the partially hepatectomized rat. Although these investigators have not directly demonstrated amino acid mobilization from the tissues to the liver in regeneration, they have shown that an increased incorporation of amino acids-^{14}C into total liver protein can be demonstrated both *in vivo* and *in vitro* in tissue slices from the liver in the early stages of regeneration (4). Indirect evidence that an increased transport of amino acids is occurring to the regenerating liver fragment per unit weight is provided by the observation that the rate of urea production is rapidly increased during the first 24 hours (30). Also, the increase in glutamic and aspartic acids and the decrease in glutamine in the liver 6 hours after partial hepatectomy may be related to this fact (10).

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MAGGI HYDROLYSED PLANT PROTEIN (HPP) 4BE:

90 DAY TOXICITY STUDY IN RATS

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MAGGI HYDROLYSED PLANT PROTEIN (HPP) 4BE:

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1. Introduction

Hydrolysed plant proteins have for long time been in use in the culinary industry for the preparation of sauces, soups, condiments, etc. The use as food adjuncts of such plant protein hydrolysates range from 0.5 to 1.0% in foods on an "as consumed" basis. Presuming that an average human adult ingests about dry 500g food a day, and all that food contains the hydrolysed plant protein as an adjunct, the daily intake of plant protein hydrolysates would amount to less than 0.1g/kg body weight, for an adult weighing 70kg. For toxicological testing, introducing a safety factor of 10:1 would then mean administration of 1g of test material per kilogram body weight, for trials with rats.

In the investigations under report, our first aim was to assess whether the Maggi HPP 4BE fed at a very high level to rats results in toxic effects, and then, whether the toxicity, if any, is only due to the high amount of sodium chloride in the material or if other additional factors need to be taken into account. In addition, it was aimed at establishing a 'no effect' level, and investigate the results of a 90 day feeding study with the HPP 4BE in rats.

2. Materials and Methods

2.1. Test product:

The Maggi hydrolysed plant protein (HPP) 4BE was obtained from Nestlé Company Inc., Food Ingredients Division, 100 Bloomingdale Road, White Plains, New York, N.Y., USA.

This product, for different markets is produced in liquid and paste forms as well, with the same chemical composition on a dry matter basis.

For practical reasons, HPP 4BE in powder form was chosen for feeding to the animals under trial. The analysis of the HPP 4BE powder is given in Appendix I. Additional analysis for heavy metals indicated that the test material contained lead (Pb) 1.0ppm, arsenic (As) 0.5ppm, mercury (Hg) 0.001ppm, cadmium (Cd) 0.02ppm, and selenium (Se) 2ppm. The amino acid composition of the HPP 4BE powder is set out in Appendix II.

2.2. Animals and Conditions:

SPF Sprague-Dawley rats, obtained from the breeding colony of Tierfarm A.G., Sisseln, Switzerland, were used in the first preliminary trial. Twenty male and twenty female rats weighing 50-60g were randomised and allotted to seven different experimental treatments. The rats were housed singly in Makrolon cages (size III) provided with internal wire-mesh grills. In the animal room the temperature was maintained at 22°C, and humidity at 55%. The animals had programmed light and darkness, each for twelve hours (Appendix IX).

In the second regular trial lasting 90 days, young SPF Sprague-Dawley rats from Süddeutsche Versuchstierfarm, Tuttlingen, W.Germany were used. These rats had an average initial weight of 85g, and once again 20 males and 20 females were randomly allotted to each of the different groups. Housing conditions were exactly the same as before. Diets and water were given ad libitum throughout the test period.

2.3. Groups and dietary treatments:

In the preliminary trial (Appendix III), the basal diet (Appendix IV) used contained 20% protein (%Nx6.25). The control group A had all of its protein coming from casein, whereas in the test groups, casein together with the protein from the HPP 4BE contributed the 20% of total protein. HPP 4BE was incorporated at levels of 10, 20 and 50% (treatments B, C and D, respectively) in the basal diet. Three further groups (E, G and H) contained as additions to the basal diet, amounts of sodium chloride, ammonium chloride and monosodium glutamate, equivalent to those provided by the highest level (50%) of HPP 4BE in test diet D. This arrangement was expected to permit distinction of effects of constituents like sodium chloride from those resulting from a synergistic action of all the other components together and present in the HPP 4BE.

The same basal casein diet cited above was used in the 90 day trial (Appendix VII). The protein content was again 20% (%Nx6.25), but the HPP 4BE powder was only incorporated at levels of 1, 2.5, 5 and 10% respectively, in treatments B, C, D and E. Appropriate adjustments were again made to equalise total protein contents of all diets. To treatment groups G and H only sodium chloride addition was made, respectively at levels provided by the 5 and 10% HPP 4BE groups D and E. In two further groups (I and K) sodium chloride, ammonium chloride and monosodium glutamate were all added together to the basal diet to provide respectively, levels present in the 5 and 10% HPP 4BE containing diets.

2.4. Determinations:

Food consumption of all the rats was recorded daily during the first four weeks, and their general behaviour throughout the trial period. Weight gain data was recorded

weekly for all animals of all groups including the control group A.

At the end of the 90 days of trial blood samples were taken from each animal for determination of red blood cells, white blood cells, haemaglobin and haematocrit (for methods used, see Appendix VIII). Two blood smears were taken from each animal but differential counts were carried out on only 5 male and 5 female rats of each of groups A, D, E, J and K.

Urines were collected at the end of the assay from 5 male and 5 female rats out of every group and examined for albumin, glucose, haemoglobin, pH and sediment.

Plasma glucose; blood urea nitrogen, cholesterol and alkaline phosphatase were determined in all animals of all groups. Transaminase activities were measured only in groups A, D, E, J and K.

At autopsy, performed after an overdose of ether, weights of liver, kidney and testicles were recorded and expressed as g/100g body weight. Liver, kidney, testicles, pancreas, lung and adrenals were then submitted to histological processing, e.g. fixation in Bouin, embedding in paraffin and finally staining with Haematoxylin-Eosin and PAS. In addition to Haematoxylin-Eosin, Alizarin-red was also used for staining of kidney sections.

3. Results of preliminary trial

3.1. General appearance and behaviour of rats:

Already after one week on the trial, animals receiving the highest level (50%) of HPP 4BE showed severe diarrhoea and were consequently in a bad state. They developed bad fur and lost weight. By the third week, several rats from this particular group had died with signs of terminal exsiccosis. In the other groups in this preliminary trial, animals

survived, but the trial was terminated at the end of six weeks. One animal in group E had to be killed when it showed symptoms of a middle ear infection, though this was not related to the feeding of the HPP 4BE.

3.2. Growth (Table I):

Compared to the control group A, growth in both sexes was considerably inferior in group C (20% HPP 4BE), and lowest in group E receiving the highest level (20%) of sodium chloride. Group B (10% HPP 4BE) exhibited a tendency towards retarded growth, whereas G and H containing ammonium chloride and monosodium glutamate respectively, were comparable in growth to the control group A. Since several animals of group D (50% HPP 4BE) died, this group was not comparable with the others.

3.3. Haematology (Table II):

Haematological examination indicated the absence of pathological values for groups A, B, C, E, G and H for both sexes. However, significantly different values were obtained for animals of group E receiving the high amount of sodium chloride.

3.4. Blood chemistry (Table III):

Only values for blood urea nitrogen showed significant ($p \leq 0.01$) as well as dose related elevations in both sexes of groups B, C and E, compared to the control group A. Other parameters such as glucose and transaminase activities remained in the normal range and showed no correlations to the dose of HPP 4BE incorporated. Results for animals of group D (50% HPP 4BE) are not comparable to the others and hence are not set out in detail. However, values for blood urea nitrogen were generally very high in this group (up to 150mg%).

3.5. Autopsy:

One animal from group G which died during the trial period showed internal haemorrhages and its intestines were full of gas. This was just a single case without any relation to the test substance. In group B one animal showed distortion of the spleen which clearly was a congenital malformation. Rats of group D showed hair loss, some internal haemorrhages and generally large amounts of gas in the intestines. The intestines themselves were attached due to an haemorrhagic-catharrhalic enteritis.

Hydronephrosis in kidneys, a common congenital damage in this rat strain, was observed in several of the rats.

3.6. Organ weight (Table IV):

The fresh weights of excised organs of animals in groups B, C and E reflected the established pattern. Thus, liver and kidneys of both males and females from these groups were bigger on a g/100g body weight basis when compared to the controls. Testicles were also bigger in these groups, indicating a clear dose-dependent effect.

3.7. Histopathology (Table V):

In this preliminary trial, only a certain number of animals chosen at random from each of the groups A, B, C, D, G and H were examined.

Liver: Nuclear unrest (polymorphism, hyperchromatism, two or more nucleoli per nucleus, binucleated cells) was present in a mild degree in some animals of all groups. This change was not treatment or dose related. Cytoplasmic vacuoles, predominantly in a peripherolobular location, were seen in a mild, though rarely in a moderate degree in some animals of all the groups. This change representing lipid deposition is considered unrelated to either the treatment or dose level of the test substances.

PAS positive granula, predominantly in the peripherolobular and/or peripherolobar hepatocytes, were observed slightly more frequently and to a higher degree in group D, and less frequently and to a lesser degree in group H than in the other groups. The distribution of granula is in part artificial (steam effect). The presence of increased PAS positive granula is a reversible change.

A mild to moderate decrease in size of hepatocytes (shrunken cytoplasm) was present in 9 out of 22 animals of group D (50% HPP 4BE). Shrunken cytoplasm of hepatocytes was not observed in animals of other groups and is therefore considered a dose-dependent observation.

Lympho-reticular proliferation and infiltration, mainly portal and perivascular and/or focal hepatitis and extra-medullary haematopoiesis were present in single animals of all groups. These represent unspecific responses and are unrelated to either treatment or dose.

Kidney: A mild to moderate degree of hydronephrosis was observed in single animals of several dietary groups. In some instances this was accompanied by focal tubular dilation hyalin casts and focal hypertrophy of the tubular epithelium. This type of hydronephrosis occurs in moderate to high frequency as a congenital disorder in some strains of rats. Tubular dilation can be a congenital disorder, or in the case of involvement of collecting tubules, it is secondary to hydronephrosis. Minimal deposition of lipogenic pigment was noted in a focus of tubular hypertrophy in one animal each of group B (10% HPP 4BE) and group D (50% HPP 4BE). These isolated findings are not treatment related.

Single granulomas, probably of parasitic origin (Trichosomoides crassicauda?) were noted in two animals from each of groups C (20% HPP 4BE) and E (20% NaCl). Lymphoreticular proliferation and infiltration were present in single control and treated animals.

Testis: Incomplete spermatogenesis, and decreased or lack of spermatogenesis was seen in all the 10 animals of group D. In comparison to those of the other groups, the testes of rats in group D appeared juvenile with a moderate to severe degenerative atrophy of the seminiferous tubules. Spermiogenesis was stopped at the stage of spermids fusing to degenerate or form giant cells. Restitutional nuclei were also observed, these resulting from an incomplete division of spermids (chromosomes divide but do not separate, but cells desquamate and are secreted).

The above changes are known to occur in many species as a response to metabolic and other disturbances, as well as all febrile diseases. These changes are generally reversible unless the causatory toxic influences persist for a very long time resulting in fibrosis. There were no abnormal findings in testes of the other groups.

Pancreas: Both the exocrine and endocrine pancreatic tissue was examined from animals of groups A, B, C, E, G and H and from only 3 animals of group D. A mild to moderate decrease in zymogen granules was observed in most of the animals examined. In some cases a normal amount of zymogen or even a slight increase was noted in the periinsular acinar tissue. Since identical findings were made in both the control and treated animals, the diminution of zymogen is interpreted as unrelated either to treatment or dose.

Lung: The lungs of only rats A22, A25, B22, C25, H21 and H23 were examined. There were no abnormal findings.

Adrenal: The adrenals of rats D1, D2, D11, D12, D22 and D23 were examined. There were no abnormal findings.

4. Discussion of histopathology of preliminary trial

Group A (Basal Casein control, 20% protein): Abnormal findings were rare, and where present , mild in degree.

Group B (Basal casein diet+10% HPP 4BE): Abnormal findings were rare, and where present, only mild in degree. They were identical or compatible with those observed in the control group A. There were no HPP 4BE induced changes detectable microscopically.

Group C (Basal casein diet + 20% HPP 4BE): Abnormal findings were rare, and where present only mild in degree with the exception of moderate congenital hydronephrosis in rat C18. They were identical or compatible with those of rats in control group A. There was no HPP 4BE induced change detectable upon microscopic examination.

Group D (Basal casein diet + 50% HPP 4BE): Abnormal findings were made in the liver in the form of mild to moderate decreas in size of liver cell cytoplasm in 9 out of 22 animals. This was most likely a treatment induced change and cannot be further explained with the evidence available. Other hepatic changes in group D rats were identical or compatable with those of control group A.

Moderate to severe testicular changes as described earlier were present in all of the males examined. They represent an unspecific (probably reversible) reaction of the testicular tissue to the treatment.

There were no treatment induced changes in the kidneys.

Group E (Basal casein diet + 19.8%NaCl = amount in group D): Abnormal findings were rare, and where present, only mild in degree. They were identical or compatible with those of rats in control group A. There were no treatment induced changes detectable upon microscopical examination.

Group G (Basal casein diet + 0.65% NH_4Cl = amount in group D) Abnormal findings were rare, and where present, only mild in degree. There were identical or compatible with those of control group A. There were no treatment induced changes detectable upon microscopic examination.

Group H (Basal casein diet + 6.8% MSG = amount in group D): Abnormal findings were rare, and where present, only mild in degree. They were identical or compatible with those of control group A. There were no treatment induced changes detectable upon microscopic examination.

Treatment induced changes were present in livers of 9 out 22 rats and in the testes of all of the 10 males of group D. The hepatic changes consisted chiefly of mild to moderate decrease in the size of hepatocellular cytoplasm. The testicular lesions consisted of moderate to severe degenerative atrophy.

5. Results of 90 day trial

5.1. General appearance and behaviour of rats:

No clinical symptoms appeared during the 90 day trial period. The animals in all groups were normal and unlike in the preliminary trial showed, especially, no hair loss or diarrhoea at any stage during the trial.

5.2. Growth (Table VI):

Growth performance in males recorded during the test period indicated a tendancy to a retarding influence of the test diets. Increasing amounts of HPP 4BE and sodium chloride, and especially, the highest dose of 10% HPP 4BE (group E) lowered the net weight gain.

Food conversion efficiency calculated after 4 weeks of trial was lowest in the 10% HPP 4BE group E, but significantly ($p<0.01$) lower also in groups D (5% HPP 4BE), H (4% NaCl) and K (4%NaCl + 0.13% NH_4Cl + 1.4% MSG), compared to the control group A. In females as well net weight gain after 90 days was lowest in groups E, H and K, whilst food conversion efficiency calculated after 4 weeks was significantly lower in D and E ($p<0.01$) and H and K ($p<0.05$)

5.3. Haematology (Table VII):

Values for red blood cells, white blood cells, haemoglobin and haematocrit showed no changes and remained in the normal range. No major differences in values were observed between the different test groups and the control at the end of the test period. Within treatment variations were minimal with no differences in values between the sexes. Differential counts carried out only for animals of groups A, D, E, J and K showed a normal distribution.

5.4. Urine analysis (Table VIII):

The urines were negative for haemoglobin and glucose. The pH was normal. No abnormal amounts of red blood cells and white blood cells were found in the sediment. The albuminuria observed is common in most rat strains, and no dose effect or other major differences could be detected between the different groups or the sexes. Some cylinders were found in single animals but seem to be of no importance.

5.5. Blood chemistry (Table IX):

At the end of the 90 day trial, blood glucose levels, blood urea nitrogen and cholesterol were in the normal range in all the groups. Slight decrease in glucose levels in test groups and also the slight increase in blood urea nitrogen on the higher doses of HPP 4BE may be interpreted as a functional and compensatory effect. Significant differences, however, appeared between the control and the test groups, especially those groups which had either high levels of the test material or the high level of sodium chloride alone or sodium chloride in addition to ammonium chloride and MSG. No significant differences were noticed between groups on the HPP containing diets D (5% HPP 4BE) and E (10% HPP 4BE) and groups receiving the diet with sodium chloride at 4% or the diet with sodium chloride, ammonium chloride and MSG.

Alkaline phosphatase values seemed to be only slightly elevated with the highest dose level of HPP 4BE in diet.

5.6. Autopsy:

One female rat (H7) died at the beginning of the trial for unknown reasons and was found in an autolysed state.

Macroscopic inspection during autopsy showed single cases of congenital malformations in male rats only, the manifestations being hernia of the diaphragm (E6) and distortion of the spleen (H28, K30). These manifestations are considered to be unrelated to the test products. In 1 male rat (G34) the liver had a pale yellow appearance and looked dystrophic and one testicle was atrophied. This was just a single observation and hence has no correlation to the test substance

5.7. Organ Weights (Table X):

Comparison of the test groups showed that the relative weight of livers of rats of group E (10% HPP 4BE) were slightly greater than those of the control group A, whereas livers of rats of group B (1% HPP 4BE) and group H (4% NaCl) were slightly lower. Rats of group J (2% NaCl + NH_4Cl + MSG) had the lowest relative liver weight which was highly significant ($p<0.01$) compared with the control group A.

Kidney weights were highest in group E but rats in groups C, D, G, H, J and K also showed significant increases compared to those of the control group A.

Relative weights of testicles were found to be increased compared to those of the control group A in the animals of all the groups with the highest dose levels of the test substances, i.e. groups E, H and K.

Liver weights of female rats of the test groups showed no differences compared to those of female controls. Relative weights of kidneys, however, were significantly increased in groups D, E, H and K, compared to those of the females in the control group A.

5.8. Histopathology (Table XI):

Liver: Nuclear unrest (polymorphism, hyperchromatism, two or more nucleoli per nucleus, binucleated cells) was present in a mild degree in several animals, but more frequently in females of the control and test groups. Frequency and degree of this increased nuclear activity were not treatment or dependent. Vacuoles in hepatocytes, predominantly small, were observed in some animals, again mostly females of all groups. This finding, representing lipid deposition, was very mild in degree and was equally frequent in the control group A as in group E with the 10% HPP 4BE. It was not a treatment or dose effect.

Pigment, probably of lipogenic nature, was present in a small quantity in 1 male each of the control group A and test groups D, E and H and in some females of the control group A (2 out of 20) as well as of the treatment groups D (3 out of 10), E (5 out of 20), G (2 out of 10), H (9 out of 19), J (8 out of 10) and K (7 out of 20). This pigment was not present in either the control or treatment groups in the preliminary trial. This observation is interpreted as strain, and to some degree sex related, rather than as a treatment or a dose effect.

Degeneration of single perivascular hepatocytes was seen in one rat each of control group A and test groups D, E, G and J, and 2 rats of group K. This change, as well as mild lympho-reticular proliferation and infiltration in some animals of all groups, focal chronic hepatitis (E27, G34), and extramedullary haematopoiesis (normoblast nests) in single animals of most most groups were inflammatory or unspecific responses. They were unrelated to treatment or dose. (Other hepatic changes seen in G34 are discussed with group G).

Kidney: In most kidneys from the control and treated male rats there was evidence of mild to moderate dilation of tubules. The dilated tubules usually contained hyaline casts.

The degree of change (mild=1, moderate=2) reflected the number of tubules dilated and the degree of dilation. Identical changes occurred less frequently, and generally in slightly milder degrees in the female rats of all groups. Concommitant with tubular dilation, a focal tubular epithelial hypertrophy was frequently seen. In a few cases the latter was accompanied by deposition of a minimal amount of pigment of a lipogenic nature (morphologically compatible with the pigment observed in livers). Epithelial hypertrophy with or without deposition of pigment was often topographically and degree-wise related to tubular dilation. These changes are interpreted as secondary to tubular dilation which itself was strain and to some degree sex related. It is known to occurr in moderate to high frequency in some strains of rats as a congenital hereditary disorder.

A mild degree of lymphoreticular proliferation and infiltration was noted in single animals of the control group, and in most animals of the treated groups.

Testis: Severe degenerative tubular atrophy was noted in G34 (see under discussion of group G). No microscopic changes were present in any other animal of the control or test groups.

6. Discussion of histopathology of 90 day trial

Group A (Basal casein control, 20% protein): Abnormal findings in the liver were rare, and where present, mild in degree.

Dilation of renal tubules with or without subsequent focal epithelial hypertrophy was present in most males and in several females. Other renal changes were rare, and where present, only mild in degree.

There was no testicular lesion.

Group D (Basal casein diet + 5% HPP 4BE): Abnormal findings in the liver were rare, and where present, mild in degree.

Dilation of renal tubules with or without subsequent focal epithelial hypertrophy was present in most male and in several female rats of this group. Other renal changes were rare, and where present (D6), mild in degree.

There was no prominent testicular lesion. All lesions observed were identical or compatible with those of the control group A.

There was no HPP 4BE induced change detectable upon microscopic examination.

Group E (Basal casein diet + 10% HPP 4BE): Abnormal findings in the liver were rare, and where present, mild in degree.

Dilation of renal tubules with or without subsequent focal epithelial hypertrophy was present in most male and half the number of female rats in the group. Other renal changes were rare, and where present, mild in degree.

There was no prominent testicular lesion. All lesions observed were identical or compatible with those noted in control group A.

There was no HPP 4BE induced change detectable upon microscopic examination.

Group G (Basal casein diet + 2% NaCl=amount in group D): Abnormal findings in the liver were rare, and where present, mild in degree, except in rat G34. In this animal, severe vacuolation of hepatocytes (fatty change), moderate fatty hepatocellular degeneration, moderate hepatocytomegaly and hepatocellular degeneration and severe bile duct proliferation were observed. These lesions represent a severe (dietetic?) hepatic dystrophy.

Dilation of renal tubules with or without focal epithelial hypertrophy was present in most males and in half the

number of female rats. Other renal changes were rare, and where present, mild in degree.

There was no prominent testicular lesion. All lesions observed were identical or compatible with those of control group A.

There was no treatment induced change detectable upon microscopic examination.

Group J (Basal casein diet + NaCl + NH_4Cl + MSG = amount in group D): Abnormal findings in the liver were rare, and where present, mild in degree. The exception was the observation of lipogenic pigment in 8 out of 10 female rats examined.

Dilation of renal tubules with or without subsequent focal epithelial hypertrophy was present in most males and in a single female animal. Other renal changes were rare, and where present, mild in degree.

There was no prominent testicular lesion. All lesions observed were identical or compatible with those of control group A.

There was no treatment induced change detectable upon microscopic examination.

Group K (Basal casein diet + NaCl + NH_4Cl + MSG = amount in group E): Abnormal findings in the liver were rare, and where present, mild in degree. The exception was the observation of lipogenic pigment in 7 out of 20 female rats examined.

Dilation of renal tubules with or without subsequent focal epithelial hypertrophy was present in most males and half the number of female rats. There were no other renal changes excepting for minimal lipogenic pigment deposition in the kidneys of rats K19 and K29.

There was no prominent testicular lesion. All lesions

observed were identical or compatible with those of control group A.

There was no treatment induced change detectable upon microscopic examination.

In short all histopathological changes observed in both male and female rats of the various treatment groups with the exception of rat G34 were identical or compatible with those present in the animals of the control group A. The hepatic dystrophy and degenerative testicular atrophy observed in G34 are interpreted as not induced by the treatment since no comparable changes were present in any other animal. Overall there was no treatment or dose related abnormal finding in any animal of any of the test groups.

Histopathological examination of sections of organs in both the preliminary and the 90 day trial was carried out by Prof. Dr. H. Luginbühl, Director, Institute for Tierpathologie, University of Berne, 3043 Uettligen, Berne (Switzerland).

7. Summary and Conclusions

Maggi hydrolysed plant protein (HPP) 4BE was incorporated at high levels (10, 20 and 50%) in a casein based basal, semi-synthetic diet and fed to both male and female SPF, Sprague-Dawley rats, for a period of six weeks in a preliminary trial. This trial, undertaken out of theoretical considerations, demonstrated that the HPP 4BE, administered at excessive levels (50% in diet) caused severe clinical symptoms and death in the rats. The test product contained almost 40% chloride, calculated as sodium chloride. The results of the preliminary trial further indicated that the test product was apparently more toxic to the experimental animals at the high dose level of 50% in the diet, than an amount of sodium chloride alone, equivalent to that contributed by 50% of the HPP 4BE.

The preliminary trial was followed by a regular 90 day feeding study, but with the test product incorporated at lower levels in the basal casein diet. In this second trial, 1%, 2.5%, 5% and 10% of the Maggi HPP 4BE was incorporated in the same casein-based, semi-synthetic diet for feeding to both male and female SPF, Sprague-Dawley rats. These levels correspond to an ingestion of 1, 2.5, 5 and 10g Maggi HPP 4BE per kg body weight by the rats. In general, no clinical symptoms of significance developed in the rats at these levels during the trial period of 90 days.

Weight gain data for the first four weeks of trial indicated lower gains by both sexes of groups D (5% HPP), E (10% HPP), H (4% NaCl) and K (4% NaCl+1.4% MSG+0.13% NH_4Cl), when compared to the control group A (basal). However, the differences proved to be significant with regard only the data for group D males (P 0.05) and group E females (P 0.05). The lower weight gains were associated with higher food intakes, resulting in significantly lower food conversion efficiency for both sexes of groups D, E, H and K. After 90 days of trial, the three groups E, H and K were still lower in body weight compared to control group A or the other

treatment groups. Now, however, rats of group E were not significantly different from those of groups H and K when growth was compared. In fact, the females of group E were even marginally superior with regard to body weight, than the groups H and K receiving the sodium chloride containing diets. It therefore seems appropriate to attribute these effects to the high sodium chloride content (40%) of the product HPP 4BE, this amount of sodium chloride itself naturally being a self-limiting factor in human consumption of the hydrolysed plant protein.

Haematological values, analytical values for urine and biochemical parameters were all within the normal and acceptable range for all the test groups, with minimum variation between values for replicates within each treatment group. The only exception to the normal pattern was the single animal (G34) in group G with a severe hepatic dystrophy. The animals in group G were fed on the low (2%) sodium chloride containing basal diet, which was comparable to the 5% HPP 4BE diet. The elevated value for blood urea nitrogen (BUN, 23mg%) and a value of 84 I.U. for alkaline phosphatase obtained for this particular animal, corresponded to the unusual histological picture.

Although statistical evaluation showed some significant differences in values for certain of the above mentioned parameters between the control group A and the various test groups, there existed no significant differences between the HPP containing, and sodium chloride containing treatment groups. It is concluded that these variations and the significant differences from the controls were a direct result of the level of sodium chloride in the diets, irrespective of whether it was incorporated as the salt, or indirectly along with the test product.

When fresh weights of excised organs from the test groups were compared with those of the control group, differences were only slight, and significant in both sexes only for

the kidneys at the highest dose level of either the HPP or sodium chloride. Since this finding was not accompanied by biochemical or histopathological alterations, it would seem to reflect just a functional adaptation. Simple water retention due to an accelerated electrolyte regulation would then seem to be the most logical explanation for this observation.

In both the preliminary trial terminated after six weeks and the subsequent 90 day trial, the absence of any deleterious effects of the Maggi HPP 4BE was established beyond doubt, by the histopathological examination of the various tissues. The rare lesions in some tissues were always identical or compatible with those encountered in corresponding tissues of animals in the control casein group.

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MAGGI HYDROLYSED PLANT PROTEIN (HPP) 4BE:

90 DAY TOXICITY STUDY IN RATS

T A B L E S

Assay 14

Magi hydrolysed plant protein 4 BE

Table I Mean body weight and mean weight gain of male/female rats (4 weeks)

Groups	Males		Females	
	Body weight	weight gain	Body weight	Weight gain
A	195.9	129.2	163.9	101.0
B	182.3	117.4	153.8	91.2
C	158.3	93.7	136.2	93.6
E	120.9	56.2	115.7	53.0
G	196.8	132.9	165.8	103.2
H	196.1	131.2	162.5	99.7
S.E. of treatment mean	± 1.93	± 2.02	± 1.91	± 1.91
L.S.D. ($P \leqslant 0.05$)	± 5.38	± 5.64	± 5.33	± 5.33

Assay 14

Maggi hydrolyzed plant protein 4 BE

Table II Mean haematological results

Groups	Males				Females			
	RBC $10^6/mm^3$	WBC $10^3/mm^3$	Hb g%	Htc %	RBC $10^6/mm^3$	WBC $10^3/mm^3$	Hb g%	Htc %
A	5.22	14.7	16.1	51	5.03	10.4	16.4	51
B	5.31	13.9	16.1	51	4.92	9.8	16.1	50
C	5.35	13.6	16.2	51	5.10	9.7	15.7	49
E	5.49	11.2	16.8	52	5.56	9.2	16.7	53
G	5.20	13.2	16.3	50	5.34	9.1	16.1	51
H	5.25	12.8	15.9	50	5.13	8.9	16.1	51
S.E. of treatment mean	±0.10	±0.42	±0.14	±0.43	±0.13	±0.40	±0.16	±0.51
L.S.D. ($P \leqslant 0.05$)	±0.26	±1.17	±0.38	±1.20	±0.36	±1.11	±0.44	±1.42

Assay 14

Maggi hydrolyzed plant protein 4 BE

Table III Mean blood chemistry results

Groups	Males			Females		
	BUN mg%	Glucose mg%	SGPT IU	BUN mg%	Glucose mg%	SGPT IU
A	10.1	114.5	8.8	14.8	124.3	8.1
B	16.7	123.5	7.9	15.4	116.7	7.0
C	28.7	130.1	8.5	26.3	115.4	6.9
E	52.1	111.9	9.8	53.1	113.6	7.5
G	11.0	109.6	8.2	13.1	117.9	7.9
H	12.4	110.9	8.4	15.6	124.3	7.8
S.E. of treatment mean	± 1.21	± 3.95	± 0.39	± 1.28	± 3.25	± 0.31
L.S.D. ($P \leq 0.05$)	± 3.40	±11.06	± 1.10	± 3.58	± 9.09	±00.86

Assay 14

Maggi hydrolysed plant protein 4 BE

Table IV Mean organ weights

Groups	Males			Females	
	Liver g	Kidney g	Gonads g	Liver g	Kidney g
A	3.21	0.79	1.34	2.93	0.78
B	3.37	0.97	1.44	3.20	1.06
C	3.69	1.26	1.61	3.63	1.22
E	3.60	1.17	1.71	3.64	1.23
G	3.08	0.78	1.38	2.94	0.80
H	3.12	0.81	1.31	2.96	0.86
S.E. of treatment mean	± 0.05	± 0.02	± 0.03	± 0.04	± 0.02
L.S.D. ($P < 0.05$)	± 0.15	± 0.05	± 0.10	± 0.13	± 0.07

le V

Liver

Group A and B

Assay 14

Maggi hydrolysed plant protein 4 BE

Animal number	group A, males and females										group B, males and females									
	4	8	10	14	19	22	25	30	36	38	3	4	10	13	20	22	27	32	36	40
Nuclear unrest	1	1	0	0	0	1	0	0	0	1	1	1	0	0	0	0	0	1	1	0
Cytoplasmic vacuoles, small predominantly peripherolobular (probably lipid)	0	0	0	0	0	0	1	1	2	1	0	0	0	0	0	0	1	1	1	1
Increased PAS + granula in hepatocytes, predominantly peripherolobular or peripherolobar	0	0	0	2	1	1	0	0	0	-	1	0	0	2	1	-	0	0	0	0
Shrunken cytoplasm, hepatocytes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lymphoreticular proliferation and infiltration, portal and perivascular	0	1	0	0	0	1	1	0	0	1	1	1	0	0	1	1	0	0	0	0
Hepatitis, focal	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0
Extramedullary hematopoiesis (Normoblast nests)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

0 = no change

1 = minimal to mild change

2 = moderate change

3 = severe change

- = no section

Assay 14

Maggi hydrolysed plant protein 4 BE

Table V (contd.)

Liver

Group C, males and females

Animal number	2	6	11	14	18	20	25	31	36	39
Nuclear unrest	0	0	1	0	0	1	0	1	1	0
Cytoplasmic vacuoles, small, predominantly peripherolobular (probably lipid)	0	0	0	0	0	0	0	0	1	0
Increased PAS + granula in hepatocytes, predominantly peripherolobular or periphero-lobar	-	0	1	0	1	1	0	-	0	0
Shrunken cytoplasm, hepatocytes	0	0	0	0	0	0	0	0	0	0
Lymphoreticular proliferation and infiltration, portal and peri-vascular	0	0	0	0	0	1	0	0	1	0
Hepatitis, focal	0	0	0	0	0	1	0	1	0	1
Extramedullary hematopoiesis (Normoblast nests)	0	0	0	0	0	1	1	0	1	0

Assay 14

Maggi hydrolysed plant protein 4 BE

Table V (condt.)

Liver

Group D, males and females

Animal number	1	2	3	8	9	11	12	15	16	17	19	21	22	23	24	25	30	31	35	36	37	38
Nuclear unrest	1	1	0	0	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0	0	1	0
Cytoplasmic vacuoles, small, predominantly peripherolobular (probably lipid)	0	0	0	1	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Increased PAS + granula in hepatocytes, predominantly peripherolobular or peripherolobar	0	0	2	1	0	0	0	0	0	0	0	0	2	2	2	1	1	1	1	2	0	0
Shrunken cytoplasm, hepatocytes	2	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	1	0	1	2
Lymphoreticular proliferation and infiltration, portal and perivascular	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0
Hepatitis, focal	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Extramedullary hematopoiesis (Normoblast nests)	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0

Table V (condt.)

Assay 14

Maggi hydrolysed plant protein 4 BE

Liver

Group H, males and females

Animal number	1	3	12	17	20	21	23	31	36	40
Nuclear unrest	0	1	0	0	0	0	1	0	0	1
Cytoplasmic vacuoles, small, predominantly peripherolobular (probably lipid)	0	0	0	0	0	1	0	1	1	1
Increased PAS + granula in hepatocytes, predominantly peripherolobular or peripherolobar	0	0	0	0	0	0	1	0	0	0
Shrunken cytoplasm, hepatocytes	0	0	0	0	0	0	0	0	0	0
Lymphoreticular proliferation and infiltration, portal and perivascular	0	0	0	0	0	0	0	0	0	1
Hepatitis, focal	0	0	0	0	0	0	0	0	0	0
Extramedullary hematopoiesis (Normoblast nests)	0	0	0	0	0	0	0	0	0	0

Table V (condt.)

Assay 14

Maggi hydrolysed plant protein 4 BE

Liver

Group E and G

Animal number	group E, males and females										group G, males and females									
	4	7	12	18	19	22	28	33	36	40	4	5	10	14	16	21	23	28	30	37
Nuclear unrest	0	0	0	1	0	1	1	1	0	1	1	-	0	1	0	0	0	0	1	0
Cytoplasmic vacuoles, small, predominantly peripherolobular (probably lipid)	0	0	0	0	0	0	0	0	0	0	0	-	1	0	0	0	0	0	1	1
Increased PAS + granula in hepatocytes, predominantly peripherolobular or peripherolobar	0	0	1	0	0	1	0	-	0	0	0	-	0	0	1	0	0	1	-	0
Shrunken cytoplasm, hepatocytes	0	0	0	0	0	0	0	0	0	0	0	-	0	0	0	0	0	0	0	0
Lymphoreticular proliferation and infiltration, portal and perivascular	0	0	1	0	0	0	0	0	1	1	0	-	0	0	0	0	0	0	0	0
Hepatitis, focal	0	0	1	0	0	0	0	0	0	0	0	-	0	0	0	0	0	0	1	0
Extramedullary hematopoiesis (Normoblast cells)	0	0	0	0	1	1	0	0	1	0	1	-	0	0	0	0	0	0	0	0

Table V (contd.)

Assay 14

Maggi hydrolysed plant protein 4 BE

Kidney

Group C, males and females

Animal number	2	6	11	14	18	20	25	29	31	36	39
Hydronephrosis	0	0	0	0	2	1	0	0	0	2	0
Dilated tubules, some with hyalin casts	0	0	0	0	0	1	0	0	0	1	0
Dilated collecting tubules, some with hyalin casts	0	0	0	0	1	1	0	0	0	0	0
Tubular hypertrophy, focal	0	0	0	0	1	0	0	0	0	1	0
Pigment (lipogenic) in foci of tubular hypertrophy	0	0	0	0	0	0	0	0	0	0	0
Granuloma (parasitic?)	0	0	0	0	0	1	1	0	0	0	0
Lympho-reticular proliferation and infiltration	0	1	0	1	0	0	0	0	0	1	0
Testis											
Tubular atrophy, degenerative	0	0	0	0	0	0					
Incomplete spermatogenesis	0	0	0	0	0	0					
Decreased spermatogenesis	0	0	0	0	0	0					

Table V (Contd.)

Group A and B

Assay 14

Maggi hydrolysed plant protein 4 BE

Kidney

Animal number	group A, males and females										group B, males and females									
	4	8	10	14	19	22	25	30	36	38	3	4	10	13	20	22	27	32	36	40
Hydronephrosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dilated tubules, some with hyalin casts	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dilated collecting tubules some with hyalin casts	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tubular hypertrophy focal	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	1
Pigment (lipogenic) in foci of tubular hypertrophy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Granuloma (parasitic)?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho reticular proliferation and infiltration	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
Testis																				
Tubular atrophy degenerative	0	0	0	0	0						0	0	0	0	0					
Incomplete spermatogenesis	0	0	0	0	0						0	0	0	0	0					
Decreased spermatogenesis	0	0	0	0	0						0	0	0	0	0					

Table V (contd.)

Kidney

Group D

Assay 14

Maggi hydrolysed plant protein 4 BE

Animal number	21	21	22	22	23	23	24	24	25	25	30	30	31	31	35	35	36	36	37	37	38	38
Hydronephrosis	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dilated tubules, some with hyalin casts	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dilated collecting tubules some with hyalin casts	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tubular hypertrophy, focal Pigment (lipogenic) in foci of tubular hypertrophy	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Granuloma (parasitic) ?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table V (contd.)

Group D

Assay 14

Maggi hydrolysed plant protein 4 BE

Kidney																				
Animal number	1	1	2	2	3	3	8	8	9	9	11	11	12	12	16	16	17	17	19	19
Hydronephrosis	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dilated tubules, some with hyalin casts	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dilated collecting tubules some with hyalin casts	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tubular hypertrophy, focal	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Pigment (lipogenic) in foci of tubular hypertrophy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Granuloma (parasitic ?)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Testis																				
Tubular atrophy, degenerative	3		3		2		3		3		3		3		2		3		3	
Incomplete spermatogenesis	3		3		2		3		3		3		3		2		3		3	
Decreased spermatogenesis	3		3		2		3		3		3		3		2		3		3	

Table V (contd.)

Assay 14

Maggi hydrolysed plant protein 4 BE

Kidney

Group E and G

Animal number	group E, males and females										group G, males and females									
	4	7	12	18	19	22	28	33	36	40	4	5	10	14	15	21	23	28	30	37
Hydronephrosis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dilated tubules, some with hyalin casts	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Dilated collecting tubules some with hyalin casts	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tubular hypertrophy, focal*	0	0	0	1	1	1	0	1	1	0	0	0	1	0	0	0	0	0	0	0
Pigment (lipogenic) in foci of tubular hypertrophy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Granuloma (parasitic?)	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Testis																				
Tubular atrophy, degenerative	0	0	0	0	0						0	0	0	0	0					
Incomplete spermatogenesis	0	0	0	0	0						0	0	0	0	0					
Decreased spermatogenesis	0	0	0	0	0						0	0	0	0	0					

*mostly collecting tubules, deep cortex (minimal degree)

Table V (condt.)

Assay 14

Maggi hydrolysed plant protein 4 BE

Kidney

Group H, males and females

Animal number	1	3	12	17	20	21	23	31	36	40
Hydronephrosis	0	0	0	0	0	0	0	0	1	0
Dilated tubules, some with hyalin casts	0	0	0	0	0	0	0	0	0	0
Dilated collecting tubules some with hyalin casts	1	0	0	0	0	0	0	0	0	0
Tubular hypertrophy, focal	0	0	1	0	0	0	0	0	1	0
Pigment (lipogenic) in foci of tubular hypertrophy	0	0	0	0	0	0	0	0	0	0
Granuloma (parasitic?)	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	0	0	0	1	0	0	0
Testis										
Tubular atrophy, degenerative	0	0	0	0	0					
Incomplete spermatogenesis	0	0	0	0	0					
Decreased spermatogenesis	0	0	0	0	0					

Table V (contd.)

Assay 14

Maggi hydrolysed plant protein 4 BE

Pancreas

	group A									
Animal number	4	8	10	14	19	22	25	30	36	38
Decrease in cymogene granules	0	2	0	1	0	1	2	1	1	1
Langerhans islands	0	0	0	0	0	0	0	0	0	0

	group B									
Animal number	3	4	10	13	20	22	27	32	36	40
Decrease in cymogen granules	1	0	2	-	1	1	1	1	1	1
Langerhans islands	0	0	0	0	0	0	0	0	0	0

	group C										
Animal number	2	6	11	14	18	20	25	29	31	36	39
Decrease in cymogen granules	1	1	1	1	0	1	1	1	1	1	0
Langerhans islands	0	0	0	0	0	0	0	0	0	0	0

	group D		
Animal number	2	21	22
Decrease in cymogen granules	2	2	2
Langerhans islands	0	0	0

Table V (concld.)

Assay 14

Maggi hydrolysed plant protein 4 BE

Pancreas

	group E									
Animal number	4	7	12	18	19	22	28	33	36	40
Decrease in cymogen granules	1	2	2	2	2	1	0	0	1	1
Langerhans islands	0	0	0	0	0	0	0	0	0	0

	group G									
Animal number	4	5	10	14	16	21	23	28	30	37
Decrease in cymogen granules	0	0	2	1	1	1	1	1	0	1
Langerhans islands	0	0	0	0	0	0	0	0	0	0

	group H									
Animal number	1	3	12	17	20	21	23	31	36	40
Decrease in cymogen granules	1	-	1	1	2	1	1	2	1	2
Langerhans islands	0	-	0	0	0	0	0	0	0	0

Table VI: Weight increases of male rats

Assay 28

Maggi hydrolysed plant protein 4 BE

Treatment Diet	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12
A	44.7	86.3	123.9	149.7	178.4	199.1	218.1	237.6	258.6	271.3	280.8	294.3
B	44.7	54.8	125.1	147.9	178.0	198.4	218.6	235.2	255.4	263.6	272.9	278.6
C	44.5	84.4	121.5	146.0	171.0	190.5	209.8	227.3	248.9	261.4	272.4	277.3
D	44.1	81.9	118.1	138.2	165.2	182.4	203.4	219.0	240.4	253.6	261.2	266.1
E	39.6	75.6	107.2	129.8	152.3	169.1	195.6	205.3	232.8	241.2	248.5	255.8
G	44.5	84.7	121.2	149.0	174.6	193.5	210.3	227.4	249.7	261.5	269.0	273.8
H	42.7	81.4	116.3	141.1	164.7	175.9	195.9	210.7	232.0	243.0	251.6	254.1
J	44.1	77.1	115.6	141.4	166.1	180.0	199.0	219.8	243.5	254.3	262.9	274.0
K	49.3	83.6	120.0	145.4	161.7	189.3	208.9	224.2	246.4	258.2	263.5	269.6

Table VI: Weight increases of female rats

(contd.)

Assay 28

Maggi hydrolysed plant protein 4 BE

Treatment Diet	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11	Week 12
A	24.9	43.5	60.7	73.0	87.8	100.7	110.4	121.2	128.3	131.1	132.7	133.5
B	24.1	44.8	63.6	78.2	89.6	104.5	119.7	127.7	133.0	139.5	138.5	139.7
C	26.8	45.2	61.9	74.0	88.0	99.5	112.0	120.3	127.2	131.3	132.7	132.6
D	25.8	41.9	59.6	70.0	84.5	96.0	106.8	116.3	125.0	130.2	128.8	129.3
E	22.8	39.1	54.9	65.8	79.5	92.4	102.7	108.9	116.4	124.1	123.0	126.6
G	26.1	43.6	60.8	74.2	90.1	95.6	108.9	117.1	125.6	128.9	129.0	130.7
H	25.7	42.6	59.5	70.6	85.1	93.8	100.5	115.1	119.7	123.8	123.8	125.6
J	26.0	42.8	60.7	72.9	89.4	98.3	114.8	123.1	121.8	125.5	127.3	128.3
K	23.3	41.4	55.6	67.2	81.6	92.6	102.9	109.2	116.9	122.1	120.8	123.4

Table VI : Data on weight gain, food intake and food conversion efficiency for 4 weeks (males) (contd.)

Assay 28

Maggi hydrolysed plant protein 4 BE

Treatment /Diet	Weight gain	Food intake	Food conversion efficiency
A	149.8 ±17.8	389.0 ±23.7	0.383 ± 0.028
B	148.0 ±11.5	406.1 ±28.7	0.364 ± 0.028
C	148.0 ±18.7	397.2 ±23.9	0.372 ± 0.037
D	138.4 ± 9.3	417.5 ±18.2	0.331 ± 0.192
E	134.9 ±24.1	419.2 ±47.7	0.323 ± 0.044
G	149.0 ±15.4	401.7 ±21.1	0.371 ± 0.033
H	141.2 ±14.4	398.4 ±19.8	0.354 ± 0.024
J	141.4 ±14.8	395.0 ±30.2	0.358 ± 0.032
K	145.4 ±16.5	418.2 ±18.7	0.347 ± 0.028

1296.1

91.62

235.62 g

3642.30

404.7

$\frac{404.7}{28}$ = 14.45 g food/day

0.356

14.45 g/day × 1 rat / 0.2356 kg × 10 g HPP / 100 g food = 6.17 g HPP / kg body weight

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Table VI: Data on weight gain, food intake and food conversion efficiency for 4 weeks (females) (contd.)

Assay 28

Maggi hydrolysed plant protein 4 BE

Treatment/ Diet	Weight gain	Food intake	Food conversion efficiency
A	73.0 ± 9.9	291.7 ±19.5	0.249 ± 0.010
B	78.2 ±13.6	295.3 ±30.2	0.264 ± 0.032
C	73.5 ±12.5	307.6 ±26.6	0.238 ± 0.030
D	70.1 ±10.5	324.7 ±20.0	0.215 ± 0.026
E	65.9 ±10.5	317.8 ±20.2	0.206 ± 0.024
G	74.2 ±11.7	310.6 ±28.4	0.238 ± 0.024
H	70.6 ±13.1	310.1 ±20.1	0.226 ± 0.032
I	72.9 ±15.4	288.8 ±30.9	0.251 ± 0.040
K	67.3 ±10.3	297.6 ±25.2	0.226 ± 0.026

Table VI: Analysis of significant differences

(contd.)

Assay 28

Maggi hydrolysed plant protein 4 BE

T-test	Weight gain	Food intake	Food conversion Efficiency	Weight gain	Food intake	Food conversion Efficiency
		Male			Female	
A-B		*	*			
A-C					*	
A-D	*	**	**		**	**
A-E		*	**	*	**	**
A-G					*	
A-H			**		*	*
A-J			*			
A-K		**	**			*

* $P \leqslant 0.05$

** $P \leqslant 0.01$

Table VI : Data on weight gain for experimental period of 90 days (males)

(contd.)

Treatment/Diet	Mean Initial Weight (g)	Mean Weight at 90 days (g)	Mean net Weight gain (g)
A	91.62 ± 5.42	375.15 ± 30.34	283.87 ± 30.70
B	91.70 ± 5.27	375.90 ± 26.95	278.60 ± 29.46
C	9o.80 ± 4.97	369.30 ± 29.72	277.35 ± 29.52
D	91.77 ± 5.03	357.95 ± 22.45	266.15 ± 21.55
E	91.77 ± 4.84	337.60 ± 40.33	255.82 ± 39.81
G	91.75 ± 4.82	366.05 ± 36.27	273.80 ± 37.67
H	91.72 ± 4.72	350.75 ± 25.64	254.07 ± 28.77
J	91.72 ± 4.70	360.75 ± 35.51	269.02 ± 34.65
K	91.70 ± 4.73	361.90 ± 26.95	269.60 ± 28.60

x=4.55

91.62

Table VI : Data on weight gain for experimental period of 90 days (females)

(concld.)

Treatment/Diet	Mean Initial Weight (g)	Mean Weight at 90 days (g)	Mean net Weight gain (g)
A	92.72 ± 5.57	225.75 ± 17.58	133.52 ± 15.17
B	92.72 ± 5.61	231.80 ± 18.42	139.67 ± 20.43
C	92.77 ± 5.47	225.85 ± 19.08	132.57 ± 17.90
D	92.80 ± 5.45	221.70 ± 15.45	129.30 ± 16.15
E	92.85 ± 5.36	218.75 ± 20.40	126.55 ± 20.40
G	92.87 ± 5.25	224.05 ± 15.08	130.72 ± 17.20
H	92.90 ± 5.22	218.55 ± 19.44	125.65 ± 18.06
J	92.87 ± 5.30	226.40 ± 22.42	128.27 ± 21.80
K	93.00 ± 5.20	216.45 ± 16.72	123.40 ± 16.34

Table VII: Haematological results/males

Assay 28

Maggi hydrolysed plant protein 4 BE

Group	Red blood cells 10^6 /mm^3	Haemoglobin g %	Haematocrit %	White blood cells 10^3 /mm^3	Lympho %	Neutro %	Eos %	Baso %	Mono %
A	5.56 ±0.5	16.9 ±1.0	51 ±2.5	11.6 ±2.4	86	11	0-3	-	0-3
B	5.34 ±0.6	16.9 ±1.0	50 ±2.4	12.1 ±1.9					
C	5.40 ±0.6	16.9 ±1.0	50 ±2.1	10.9 ±2.0					
D	5.58 ±0.5	17.3 ±0.8	50 ±2.3	11.06 ±2.4	93	5	0-1	-	0-4
E	5.45 ±0.7	17.3 ±0.6	51 ±2.0	10.49 ±2.0	88	9	0-2	-	0-2
G	5.55 ±0.7	16.8 ±0.9	50 ±2.3	10.56 ±3.2					
H	5.52 ±0.7	17.3 ±0.8	50 ±1.1	10.42 ±2.3					
J	5.69 ±0.6	17.3 ±1.1	50 ±1.9	10.90 ±2.0	91	6	0-4	-	0-3
K	5.65 ±0.6	17.5 ±0.8	50 ±2.0	9.9 ±2.3	88	10	0-4	-	0-4

Table VII : Haematological results/females

Assay 28

Maggi hydrolysed plant protein 4 BE

Group	Red blood cells 10^6 /mm^3	Haemoglobin g %	Haematocrit %	White blood cells 10^3 /mm3	Lympho %	Neutro %	Eos %	Baso %	Mono %
A	4.31 ±0.5	15.9 ±0.6	47 ±2.0	9.59 ±2.1	87	7	0-5	-	0-4
B	4.34 ±0.6	17.0 ±0.7	47 ±2.9	10.1 ±2.4					
C	4.54 ±0.7	16.6 ±0.9	47 ±1.8	10.0 ±2.7					
D	4.43 ±0.7	16.6 ±0.9	48 ±1.7	9.1 ±2.3	90	7	0-3	0-1	0-2
E	4.16 ±0.7	16.3 ±0.8	47 ±1.8	7.4 ±1.3	91	7	0-3	0-1	0-2
G	4.40 ±0.7	16.3 ±0.8	46 ±1.5	8.7 ±2.1					
H	4.57 ±0.8	16.8 ±0.7	47 ±2.2	9.11 ±2.2					
J	4.60 ±0.8	16.9 ±0.8	47 ±2.2	8.9 ±2.1	89	9	0-4	-	0-1
K	4.73 ±0.5	16.6 ±0.8	47 ±1.7	7.9 ±2.3	91	7	0-3	-	0-3

Table VIII: Urines/males

Assay 28

Maggi hydrolysed plant protein 4 BE

Group	pH	Hb	Glucose	Albumin			Cylinder			WBC			RBC		
				++	+	(+)	++	+	(+)	++	+	(+)	++	+	(+)
A	6-7	-	-	6/20	5/20	9/20	-	-	-	-	-	-	-	-	-
B	6-7	-	-	9/20	5/20	6/20	-	-	-	-	-	-	-	-	-
C	6-7	-	-	10/20	4/20	6/20	-	-	-	-	-	-	-	-	-
D	6-7	-	-	6/20	4/20	10/20	-	-	-	-	-	-	-	-	-
E	6-7	-	-	9/19	4/19	5/19	-	-	-	-	-	1/6	-	-	-
G	6-7	-	-	2/19	9/19	8/19	-	-	-	-	-	2/4	-	-	1/4
H	6-7	-	-	6/20	6/20	7/20	-	1/7	4/7	-	-	3/7	-	-	-
J	6-7	-	-	8/19	3/19	8/19	-	-	-	-	-	1/6	-	-	-
K	6-7	-	-	4/20	12/20	4/20	-	-	2/5	-	-	1/5	-	-	-

Table VIII:Urines/females

Assay 28

Maggi hydrolysed plant protein 4 BE

Group	pH	Hb.	Glucose	Albumin			Cylinder			WBC			RBC		
				++	+	(+)	++	+	(+)	++	+	(+)	++	+	(+)
A	6-7	-	-	5/20	9/20	6/20	-	-	-	-	-	-	-	-	-
B	6-7	-	-	6/19	7/19	5/19	-	-	-	-	-	-	-	-	-
C	6-7	-	-	8/20	4/20	8/20	-	-	-	-	-	-	-	-	-
D	6-7	-	-	5/20	8/20	4/20	-	-	-	-	-	-	-	-	-
E	6-7	-	-	6/20	8/20	5/20	-	-	1/6	-	-	2/6	-	-	-
G	6-7	-	-	5/19	7/19	7/19	-	-	-	-	-	-	-	-	-
H	6-7	-	-	1/20	7/20	12/20	-	-	-	-	-	1/6	-	-	1/6
J	6-7	-	-	6/19	4/19	9/19	-	-	-	-	-	-	-	-	-
K	6-7	-	-	1/18	4/18	13/18	-	-	3/5	-	-	2/5	-	-	-

Table IX : Blood chemistry results/males

Assay 28

Maggi hydrolysed plant protein 4 BE

Group	Glucose mg%	Blood urea nitrogen mg%	Cholesterol mg%	Transaminase-GP IU	Alkaline Phosphatase IU
A. Bd (basal diet/ 20% protein)	126 ±15.6	15.4 ±1.46	77.3 ±13.84	11.0 ±1.47	27.9 ±5.58
B. Bd + 1% 4BE	117 ±10.5	15.7 ±1.80	78.9 ±16.74		29.4 ±6.95
C. Bd + 2.5% 4BE	114 ± 9.9	16.0 ±2.52	80.2 ±11.83		29.8 ±6.12
D. Bd + 5% 4BE	115 ±16.0	16.1 ±2.52	81.1 ±15.25	14.2 ±0.79	28.4 ±5.41
E. Bd + 10% 4BE	107 ±14.2	18.2 ±3.98	92.6 ±15.66	18.5 ±4.82	34.3 ±7.28
G. Bd + NaCl equal to diet D	114 ±24.1	16.7 ±2.42	84.3 ±18.25		34.0 ±13.8
H. Bd + NaCl equal to diet E	106 ±12.1	18.0 ±2.12	84.5 ±16.17		28.6 ± 3.0
J. Bd + NaCl, NH_4Cl and MSG equal to diet D	108 ±13.1	16.7 ±2.00	86.9 ±18.17	14.3 ±3.83	35.1 ±12.21
K. Bd + NaCl, NH_4Cl and MSG equal to diet E	111 ±13.0	19.6 ±2.50	91.0 ±22.14	14.0 ±3.12	31.7 ± 8.2
t-test					
A - B	*				
A - C	**				
A - D	*			**	
A - E	**	**	**	**	*
A - G		*			
A - H	**	**			
A - I	**	*			*
A - K	**	**	*		
D - G					
D - I					*
E - H					**
E - K					

** $p \leq 0.01$ * $p \leq 0.05$

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Table IX: Blood chemistry results/females

Assay 28

Maggi hydrolysed plant protein 4 BE

Group	Glucose mg%	Blood urea nitrogen mg%	Cholesterol mg%	Transaminase-GP IU	Alkaline Phosphatase IU
A.Bd (basal diet/ 20% protein)	126 ± 26.7	16.9 ±2.26	85.5 ± 14.6	7.9 ±2.48	26.2 ±9.28
B.Bd + 1% 4BE	121 ± 20.8	16.7 ±2.95	77.6 ± 11.05		25.7 ±4.79
C.Bd + 2.5% 4BE	121 ± 26.4	16.6 ±2.62	78.6 ± 16.8		25.6 ±5.72
D.Bd + 5% 4BE	118 ± 22.1	16.6 ±2.66	80.2 ± 9.63	8.3 ±1.05	25.1 ±5.25
E.Bd + 10% 4BE	115 ± 17.7	17.3 ±3.20	84.1 ± 14.14	8.7 ±1.71	28.3 ±6.24
G.Bd + NaCl equal to diet D	120 ± 16.5	16.3 ±3.40	81.9 ± 17.8		26.2 ±6.45
H.Bd + NaCl equal to diet E	109 ± 10.5	18.4 ±3.73	87.5 ± 16.1		30.0 ±5.32
J.Bd + NaCl, NH_4Cl and MSG equal to diet D	116 ± 18.4	16.4 ±3.54	85.0 ± 14.7	8.8 ±1.06	30.3 ±5.25
K.Bd + NaCl, NH_4Cl and MSG equal to diet E	127 ± 22.1	17.3 ±2.44	92.4 ± 15.8	11.5 ±2.22	29.0 ±6.66
t-test					
A - B					
A - C					
A - D					
A - E					
A - G					
A - H	**				
A - I					
A - K				*	
D - G					
D - I					*
E - H					
E - K				*	

** $p \leq 0.01$ * $p \leq 0.05$

Assay 28
Maggi hydrolysed
plant protein 4 BE

Table X : Mean organ weights

Groups	Males			Females	
	Liver g	Kidney g	Gonads g	Liver g	Kidney g
A	3.33	0.64	0.87	2.67	0.66
B	3.22	0.65	0.88	2.59	0.66
C	3.37	0.69	0.90	2.59	0.71
D	3.33	0.73	0.92	2.69	0.73
E	3.46	0.81	0.94	2.81	0.82
G	3.27	0.71	0.89	2.56	0.71
H	3.22	0.71	0.92	2.62	0.73
J	3.12	0.69	0.90	2.57	0.69
K	3.20	0.73	0.93	2.66	0.75

Assay 28

Maggi hydrolysed plant protein 4 BE

Table XI

Liver

Group A, male rats

Animal number	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40
Nuclear unrest	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
Vacuoles in hepatocytes, predominantly small	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Pigment (lipogenic) in phagocytes and Kupffer cells, predominantly perivascular	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Degeneration of single hepatocytes, perivascular	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0
Normoblast nests	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Hepatitis, chronic, single focus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

0 = no change

1 = minimal to mild change

2 = moderate change

3 = severe change

- = no section

Assay 28

Maggi hydrolysed plant protein 4 BE

Table XI (contd.)

Liver

Group A, female rats

Animal number	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39
Nuclear unrest	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1
Vacuoles in hepatocytes, predominantly small	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
Pigment (lipogenic) in phagocytes and Kupffer cells, predominantly perivascular	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0
Degeneration of single hepatocytes, perivascular	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Lympho-reticular proliferation and infiltration	0	0	0	0	0	1	1	0	1	0	0	0	0	1	1	0	0	0	1	0
Normoblast nests	1	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
Hepatitis, chronic, single focus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Assay 28

Maggi hydrolysed plant protein 4 BE

Table XI (contd.)

Liver

Group D

Animal number	males											females									
	2	4	6	8	10	12	14	20	32	38	40	1	3	5	21	23	25	27	35	37	39
Nuclear unrest	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	1
Vacuoles in hepatocytes, predominantly small	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Pigment (lipogenic) in phagocytes and Kupffer cells, predominantly perivascular	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0
Degeneration of single hepatocytes, perivascular	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	1	0	1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1
Normoblast nests	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Hepatitis, chronic, single focus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Assay 28

Maggi hydrolysed plant protein 4 BE

Table XI (contd.)

Liver

Group E, male rats

Animal number	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40
Nuclear unrest	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
Vacuoles in hepatocytes, predominantly small	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pigment (lipogenic) in phagocytes and Kupffer cells, predominantly perivascular	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Degeneration of single hepatocytes, perivascular	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Normoblast nests	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0
Hepatitis, chronic, single focus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Assay 28

Maggi hydrolysed plant protein 4 BE

Table XI (contd.)

Liver

Group E, female rats

Animal number	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39
Nuclear unrest	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	1	1	1
Vacuoles in hepatocytes, predominantly small	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1
Pigment (lipogenic) in phagocytes and Kupffer cells, predominantly perivascular	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1
Degeneration of single hepatocytes, perivascular	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Lympho-reticular proliferation and infiltration	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
Normoblast nests	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Hepatitis, chronic, single focus	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0

Assay 28

Maggi hydrolysed plant protein 4 BE

Table XI (contd.)

Liver

Group G

Animal number	males										females									
	6	8	20	22	30	32	34*	36	38	40	1	3	5	11	21	23	25	35	37	39
Nuclear unrest	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1
Vacuoles in hepatocytes, predominantly small	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	1	0
Pigment (lipogenic) in phagocytes and Kupffer cells, predominantly perivascular	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1
Degeneration of single hepatocytes, perivascular	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	1	1	0	0	0	0	3	0	0	0	0	0	0	0	0	1	0	0	0	0
Normoblast nests	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hepatitis, chronic, single focus	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0

*see extra table

Assay 28

Maggi hydrolysed plant protein 4 BE

Table XI (contd.)

Group G

Liver (continued)

Animal number	male 34
Fatty change, predominantly centrolobular	3
Fatty hepatocellular degeneration	2
Hepatocytomegaly and hepatocellular regeneration	2
Bile duct proliferation	3
Polymorphonuclear cell infiltration	1
Adrenal	
Vacuoles, small and large in fasciculata	2
Medulla	0
Heart	0
Spleen	0

Assay 28

Maggi hydrolysed plant protein 4 BE

Table XI (contd.)

Group H, male rats

Liver

Animal number	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40
Nuclear unrest	1	0	0	0	0	0	0	-	0	1	0	0	0	0	0	0	0	0	0	0
Vacuoles in hepatocytes, predominantly small	0	0	0	0	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0
Pigment (lipogenic) in phagocytes and Kupffer cells, predominantly perivascular	0	0	0	0	0	0	0	-	0	0	0	0	0	1	0	0	0	0	0	0
Degeneration of single hepatocytes, perivascular	0	0	0	0	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	1	1	0	0	0	0	-	0	0	0	0	1	1	0	0	0	0	0	0
Normoblast nests	0	0	0	1	0	0	1	-	0	0	0	0	0	0	0	0	0	0	0	0
Hepatitis, chronic, single focus	0	0	0	0	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0

Assay 28

Maggi hydrolysed plant protein 4 BE

Table XI (contd.)

Liver

Group H, female rats

Animal number	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39
Nuclear unrest	0	0	0	-	0	1	0	1	0	0	0	0	0	0	0	1	1	1	1	0
Vacuoles in hepatocytes, predominantly small	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1
Pigment (lipogenic) in phagocytes and Kupffer cells, predominantly perivascular	1	0	0	-	0	0	1	1	1	0	1	0	1	0	0	1	1	0	0	1
Degeneration of single hepatocytes, perivascular	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	-	0	0	1	1	0	0	1	0	1	0	0	0	1	1	1	0
Normoblast nests	0	0	0	-	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1
Hepatitis, chronic, single focus	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Liver

Table XI (contd.)

Group J

Assay 28

Maggi hydrolysed plant protein 4 BE

Animal number	males										females									
	2	4	6	14	22	24	26	36	38	40	1	3	5	13	21	23	25	35	37	39
Nuclear unrest	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	1
Vacuoles in hepatocytes predominantly small	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Pigment (lipogenic) in phagocytes and Kupffer cells, predominantly perivascular	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1	1	1
Degeneration of single hepatocytes, perivascular	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	0	1	1	1	0	0	1	0	1	0	1	0	1	0	1	0	1
Normoblast nests	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hepatitis, chronic, single focus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Assay 28

Maggi hydrolysed plant protein 4 BE

Table XI (contd.)

Group K, male rats

Liver

Animal number	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40
Nuclear unrest	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Vacuoles in hepatocytes, predominantly small	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pigment (lipogenic) in phagocytes and Kupffer cells, predominantly perivascular	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Degeneration of single hepatocytes, perivascular	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0
Normoblast nests	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
Hepatitis, chronic, single focus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table XI (contd.)

Group K, female rats

Assay 28

Maggi hydrolysed plant protein 4 BE

Liver

Animal number	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39
Nuclear unrest	1	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	1	1	1	1
Vacuoles in hepatocytes, predominantly small	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0
Pigment (lipogenic) in phagocytes and Kupffer cells, predominantly perivascular	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	1	1	1	1
Degeneration of single hepatocytes, perivascular	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Normoblast nests	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Hepatitis, chronic, single focus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table XI (contd.)

Group A, male rats

Assay 28

Maggi hydrolysed plant protein 4 BE

Kidney

Animal number	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40
Dilated tubules with hyalin casts	1	2	1	2	1	0	1	1	1	1	1	1	1	1	1	0	1	1	0	1
Tubular hypertrophy, focal	0	2	0	1	0	0	1	1	1	1	1	1	0	0	0	1	0	1	0	0
Pigment (lipogenic) in foci of tubular hypertrophy (intra- and extracellular)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Testis																				
Tubular atrophy, degenerative	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Assay 28

Maggi hydrolysed plant protein 4 BE

Table XI (contd.)

Group A, female rats

Kidney

Animal number	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39
Dilated tubules with hyalin casts	1	0	1	2	0	1	0	0	0	1	0	1	0	0	0	1	0	0	1	0
Tubular hypertrophy, focal	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Pigment (lipogenic) in foci of tubular hypertrophy (intra-and extracellular)	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0

Table XI (contd.)

Assay 28

Maggi hydrolysed plant protein 4 BE

Kidney

Group D

Animal number	males											females									
	2	4	6	8	10	12	14	20	32	38	40	1	3	5	21	23	25	27	35	37	39
Dilated tubules with hyalin casts	1	1	1	-	1	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	1
Tubular hypertrophy, focal	1	1	1	-	1	2	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0
Pigment (lipogenic) in foci of tubular hypertrophy (intra- and extracellular)	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	1	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Testis																					
Tubular atrophy, degenerative	0	0	0	0	0	0	0	0	0	0	0										

Assay 28

Maggi hydrolysed plant protein 4 BE

Table XI (contd.)

Group E, male rats

Kidney

Animal number	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40
Dilated tubules with hyalin casts	0	2	1	2	1	2	0	1	1	1	0	1	1	1	1	1	1	1	1	1
Tubular hypertrophy, focal	0	1	1	2	1	1	1	1	0	1	0	1	0	1	1	1	0	1	1	0
Pigment (lipogenic) in foci of tubular hypertrophy (intra- and extracellular)	0	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0
Lympho-reticular proliferation and infiltration	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Testis																				
Tubular atrophy, degenerative	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table XI (contd.)

Assay 28

Maggi hydrolysed plant protein 4 BE

Kidney

Group E, female rats

Animal number	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39
Dilated tubules with hyalin casts	1	0	1	0	0	0	1	1	1	0	0	1	1	0	1	1	0	1	0	0
Tubular hypertrophy, focal	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
Pigment (lipogenic) in foci of tubular hypertrophy (intra- and extracellular)	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table XI (contd.)

Assay 28

Maggi hydrolysed plant protein 4 BE

Kidney

Group G

Animal number	males										females									
	6	8	20	22	30	32	34	36	38	40	1	3	5	11	21	23	25	35	37	39
Dilated tubules with hyalin cast	1	1	1	1	1	1	0	2	0	1	0	1	1	1	1	1	0	0	0	0
Tubular hypertrophy, focal	0	1	1	1	1	1	0	1	0	1	0	1	1	0	0	0	0	0	0	0
Pigment (lipogenic) in foci of tubular hypertrophy (intra- and extracellular)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Testis																				
Tubular atrophy, degenerative	0	0	0	0	0	0	3	0	0	0										

Table XI (contd.)

Group H, male rats

Assay 28

Maggi hydrolysed plant protein 4 BE

Kidney																				
Animal number	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40
Dilated tubules with hyalin casts	1	1	1	2	1	1	1	-	1	1	1	2	1	1	1	1	2	1	1	1
Tubular hypertrophy, focal	1	1	1	1	1	0	1	-	1	1	1	2	1	1	1	1	2	1	0	0
Pigment (lipogenic) in foci of tubular hypertrophy (intra- and extracellular)	1	0	0	0	0	0	0	-	0	0	0	0	1	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	1	1	0	1	0	0	-	0	0	0	0	0	0	0	0	0	1	0	0
Testis																				
Tubular atrophy, degenerative	0	0	0	0	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0

Table XI (contd.)

Assay 28

Maggi hydrolysed plant protein 4 BE

Kidney

Group H, female rats

Animal number	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39
Dilated tubules with hyalin casts	0	0	0	-	0	0	0	1	0	0	1	0	0	1	0	1	1	1	0	0
Tubular hypertrophy, focal	0	1	0	-	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
Pigment (lipogenic) in foci of tubular hypertrophy (intra- and extracellular)	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table XI (contd.)

Assay 28

Maggi hydrolysed plant protein 4 BE

Kidney

Group J

Animal number	males										females									
	2	4	6	14	22	24	26	36	38	40	1	3	5	13	21	23	25	35	37	39
Dilated tubules with hyalin casts	0	1	1	0	1	1	0	2	1	2	0	0	0	0	0	1	0	0	1	0
Tubular hypertrophy, focal	0	0	0	1	0	0	1	1	0	2	0	0	0	0	0	1	0	0	0	0
Pigment (lipogenic) in foci of tubular hypertrophy (intra- and extracellular)	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	C	0	0	0
Testis																				
Tubular atrophy, degenerative	0	0	0	0	0	0	0	0	0	0										

Assay 28

Maggi hydrolysed plant protein 4 BE

Table XI (contd.)

Group K, male rats

Kidney

Animal number	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40
Dilated tubules with hyalin casts	1	1	1	1	2	1	2	1	0	1	1	1	1	1	1	1	2	2	1	0
Tubular hypertrophy, focal	1	1	1	0	2	1	1	1	0	1	1	1	1	1	2	0	2	1	0	0
Pigment (lipogenic) in foci of tubular hypertrophy (intra- and extracellular)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Testis																				
Tubular atrophy, degenerative	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table XI (concld.)

Assay 28

Maggi hydrolysed plant protein 4 BE

Kidney

Group K, female rats

Animal number	1	3	5	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35	37	39
Dilated tubules with hyalin casts	0	1	0	1	1	0	0	0	0	1	1	1	1	1	1	0	0	1	0	0
Tubular hypertrophy, focal	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
Pigment (lipogenic) in foci of tubular hypertrophy (intra- and extracellular)	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
Lympho-reticular proliferation and infiltration	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

MAGGI HYDROLYSED PLANT PROTEIN (HPP) 4BE:

90 DAY TOXICITY STUDY IN RATS

A P P E N D I C E S

APPENDIX I

ANALYSIS OF HPP 4BE POWDER

Total solids	97.5	%
Ash	51.7	%
Arganic solids	45.8	%
Chloride, calc. as NaCl	39.6	%
Total Nitrogen	4.74	%
Protein (N% x 6.25)	29.6	%
MSG	13.6	%
Ammoniumchloride	1.25	%
pH	5.10	%

BACTERIOLOGICAL SPECIFICATION

Standard plate count	7500	(/8 g)
Yeast and mold	50	(/ g)
Total Thermophiles	150	(/10g)
Flat	50	(/10g)
Coliform	negative	
Salmonella	negative	

(Above Figures represent maximum limits)

APPENDIX II

AMINO ACID COMPOSITION OF POWDERED HYDROLYSATE 4BE

Lysine	1.02	%
Histidine	0.39	%
NH_3	0.45	%
Arginine	1.40	%
Aspartic acid	2.32	%
Threonine	1.02	%
Serine	1.15	%
Glutamic acid	11.90	%
Proline	1.77	%
Glycine	1.25	%
Alanine	2.08	%
Valine	1.38	%
Methionine	0.24	%
Isoleucine	0.71	%
Leucine	1.17	%
Tyrosine	0.27	%
Phenylalanine	0.87	%
Total Amino Acids	29.39	%

Appendix III

Preliminary Trial

Groups :

A. Basal casein control (20% protein)
B. Basal casein diet + 10% Maggi powder 4BE
C. Basal casein diet + 20% Maggi powder 4BE
D. Basal casein diet + 50% Maggi powder 4BE
E. Basal casein diet containing 19.8 % NaCl equal to D
G. Basal casein diet containing 0.65 % NH_4Cl equal to D
H. Basal casein diet containing 6.8 % Monosodium glutamate equal to D

All diets contain 20% protein; appropriate adjustments were made in the amounts of casein in diets B, C and D, to take into account the protein contributed by Maggi powder 4BE.

Appendix IV

BASAL DIET

Casein	(Nutritional Biochemicals Corporation, Cleveland, Ohio, USA, high nitrogen casein)	20 %
Arachis oil	(Denrées coloniales, S.A., Yverdon Switzerland)	10 %
Potato starch	(Siegfried A.G., Zofingen, Switzerland)	5 %
Corn starch	(Siegfried A.G., Zofingen, Switzerland)	q.s.ad 100
Vitamin mix	(For detail see appendix V)	1 %
Mineral mix	(Nutritional Biochemicals corporation, Cleveland, Ohio, USA, For detail see appendix VI)	4 %

Appendix V

COMPOSITION OF VITAMIN CORNSTARCH PREMIX FOR RATS

(containing water-soluble and fat-soluble vitamins and included at 1 % level in diets)

Water-soluble vitamins	g/1000g	µg/1g diet	
Thiamine (B1) HCL	0.600	6	µg/g diet
Riboflavin (B2)	1.000	10	"
Pyridoxine (B6) HC1	0.400	4	"
Pantothenic acid (Ca)	4.000	40	"
Nicotinic acid	4.000	40	"
Inositol	25.000	250	"
Para-amino benzoic acid	10.000	100	"
Biotin	0.020	0.2	"
Folic acid	0.200	2	"
Vitamin B 12	0.002	0.02	"
Choline chloride	200.000	2000.0	"

Fat-soluble vitamins in 20 g oil			
Vit. A palmitate (1.75×10^6 ui/1g)	0.286	5	ui/g diet
Vitam. D3., calciferol (40×10^6 ui/1g)	0.003125	1.25	ui/g diet
Vitamine E (D1-α- tocopheryl acetate)	6.7	67	µg/g diet
Vitamin K1 (2-methyl-3-phytyl-1-4-naphthoquinone) or Vitamin K3 (2-methyl-1-4-naphthoquinone)	0.50	5.0	µg/g diet
Corn starch	734.78 g		
	1000.0 g		

Appendix VI

COMPOSITION OF MINERAL PREMIX

Reference: Hawk, Oser & Summerson, - Salt Mix 3 (Science, 74, 369, (1931), with addition of basic zinc carbonate ($ZnCO_3$)

A) Components		g/1000 g
Ca citrate, $4H_2O$	$Ca(C_6H_5O_7).\ 4H_2O$	308.2
Ca $(H_2PO_4)_2.\ H_2O$		112.8
KH_2PO_4		218.7
KCL		124.7
NaCL		77.0
$CaCO_3$		68.5
$3M_gCO_3.\ M_g\ (OH)_2.\ 3H_2O$		35.1
$M_gSO_4.\ H_2O$ (dried)		38.3
$ZnCO_3$ (basic)		1.0
Trace salt mixture *		15.7
		1000.0

B) *Trace salt mixture	g/100 g
Ferric citrate	86.50
$CuSO_4.5H_2O$	5.98
NaF	0.76
$MnSO_4.\ 5H_2O$	1.07
K Al $(SO_4)_2.\ 12H_2O$	0.54
KIO_3	0.30
Corn starch	4.85
	100.0

Appendix VII

MAGGI HPP 4BE: 90 day trial with rats.

Groups :

A Basal casein diet/20% protein

B " " " + 1% Maggi powder 4BE

C " " " +2,5% " " "

D " " " + 5% " " "

E " " " + 10% " " "

G " " " +NaCl, in amounts equal to 5% diet D

H " " " +NaCl, in amounts equal to 10%diet E.

J " " " +NaCl, NH_4Cl and MSG in amounts equal to 5% diet D

K " " " +NaCl, NH_4Cl and MSG in amounts equal to10% diet E

Grpups :	A	B	C	D	E	G	H	I	K
Casein	23.85	23.45	22.92	22.05	20.30	23.85	23.85	23.85	23.85
Salt	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Vitamins	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Oil	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Sugar	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Maggi	-	1.00	2.50	5.00	10.00	-	-	-	-
Corn Starch	46.15	45.55	44.58	42.95	39.70	44.17	42.19	43.43	40.70
Potato Starch	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
NaCl	-	-	-	-	-	1.98	3.96	1.98	3.96
NH_4Cl	-	-	-	-	-	-	-	0.06	0.13
MSG	-	-	-	-	-	-	-	0.68	1.36

All diets contain 20% protein; appropriate adjustments were made in the amounts of casein in diets B, C, D and E, to take into account the protein contributed by Maggi powder 4BE.

Appendix VIII

METHOD USED IN HAEMATOLOGY

Determination	sample	Method	Reference
Haemoglobin	whole blood	Cyanmethaemoglobin	W.H. Crosby, J.I. Munn and F.W. Furth (1954) A. Armed Forces Medical Journal 5, 693-703.
Haematocrit	whole blood	Microcentrifuge	Mc. Inroy, R.A. (1954) J. Clin. Path. 7, 32.
RBC	whole blood	Coulter Counter	Technical Bulletin
WBC	whole blood	Coulter Counter	Technical Bulletin
Differential Count	whole blood	Staining of Pappenheim	

METHODS USED IN BLOOD CHEMISTRY

Determination	sample	Method	Reference
Alkaline Phosphatase	plasma	Boehringer/ colorimetric	Bessey, O.A.; Lowry, O.H. and Brock, M.J.; J. Biol. Chem. 164 (1964) 321.
Blood urea nitrogen	plasma	Auto-Analyzer	Technicon modification of the method of Marsh, W.H. Fingerhut, B. and Miller, H.; Clinical Chemistry 11 (1965) 624.
Cholesterol	plasma	Liebermann-Burchard	Bloor, W.R.; J. Biol. Chem. 24 (1961) 227.
Glucose	plasma	Auto-Analyzer	Hoffmann, W.S.; J. Biol. Chem. 120 (1937) 51.
Glutamic-pyruvic transaminase	plasma	Boehringer/ U.V.	Wroblewski, F. and La Due, J.S. Proc. Soc. exp. Biol. Med. 91 (1956) 569.

Appendix IX

The SPF-animals used were free and controlled from the following micro-organisms:

Micro-organism	Effects in rats
1. Klebsiella	latent, mortality in stress,
2. Staphylococcus aurius (haemolytic, coagulase +)	abscesses.
3. Streptococcus pyogenes	septicaemia.
4. Bordetella bronchiseptica	general irritation, lung.
5. Pasteurella multocida	septicaemia, mortality.
6. Yersinia pseudotuberculosis	pseudotuberculosis.
7. Pseudomonas aeruginosa	pneumonia, infection of urinary tract.
8. Salmonella	general, liver, mortality.
9. Listeria monocytogenes	C.N.S. blood composition, liver.
10. Streptobacillus moniliformis	polyarthritis, paralysis, anaemia.
11. Mycoplasma (PPLO) various serotypes	latent, growth.
12. Trichophyton	hairloss, multiple abscesses, mortality.
13. Leptospira icterohaemorrhagiae	growth, blood composition, liver.
14. Endo-and ectoparasites	growth, general irritation.

Chapter 6 Various Properties of Flavor Enhancers

6.1.2 Stability of Monosodium Glutamate

a) Changes by chemical reaction

1) Racemization

The change of L-amino acid to its antipode (D-amino acid) is called racemization. It is usually a partial change. L-Glutamic acid loses its flavor enhancing effect as a result of racemization because D-Glutamic acid has no flavor enhancing property. The racemization tends to occur in storongly acidic or alkaline conditions, especially in the latter, and is promoted when heated.

Fig.6.1 shows the racemization tendency of L-Glutamic acid in alkaline conditions at 100°C. As shown in this figure, racemization is remarkable at pH 8.5 or above. However, actually, no fool being placed under such conditions in its usual manufacturing or cooking process, there is no need to give a particular consideration on racemization.

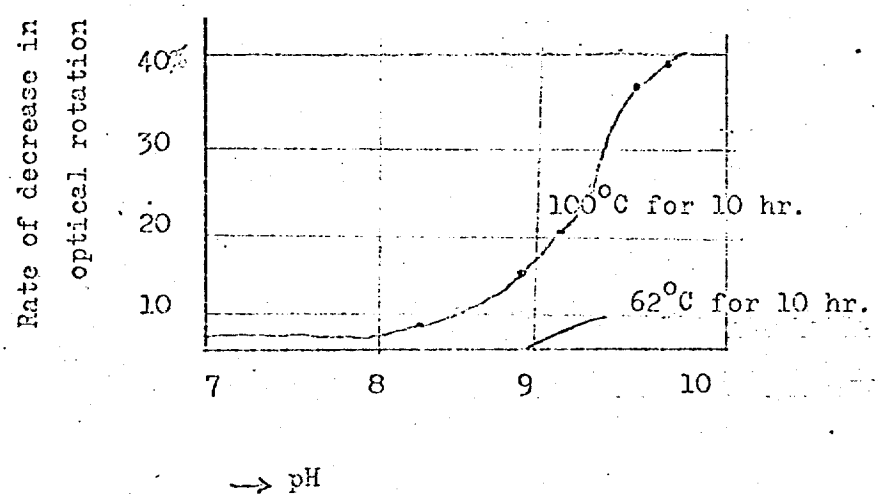
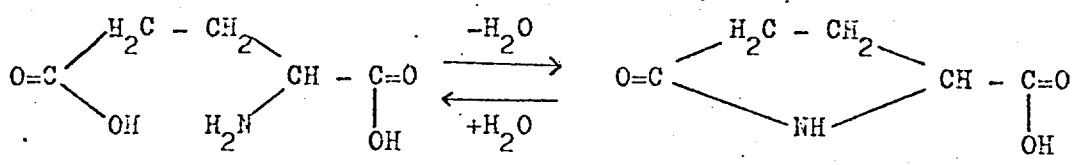


Fig.6.1 Racemization of sodium L-glutamate [1)]

2) Pyrrolidone formation

Glutamic acid has a horseshoe-shaped molecular structure with a carboxyl group on one end and an amino group on the other. So it is easily converted into pyrrolidonecarboxylic acid by cyclization due to dehydration from these two groups. This reaction is called pyrrolidone formation.



Glutamic acid Pyrrolidonecarboxylic acid

The pyrrolidone formation is promoted with the increase of acidity as shown in Fig.6.2 and the reaction is extremely retarded near the neutral point. Therefore, there is no possible loss of monosodium glutamate resulted from pyrrolidone formation expect such an extraordinary case that acidic food is cooked for many hours at high temperatures.

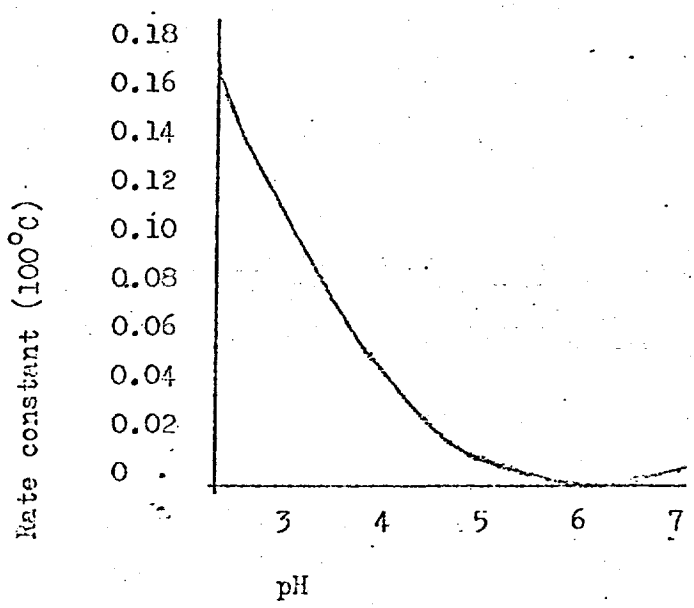


Fig.6.2 Velocity of pyrrolidone formation [2)]

3) Browning Reaction

When amino acid is heated with reducing sugar, so-called browning is induced by the Maillard reaction.

In comparison with basic amino acids, the degree of Maillard reaction is less in glutamic acid. However, precautions must be taken when the food containing a large amount of reducing sugar is processed at high temperatures.

b) Changes by enzyme action

L-Glutamic acid is metabolized by such enzymes as transaminase, dehydrogenase, decorboxylase, glutamine synthetase and glutamate racemase. Accordingly it is necessary to know that these enzymes, when existed, will transform L-Glutamic acid and thereby reduce the good taste of same by the following reactions. These enzyme actions tends to occur in the case of the manufacture of "miso" (bean paste) or Japanese soy-sauce, when a small amount of glutamine is produced from L-Glutamic acid. Beside the above, there are very few instance of the action in the actual food frocessing.

Transaminase:

L-glutamic acid + pyruvic acid --- α-ketoglutaric acid + L-alanine

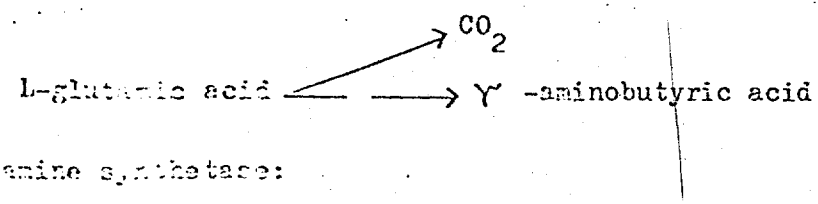
L-glutamic acid + oxalacetic acid --- α-ketoglutaric acid + L-aspartic acid

Dehydrogenase:

L-glutamic acid $\longrightarrow$ $-NH_2$

$\longrightarrow$ α-ketoglutaric acid

Decarboxylase:



Glutamine synthetase:

L-glutamic acid + NH_3 ----- L-glutamine

Glutamate racemase:

L-glutamic acid ----- DL-glutamine

c) Stability in crystalline condition

As mentioned in the paragraph of Physical and Chemical Properties, monosodium glutamate is scarcely hygroscopic, so it will not change both in appearance and quality even if it is kept for a long period in a bottle or a bag of polyethylene film.

d) Stability at the time of heating

The destruction percentage of sodium L-glutamate was tested by heating a solution containing 0.2% monosodium glutamate and 2% sodium chloride at 100°, 107° and 115°C, respectively. The results obtained are shown in Table 6.5.

Table 6.5 Stability of monosodium glutamate at high temperatures[3)]

Heating time \ Temperature	100°C	107°C	115°C
30 min.	0.3%	0.4%	0.7%
1 hr.	0.6	0.9	1.4
2 hr.	1.1	1.9	2.8
3 hr.	2.1	3.6	5.7

c) Stability during the cooking or processing of food

Monosodium glutamate was tested for the stability during the cooking by adding it to small cubes of tuna fish. The results obtained (Table 6.6) showed that the stability was nearly perfect under the conditions.[4)]

The stability of monosodium glutamate during processing of food was tested on the canning process of tuna fish in salt water.

The results obtained (Tables 6.7 and 6.8) indicate that monosodium glutamate was stable not only during the sterilization by heating but also during a long period of storage at a room temperature after the completion of processing.

Similar tests were made also on the canning of hairy crub[6)]. The meat was contained in cans of Type No.2 together with 0.3g per can of monosodium glutamate and sterilized for 80 - 90 min. under pressures of 5.0 - 5.5 lb. In this case also, monosodium glutamate proved to be stable not only during sterilization but also during two years' storage of the cans after the manufacture.

Table 6.6 Change in the amount of monosodium glutamate during cooking

Cooked sample.	(A)	(B)	(C)
Condition of samples	Addition before heating	Addition after heating	No addition
Weight	57.25g	59.40g	58.97g
pH	6.15	6.20	6.15

Monosodium glutamate concentration	0.337%	0.324%	0.157%
Total amount of monosodium glutamate	0.193g	0.192g	0.093g
Residue of monosodium glutamate added	0.100g	0.099g	--

Tuna fish: 6 - 7 pieces of $2cm^3$ in size

Flavoring fluid: Soy-sauce 8cc, sugar 1.0g, water 8.0cc

Heating for cooking: 15 min.

Table 6.7 Change in the amount of sodium L-glutamate by heating for sterilization[5)]

Process	No addition of sodium L-glutamate (control)						Addition of monosodium glutamate (250mg/can)					
	Free glutamic acid content per can (interms of sodium glutamate)				pH of fluid	Water content in solid	Monosodium glutamate content per can				pH of fluid	Water c in soli
	Sample No.	Fluid	Solid meat	Total			Sample No.	Fluid	Solid meat	Total		
				mg/can						mg/can		
Before sterilization	--	3.0 mg	11.6 mg	14.6	6.18	69.8%	--	99 mg	153 mg	252	6.20	69.8%
After sterilization (112.7°C, 70 min.)	A_3	3.4	12.5	15.9	6.15	69.5	A_2	77	178	255	6.22	69.5
	A_{25}	3.4	8.7	12.1		--	A_{24}	80	181	261		70.1

* Analysis under non-sterilized condition immediately before sterilization

** Shows analysis values of test piece 2 days after production of heat steralized cans.

0250

Table 6.8 Stability of monosodium glutamate during the storage of canned tuna in salt water [5]

Days at room temperatures	No addition of sodium L-glutamate (control)							Addition of sodium L-glutamate (250mg/can)					
	Free glutamic acid content per can (in terms of sodium glutamate)					pH of fluid	Water content in solid	Sodium glutamate content per can.					pH of fluid
	Sample No.	Fluid	Solid meat	total	Percentage			Sample No.	Fluid	Solid meat	Total	Percentage	
		mg	mg	mg/can	%		%		mg	mg	mg/can	%	
13	A_5	3.7	15.3	19.0	0.0092	6.12	70.8	A_4	71	187	258	0.124	6.11
	A_{23}	4.1	13.4	17.5	0.0084		70.8	A_{22}	64	190	254	0.122	
26	A_9	4.4	12.5	16.9	0.0081	6.07	69.9	A_8	72	195	267	0.127	6.05
	A_{21}	3.5	12.5	16.0	0.0076		69.9	A_{20}	70	190	260	0.125	
90	A_{11}	4.6	13.3	17.9	0.0086	6.08	70.5	A_{10}	69	188	257	0.123	6.12
	A_{19}	4.6	10.1	14.7	0.0071		68.8	A_{18}	63	196	259	0.124	
383	A_{13}	2.7	12.6	15.3	0.0073	6.15	68.6	A_{12}	69	196	265	0.127	6.09
	A_{17}	1.7	11.0	12.7	0.0061		69.7	A_{16}	61	199	260	0.123	

0250

Table 6.8 Stability of monosodium glutamate during the storage of canned tuna in salt water [5)]

o addition of sodium L-glutamate (control)							Addition of sodium L-glutamate (250mg/can)						
e glutamic acid content per can terms of sodium glutamate)					pH of fluid	Water content in solid	Sodium glutamate content per can.					pH of fluid	Water content in solid
ple o.	Fluid	Solid meat	total	Percentage			Sample No.	Fluid	Solid meat	Total	Percen-tage		
	mg	mg	mg/can	%		%		mg	mg	mg/can	%		%
	3.7	15.3	19.0	0.0092	6.12	70.8	A_4	71	187	258	0.124	6.11	70.1
3	4.1	13.4	17.5	0.0084		70.8	A_{22}	64	190	254	0.122		69.9
	4.4	12.5	16.9	0.0081	6.07	69.9	A_8	72	195	267	0.127	6.05	70.7
1	3.5	12.5	16.0	0.0076		69.9	A_{20}	70	190	260	0.125		68.7
1	4.6	13.3	17.9	0.0086	6.08	70.5	A_{10}	69	188	257	0.123	6.12	68.9
9	4.6	10.1	14.7	0.0071		68.8	A_{18}	63	196	259	0.124		68.7
3	2.7	12.6	15.3	0.0073	6.15	68.6	A_{12}	69	196	265	0.127	6.09	68.9
7	1.7	11.0	12.7	0.0061		69.7	A_{16}	61	199	260	0.123		68.8

Literature

1) Unpublished report of Ajinomoto Co., Inc.

2) "

3) "

4) "

5) Toi, B; Maeda, S.; Fukushima, K.; Eguchi, S. and Matsuda, M.

KANZUMEJIHO 36 (9), 32 (1957)

6) Toi, B.; Maeda, S. and Sasaki, Y.

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Chapter 8. Uses of Flavor Enhancer

8.1 Use of Flavor Enhancers in Home Cooking

8.1.1 General

a) Efficacy and Usage of Flavor Enhancer

i) Single Flavor Enhancers

When used in cooking food, monosodium glutamate not only improves the flavor but also produces various other good effects.

It softens the salty taste and works with it to strengthen the flavor of the food. When added to a sour taste, it mitigates its sharpness to produce a mellow taste. It is generally known that flavor enhancers are particularly effective when used for food cooked using salt as a main seasoning. It also has good effects on a sweet taste to produce a complex and rich flavor if used in very small quantities, and has a special effect of mitigating a bitter taste furthermore.

A single flavor enhancer as "AJI-NO-MOTO" (MSG) is a basic and all-round flavor enhancer which can be used very effectively in preparing food for cooking and for enhancing the taste of the cooked food on the table.

Proper quantity: Monosodium glutamate is most effective when added to the food with the main taste of saltiness. The quantity of monosodium glutamate to be used should be determined in accordance with the amount of salt contained in the particular food.

For example, when it is used in a clear soup flavored lightly with salt, the proper quantity is considered to be about 10 percent

of the salt contained in the soup. This type of Japanese-style soup usually contains about 1.0 percent salt and therefore the proper amount of monosodium glutamate to be added to the soup is 0.1 percent. When it is used in the richly flavored food such as hard-boiled vegetables and food cooked in Chinese style, the proper quantity would be twenty to thirty percent of the amount of salt contained in such food. It is needless to say that flavor of food is a matter of personal taste. Table 8.1, 8.2 and 8.3 give the proper amounts of salt and monosodium glutamate to be used in the most typical dishes of Japanese, Chinese and Western food.

The solubility of monosodium glutamate raises a problem when it is added to vinegar. The solubility of monosodium glutamate in vinegar (pH 2.2) at 20°C, is about 0.8 percent, accordingly any more amount added to the vinegar will only precipitate in the form of glutamic acid, which will have no effect on the taste. Further, the excess monosodium glutamate will neutralize the acetic acid in the vinegar and increase the pH value to spoil the original flavor of the vinegar. Therefore, it is advisable to avoid using more than 1.0 percent of monosodium glutamate to be added to vinegar.

Table 8.1 Standard amounts to be used for Japanese dishes

Name of dish	Monosodium glutamate %	Salt (%)
Clam soup flavored with salt	0.10	1.0
Chawan-mushi (thick custardy soup)	0.12	1.0
Sumashijiru (clear soup, Japanese soup)	0.10 - 0.15	1.0
Soup for tempura	0.50	3.5
Soup for sukiyaki	0.7 - 1.5	7.0
Deep-boiled taro	0.24	1.2
Vegetables boiled hard with soy sauce	0.45	1.8
Fried eggs	0.20	1.0

Table 8.2 Standard amounts to be used for Chinese dishes

Name of dish	Monosodium glutamate %	Salt %
Fork boiled with vinegar	0.45	1.5
Kanitama (crab flesh fried with egg and vegetables)	0.20	1.0
Frizzled boiled rice mixed with pork and vegetables	0.24	0.8
Chow mein (frizzle noodles) mixed with pork and vegetables	0.30	1.0
Soup with balls of pounded shrimps	0.12	1.0
Cold noodles	0.20	1.0
Pork and vegetable boiled in thick soy sauce with sugar	0.30	1.5
Shao-mai	0.20	0.8

Table 8.3 Standard amounts to be used for Western dishes

Name of dish	Monosodium glutamate %	Salt %
Chicken soup	0.12	1.0
Stew	0.30	1.0
Macaroni au gratin	0.30	1.0
Hamburg steak	0.20	1.0
Buttered green peas	0.18	1.2
Spanish rice	0.25	0.8

Time of use: Monosodium glutamate dissolves very easily in water, cold and warm, and is stable even when heated unless a pH value is below 4. Such being the case, the time when the flavor unhancer is used can be determined properly according to the nature and the cooking condition of the food. For example, it is recommendable that the flavor enhancer is added in the early stage of cooking in the case of deep boiling in which the material is soaked deeply with the seasoning soup while it is better to use the flavor enhancer in the final stage of cooking when it is intended to leave the original taste of the food intact or to improve the flavor of the seasoning soy soup. In the case of the deep boiling, it would be advisable to use the flavor enhancer in a quantity slightly larger than usual because the seasoning soup seep and spread all over the material.

ii) Compound Flavor Enhancers

This chapter explains about the use of compound flavor enhancers composed mainly of monosodium glutamate, disodium inosinate etc. These types of flavor enhancers are very popular in Japan, but the translations of this chapter is omitted as there is no direct relations to the questionnaire.

8.1.2 Recommendable ways of using compound and single flavor enhancers in domestic cooking

1. The use of monosodium glutamate in domestic cooking

Monosodium glutamate is used widely together with compound flavor enhancers in home cooking. It can be used effectively for all kinds of food and there is little need of any special considerations in the way of using it.

1) The time of using monosodium glutamate

The temperature for boiling food in domestic cooking would be $100^{\circ}C + 1^{\circ} \sim 2^{\circ}C$ at the most no matter how long it may be used.

Heating under such cooking conditions does not cause any destruction of monosodium glutamate in most cases, and therefore there is virtually no need to worry about the destruction of monosodium gultamate by the action of heat when it is used in preparing ordinary dishes of boiled food.

As to the penetration of monosodium glutamate into the food, the longer it is heated, the deeper it penerates into the food

like any other seasonings. Such being the case, it is advisable that monosodium glutamate is added from the start of cooking when relatively large pieces of food are cooked. On the other hand, in case of soups etc, it would be better to add monosodium glutamate immediately before the cooking is finished.

2) The amounts to be used

The proper amount of monosodium glutamate to be used in ordinary cooking is determined by the amount of salt contained in the food. When it is used in lightly seasoned soups, the minimum amount to be used is about 10 percent of the amount of salt content (usually about 1.0 percent). In the case of highly seasoned food such as hard-boiled vegetables and Chinese dishes, it would be necessary to use the flavor enhancer in a quantity equivalent to 20 to 30 percent of the salt content. However, it is quite natural that the "proper amount" of the flavor enhancer to be used in cooking varied considerably according to personal preference and the particular type of food.

The standard amounts of monosodium glutamate to be used in some typical dishes are given in Table 8.1, 8.2 and 8.3 mentioned before.

3) The relationship between monosodium glutamate and acid content in food

When monosodium glutamate is added to a food with a high degree of acidity and heated, a small portion of monosodium glutamate is dehydrated, converted into pyrolidon carboxylic acid and becomes tasteless. Actually, however, there are not so many kinds of dishes

that are extremely acidic and moreover such dishes are rarely cooked with heat, and no special consideration is required in the use of monosodium glutamate for vinegared food from practical standpoint. In the case of a Chinese dish of vinegared pork, it is cooked by using heat but its pH value is increased so much by the action of other materials of cooking and other seasonings that it cannot be placed under the category of vinegared food. When no heat is applied, it seldom happens that monosodium glutamate is destroyed by acid and becomes tasteless.

When using monosodium glutamate for vinegared food, it is advisable to use the standard amounts as explained previously. For example, in the case of vinegared dishes such as those which are seasoned with vinegar, sugar and soy sauce, the proper amount of monosodium glutamate would be up to 0.45% of the salt content and 0.50% of the sweetened vinegar.

4) Tempura and monosodium glutamate

From our experiments, it is presumable that about 10 percent of monosodium glutamate added to the coating of tempura (fish, vegetable and so forth fried in Japanese style) is destroyed under the heating conditions (usually about 180°C, 4 minutes) and the rate of destruction is related to the amount of water contained in the food to be fired. The more the water content in the fried food, the less the destruction of monosodium glutamate used in the coating. So, when monosodium glutamate is used in the tempura coating, it is advisable to sprinkle it directly on the food so that it may be kept out of immediate contact

with the very hot oil.

5) Monosodium glutamate enhances the flavor of cooked food

Westerners consider monosodium glutamate as an enhancer of flavor rather than taste, that is, a flavor enhancer intended not simply to strengthen the taste of food but to fully develop the original flavor of the material of cooking and enhance the flavor of the cooked food as a whole. It has been actually shown that the added monosodium glutamate to a cooked food that has cooked down to lose its taste, restores the taste of the food of freshly cooked state.

6) Degeneration during storage

Monosodium glutamate is less hygroscopic than sodium chloride that there is absolutely no need to worry about its degeenration by moisture absorption unless it is kept near highly moisture-absorbing substances such as salt and spices.

ii) The use of compound flavor enhancers : omitted

Effects of food additives remain a topic of discussion among some scientists

A scientific controversy that most people thought had been settled is still sputtering. It concerns monosodium glutamate (MSG), an additive that enhances the flavor of foods.

Some members of the scientific community contend that MSG causes brain damage in infant mice, and that there is no reason to suppose it is any safer for human infants. When this view was first expressed in 1969, spokesmen for the manufacturers of MSG—and of baby foods using the compound—countered by questioning the validity of the research. First, they said, the brain-damaged mice received massive overdoses of MSG; the substance had been injected subcutaneously, not given by mouth; and further, there had been a lack of studies where control animals were treated with the salts of other amino acids to see if similar results occurred. Others contend that there are far more important problems in toxicology that should be settled first.

The matter was referred to the Food and Drug Administration, which eventually decided that MSG apparently causes damage to the brains of infant mice, at certain rather high doses, but that the same damage probably does not occur in infant primates, including man. Baby food manufacturers, sensitive to the adverse publicity in the lay press, stopped using the substance in their products although the FDA left MSG on the list of products "generally recognized as safe."

One of the researchers whose work criticized the use of MSG was not satisfied with the results of the FDA study. He has continued to publish information that he believes implies a degree of risk to infant health disregarded in the FDA study. Concerning the failure of FDA to take regulatory action, John W. Olney, MD, assistant professor of psychiatry at Washington University School of Medicine, St. Louis, points out that although the manufacturers no longer include MSG in baby foods, human infants still get the controversial compound.

"MSG is included in a wide variety of adult commercial foods," he said in an interview. "Mothers very commonly hash these foods up—often with canned soup, which also contains MSG—and feed it to their infants."

Dr. Olney's opponents respond that his objections are based on study of the mouse—an animal

continued on next page

A section through the hypothalamus of an untreated 10-day-old mouse (top), showing normal appearance of the arcuate nucleus (A), the ventromedial nucleus (V), the median eminence (ME) and the third ventricle (III). The other microphotograph is of the hypothalamus of a 10-day-old mouse 6 hours after subcutaneous injection of MSG, showing swollen cyptoplasm and pyknotic nuclei of the neurons in the arcuate area, sharp demarcation of the lesion at the boundary between the arcuate and ventromedial nuclei, and alterations of the contour of the wall and floor of the third ventricle.
Photos courtesy of the *Journal of Neuropathology and Experimental Neurology*

continued from previous page
they regard as an inappropriate model. In fact, one group claims that MSG does not even damage mice, much less human babies.

In a recent issue of *Nature*, B. L. Oser, PhD, and his colleagues described feeding MSG to infant rodents and dogs in an attempt to reproduce the brain lesion previously demonstrated by Dr. Olney. Using both oral and subcutaneous routes of administration, they were unable to find any brain damage. When asked his reaction to this article, Dr. Olney replied that Dr. Oser's study avoided those parts of the original protocol most likely to show positive results.

A summary of Dr. Olney's reported work may help to make the argument more intelligible. First, he has attempted to answer the original questions by using various doses of MSG, by administering it both orally and subcutaneously, and by testing other compounds as well.

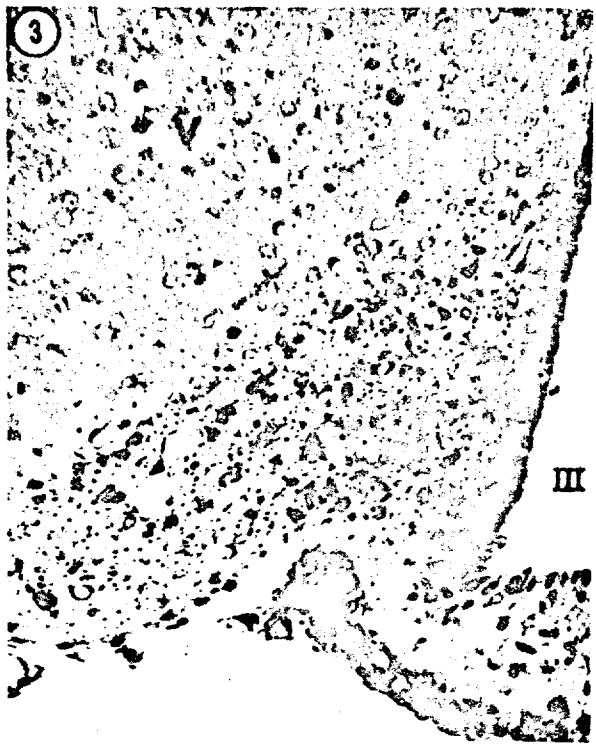
According to Dr. Olney, the brain damage caused by monosodium glutamate is a highly specific lesion in the arcuate nucleus of the hypothalamus. The route of administration appears to make no difference; oral doses of MSG lead to the same type of damage as subcutaneous injections.

Very small doses—0.25 mg per gram of body weight—produced no lesions, but the next increment—0.5 mg/g—caused damage in 12 of 23 animals tested (52%). At 1.0 mg/g, all 19 test animals were affected. The 0.5 mg/g dose, Dr. Olney maintains, is about five times the amount a baby received from a jar of baby food before the manufacturers discontinued its use. He considers this an insufficient safety factor.

Dr. Olney's experiments involved feeding (or injecting) various doses of MSG or several other compounds, then killing the animals at various intervals to check the progress of the developing lesion. He found that five or six hours after administration, the neurons of the arcuate nucleus showed extreme swelling in those animals that had received MSG or closely related compounds. The adjacent neurons in the ventromedial nucleus were not affected. The intracellular edema was so pronounced that the arcuate nucleus was expanded into the third ventricle, nearly filling it in some cases.

After 24 hours, the arcuate nucleus was no longer swollen, but many neurons were destroyed. Phagocytes had appeared and were consuming the damaged neurons. After four days, the cellular debris had been cleared away, but very few neurons were left in the area.

The damage may be long lasting. Earlier experiments (some of which led Dr. Olney to his present inquiry) showed that infant mice dosed with MSG developed into obese adults with stunted skeletons.

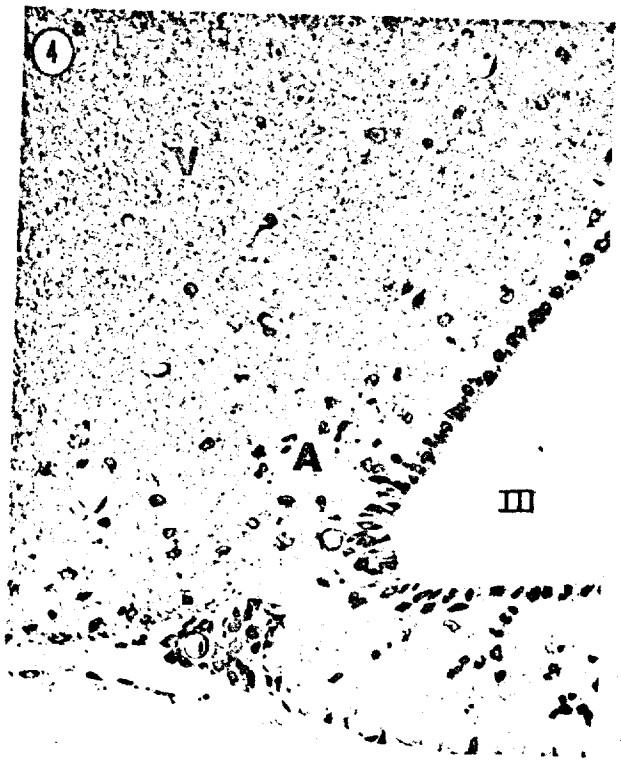


Hypothalamus of 11-day-old mouse 24 hours after subcutaneous injection of MSG. The arcuate region is no longer edematous, but is studded with dark staining bodies recognizable under electron microscopy as degenerate cells undergoing phagocytosis. Photo courtesy of the *Journal of Neuropathology and Experimental Neurology.*

The females were often sterile, and treated animals routinely suffered retinal damage. Other endocrine system disorders occurred in some animals.

Effects of this type seem to be associated with only one group of amino acids, described as "neuroexcitatory." Glutamate, aspartate, and derivatives of cysteine belong to this group. Certain compounds in this group are more powerful than glutamate—with much lower doses producing equivalent damage. Much of Dr. Olney's current research is aimed at discovering what common properties of these compounds could account for both their neuroexcitatory and neurotoxic effects.

The molecular specificity of this type of neuropathology is such, Olney says, that relatively simple manipulations of the glutamate molecule lead to changes in the effects. Lengthening the carbon chain of the molecule to six carbon atoms, changing the CO_2H terminal group to PO_3H_2, or methylating the alpha carbon atom cause the cytotoxicity to "shift" from the neurons to adjacent non-neuronal compartments. Glial or ependymal cells, on which MSG seems to have little effect, may be destroyed instead of the neurons. Dr. Olney



Hypothalamus of 14-day-old mouse 4 days after subcutaneous injection of MSG. The degeneration products have disappeared, leaving the arcuate region depleted of cells. Most of the remaining cells appear to be non-neuronal. The loss of nerve cell mass is accompanied by widening of the third ventricle. Photo courtesy of the *Journal of Neuropathology and Experimental Neurology*

hopes that such manipulations will lead to more information on how the lesion is produced.

In spite of Dr. Olney's assertions, other investigators are satisfied that the compound is safe as a food additive. In Dr. Oser's work, cited earlier, no damage was found. Another investigator, Lloyd J. Filer, MD, of the University of Iowa, Iowa City, said that "There is no question that Dr. Olney's work is valid—in mice." The more mature brains of newborn primates, he insists, are not susceptible to the lesions.

Dr. Filer worked with a series of 21 infant monkeys which had been fed 1 to 4 mg/g of MSG by a nasogastric tube. No brain damage resulted, except in a few specimens whose brains were injured by the perfusion technique used to fix the tissues.

Dr. Olney has worked with only three infant monkeys, one treated subcutaneously and two orally (he found lesions in the expected locations in all three). He agrees that a much larger sample, including appropriate control subjects, is needed in order to definitely clarify the toxic potential of MSG in infant primates. However, since he claims to have produced brain damage in every species he has treated with MSG (mice, rats, rabbits, chicks, monkeys), he questions whether differences between species are as important as others assume.

Concerning the food safety issue, Dr. Olney points out that no problem would ever have arisen if MSG had undergone appropriate safety evaluation before it was used in foods available to human infants. At present, while a safety evaluation is being conducted (which could stretch out for years), Dr. Olney maintains it would be entirely reasonable to require food companies to either desist from using MSG in foods available to human infants or to label such foods "potentially unsafe for infant consumption".

Tests—and testy comments—continue. Dr. Oser's group has begun working with monkeys, and hopes to publish its results soon. Anne Reynolds, PhD, of the Department of Anatomy at the University of Illinois, Chicago, is continuing work on both mice and monkeys which she began with Dr. Filer.

Dr. Reynolds shares the skepticism of some other investigators about the mouse as a model animal for MSG studies, but calls the problem "a dilemma" because the mouse is such a convenient, inexpensive and standardized laboratory animal. Monkeys, of course, are hard to get—and extremely expensive.

Experts discuss problems of teaching about VD

Education about venereal disease must come early to be effective; a Washington University (St. Louis) School of Medicine professor of sociology in psychiatry told a recent international symposium on venereal diseases.

Studies indicate "boys with the highest risk (of venereal disease) are not likely to be present for instruction late in the high school years," Lee N. Robins, PhD, said at the meeting in St. Louis.

Summarizing a symposium workshop, Warren A. Ketterer, MD, said the discussants thought that education about venereal disease might be offered "perhaps even down to the fifth or sixth grade." Dr. Ketterer, venereal disease chief for California's public health department, said it was generally agreed venereal disease should be taught as part of a course about communicable diseases.

James Elias, PhD, Adolph Foundation, Burbank, Calif, noted that another workshop raised the question whether authority figures or peer groups are more effective in education about VD.

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Monosodium Glutamate-Induced Brain Lesions in Mice. E. A. Arees*, and J. Mayer*, *Boston, Mass.*

Both infant and adult albino mice were injected either subcutaneously or intraperitoneally with single doses of monosodium glutamate in concentrations ranging between 2–10 mg/g body weight. Animals were killed at times varying between 30 minutes and 2 weeks and studied both with light and electron microsopy (EM). In the arcuate nucleus of hypothalamus, EM study has shown the start of degeneration in microglial cells as early as 3 hrs after treatment with necrosis being most pronounced at 24 hrs. Also at 24 hours after treatment, degeneration was observed in distal portions of axons coupled with an increase in glycogen in the processes of astrocytes.

In mice injected at 7 days of age and killed 24 hrs after treatment, light microscopy study with the Fink-Heimer stain has shown degeneration in the optic pathway with terminal degeneration being seen in the dorsal and ventral nuclei of lateral geniculate body. Also fiber and terminal degeneration was seen in the region of the subiculum. It appears that following mono-sodium glutamate injection, brain lesions are more extensive than at first suspected.

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GLUTAMIC ACID AND HUMAN INTELLIGENCE[1]

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Glutamic acid administered in supranormal quantities has been reported to enhance the intellectual functioning of mentally defective patients. Interest in this relationship was stimulated in part by the work of Weil-Malherbe (1936), who reported that l(+) glutamic acid was the only one of 12 amino acids studied which was capable of maintaining oxygen uptake in sliced brain tissue. Since that time a major literature has accumulated relating glutamic acid to human intelligence, brain function, epilepsy, audiogenic seizures, and performance of rodents in mazes. In this paper we will review the literature which evaluates glutamic acid therapy as a means of improving intellectual functioning in mental defectives.

Studies of this problem ordinarily employ institutionalized mental defectives who are placed on a high diet of glutamic acid for a specified period of time (usually two to six months). Psychological tests and/or clinical observations of intellectual functioning are made before and after treatment.

Inspection of this literature reveals a set of highly conflicting findings. The picture is somewhat clarified, however, when the studies are classified in terms of (*a*) whether or not positive results were reported, and (*b*) whether or not a control group was used. Table 1 summarizes 33 studies with mentally deficient *S*s in terms of these two variables. A study was classified as positive if significant gains in IQ scores or improvements in "intellectual functioning" were attributed to treatment with glutamic acid. Studies employing "controls" were liberally designated as those in which a similarly diagnosed group was studied in the absence of glutamic acid medication. A chi square with correction for continuity is significant at the .001 level ($\chi^2 = 12.99$), indicating that positive results tend to be related to a lack of controls. Obviously, the crucial studies occur in the Control-Positive cell of Table 1. An attempt will be made here to evaluate these studies according to the following additional methodological considerations: (*a*) adequacy of control group; (*b*) use of placebo; (*c*) control of "environmental stimulation," such that all *S*s are treated similarly except for drug administration; (*d*) ignorance of *S*s and *E*s of the medications or placebos; (*e*) statistical treatment of data; and (*f*) control of taste differences between drug and placebo (some forms of glutamic acid have a markedly bitter taste).

Albert, Hoch, and Waelsch (1946) reported positive clinical results with eight mental defectives serving as their own controls. Intelligence test performance in general seemed to improve during glutamic acid medication and drop during placebo administration. However, some tests showed both gains and losses during acid and placebo periods, and no statistical analysis was attempted.

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TABLE 1

33 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 & Gelash (1953) Zimmerman, Burgmeister, & Putnam (1948) $f=6$	Berguis (1954) Ellson, Fuller, & Urmston (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 (1950a) Zabrenko & Chambers (1952) Zublin & Lutz (1953) $f=13$
No Control	de la Fuente Muniz, Zuniga, & Yanowsky (1950) Delay, Pichot, Puech, & Perse (1951) Harney (1950) Hoven (1951) Kane (1953) Levine (1949) Müller (1953, 1954) Quinn & Durling (1950b) Schwobel (1950, 1952) Zimmerman & Burgmeister (1950) Zimmerman, Burgmeister, & Putnam (1949a, 1949b) $f=14$	$f=0$

Moreover, it was not reported if the testors had knowledge of the periods of medication, and there was no control of taste differences between acid and placebo.

The same investigators (1951) reported a more adequately designed follow-up of their first report. Controls were matched on MA, IQ, age, and sex. Significant gains in IQ scores obtained during glutamic acid medication dropped significantly during placebo periods. Testors were not informed of the medication schedules, but " . . . testing of the patients was more difficult during glutamic acid periods as later judged by the number of times it was recorded that a patient had been negativistic, distractible, etc." (p. 487). Obviously the testors possessed information which may have enabled them to differentiate the control and experimental *S*s. With regard to the taste variable, " . . . rare questions regarding differences in taste were explained as due to differences in the strength of the compound administered" (p. 475). The potential effects of such information upon the parents (who administered the medication) and the children provides an unknown, hence uninterpretable, source of variance.

Zimmerman, Burgmeister, and Putnam (1948), using 69 mentally defective children, reported significant gains in IQ scores during glutamic acid medication. Thirty-seven of these *S*s served as "controls" in the

sense that they had been given an intelligence test prior to the pretest for the study. The interval between these two testings, however, ranged from six months to eight *years*. Recognizing the potential inadequacy of such controls, the authors wrote, " . . . abrupt environmental changes might possibly influence the intelligence test score. This was not an important factor . . . since . . . the daily pattern of their lives was not appreciably changed" (p. 597). In another paragraph they stated, "Overdoses may produce distractibility . . . insomnia . . . [and] occasional gastric distress" (p. 594). Describing dosage procedures in a preliminary report (1947), "Glutamic acid was administered . . . to the point where increased motor activity was present, or where parents complained about the distractibility and noncooperativeness of the child" (p. 175). It certainly appears as though "abrupt environmental changes" *did* occur at least for some *S*s, although no method for evaluating their specific contribution in this situation was provided.

A study by Foale (1952) employed two groups of 15 mentally defective boys equated on age, IQ, and length of institutionalization. Gains in IQ scores among the treated *S*s were attributed to glutamic acid medication, although no placebos were employed for control *S*s, no control on testor knowledge was reported, and no statistical tests were attempted.

Kurland and Gelash (1953) matched 13 adult male mental defectives with 13 controls on age and IQ scores. Significantly greater gains in IQ scores were reported for the experimental *S*s. No attempts to control taste differences were made and it was not revealed if the testors knew the medication schedules.

The final and perhaps most carefully designed Control-Positive study was carried out by Head (1955). Three groups of 30 children each (schizophrenics, mental defectives, and normals) were employed in a cross-over procedure which lasted for three, one-month periods. Subgroups of 10 *S*s received different combinations and orders of medication and placebo. The author's conclusion of a significantly beneficial effect attributable to glutamic acid is based primarily on a comparison of two subgroups of mental defectives before and after their initial month in the study. Mean changes in IQ scores of 6.0 and 3.0 were reported for the experimental and control groups, respectively, during this interval. However, in computing the standard error of this mean difference (p. 120), Head incorrectly employed the standard errors of the two pre- and posttest mean differences, instead of the standard deviations of the two distributions of difference scores. When appropriate substitutions are made, the t ratio ($df = 18$) drops from 3.52 ($p < .01$) to 1.12 ($p > .05$). Head controlled taste differences, but no control over testor knowledge was reported.

The methodological criticisms of these studies do not, of course, render the authors' interpretations invalid; but the amount of contradictory experimental evidence (Control-Negative cell of Table 1) raises considerable doubt. Seven studies (Ellson, Fuller, & Urmston, 1950; Kantor & Boyes, 1951; Kerr & Szurek, 1950; Loeb & Tuddenham, 1950; Lombard, Gilbert, & Donofrio, 1955; Milliken & Standen, 1951; Zabrenko & Chambers, 1952) controlled both testor knowledge and taste differences between placebo and glutamic acid. Six studies (Ellson, Fuller, & Urmston, 1950; Loeb & Tuddenham, 1950; Lombard, et al., 1955; Milliken

& Standen, 1951; Oldfelt, 1952; Quinn & Durling, 1950a) employed matching procedures in the selection of *S*s.

Some specific points of comparison can be made between certain Control-Positive and Control-Negative studies. The negative study by Kantor and Boyes (1951) was intended as replication of the earlier Albert-Hoch-Waelsch study (1946). Improvements in the design included larger *N*s, more homogeneous and more reliable diagnoses, control of testor knowledge, and control of taste differences. Two negative studies (Ellson, et al., 1950; Loeb & Tuddenham, 1950) attempted to replicate the positive study by Zimmerman, Burgmeister, and Putnam (1947, 1948). The main improvement in the design of these replications was the use of a control group which was studied concurrently with the experimental group. Ellson, Fuller, and Urmston reported gains in IQ scores for *all Ss* which were comparable to those reported by Zimmerman, Burgmeister, and Putnam during the medication period. Loeb and Tuddenham, in attempting to explain the lack of change in IQ scores found during the six-month to eight-year control period of the Zimmerman-Burgmeister-Putnam study, point out that facilitative practice effects on IQ tests are less likely to appear over such relatively long periods of time.

Three other Control-Negative studies (Ernsting, 1949; Quinn & Durling, 1950a; Zabrenko & Chambers, 1952) also reported significant gains in IQ scores for experimental and control groups. The Zabrenko-Chambers paper studied the "environmental stimulation" variable directly. Transferring mental defectives to a setting characterized by "increased attention and emotional support" was followed by significant increases in IQ scores.

Himwich (1954) has discussed one aspect of this research which has been given little attention, that is, the form in which glutamic acid is usually administered. She points out that some studies have employed the unneutralized acid, while others have used the hydrochloride or salt (usually sodium glutamate). Presenting data from one *S* to demonstrate that the unneutralized acid does not enter the bloodstream as efficiently as the hydrochloride or salt, Himwich concludes, "It is interesting that the majority of favorable reports . . . have been obtained with sodium glutamate or the hydrochloride" (p. 352). Even though some of the papers reviewed are rather obscure on this matter, a check on those listed in Table 1 does not support Himwich's observation. As far as could be determined, all of the studies in the Control-Positive cell of Table 1 used the unneutralized acid. On the other hand, at least three of the Control-Negative papers (Ellson, et al., 1950; Loeb & Tuddenham, 1950; Milliken & Standen, 1951) used sodium glutamate.

Rogers and Pelton (1957) recently reported positive effects on IQ scores using a closely related compound, glutamine. The authors used small doses for a relatively short time (six weeks), and concluded that "some types of mental deficiency are associated with an impaired ability to synthesize glutamine from glutamic acid" (p. 88). Although *S*s were carefully matched and testor knowledge was controlled, the results were equivocal ($p < .10 > .05$) and no control of taste differences was attempted. These workers plan to publish studies with larger *N*s in the near future.

Another aspect which has received no systematic study is that of side effects. Very large doses of glutamic

acid can result in flushing (Fincle & Reyna, 1958), nausea and vomiting (Himwich, Wolff, Hunsuker, & Himwich, 1955), in addition to the distractibility, hyperactivity, etc., mentioned earlier. These effects appear to be temporary, and disappear with the cessation or reduction of medication. The importance of such effects for the present review is that their appearance enables observers to differentiate between control and experimental *S*s. Moreover, the fact that experimental *S*s who develop such side reactions will require additional care and attention introduces another "environmental stimulation" which may contaminate the findings.

A final point is that of diet. It is likely that the diets of chronic patients in certain institutions are in many respects deficient. The complexity of amino acid metabolism and the difficulty in defining which amino acids are essential for adequate nutrition are well known (Woods, 1950). The effects of glutamic acid medication may depend to a large extent upon the recent dietary history of the population under study.

In conclusion, it appears that a specific effect of supranormal amounts of glutamic acid upon human intelligence has yet to be convincingly demonstrated. The more carefully designed studies tend to be negative almost without exception. Needless to say, the classification of studies in Table 1 provides testimony in support of the demand for controlled experimentation.

Summary

A review of the literature relating glutamic acid medication to the intellectual functioning of mental defectives indicates that positive effects tend to be reported in studies not employing a control group. The few positive studies employing controls contain methodological flaws, rendering their conclusions difficult to accept. The tendency for negative findings to occur in the more adequately designed experiments sheds doubt on the hypothesis that glutamic acid medication has a specifically beneficial effect on intellectual functioning.

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FLAVORS AND NONALCOHOLIC BEVERAGES

Note on a Rapid Paper Chromatographic Method for the Estimation of Added Monosodium Glutamate in Food

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Ascending paper chromatography is proposed as a method for the estimation of monosodium glutamate. Free glutamate was determined in a variety of Chinese foods. Glutamate concentrations of 0.4 to 1.5% by weight as the monosodium salt are reported. The use of common inexpensive equipment combined with ease of manipulation makes the method ideally suited to routine laboratory operation.

The post-sino-cibal syndrome or Chinese restaurant syndrome was described in 1968 by Schaumburg and Byck (1). The symptoms include a burning sensation, facial pressure, chest pain, and headache. They appear in susceptible individuals following ingestion on an empty stomach of such Chinese specialties as Wonton soup. Monosodium-L-glutamate has been implicated as the compound responsible for the reaction (1, 2).

The receipt at this laboratory of several samples of Chinese food to be analyzed for monosodium glutamate (MSG) prompted the search for a rapid test. Bernt and Bergmeyer (3) described an enzymatically catalyzed procedure dependent on the production of DPNH. The ion exchange method of Stein and Moore (4) also affords a good means of analyzing for glutamate. Fernandez-Flores *et al.* (5) have proposed the separation of MSG by column chromatography, followed by potentiometric titration. Paper chromatography has been proposed as a method for the separation of amino acids (6), and the feasibility of its use on a semiquantitative basis was demonstrated (7–10). This latter method is both fast and inexpensive. The equipment used can be found in most local public health laboratories. For these reasons we adapted this technique for use in our laboratory.

METHOD

Apparatus and Reagents

(a) *Filter paper.*—Whatman No. 40, or equivalent.

(b) *Chromatography sheets.*—Whatman 3MM cut to 45 cm in cross-machine direction × 40 cm in machine direction.

(c) *Glass jar.*—18" high × 12" in diameter, with glass plate for cover.

(d) *Pipets.*—10 µl capacity (Fisher No. 21-157, or equivalent).

(e) *Chromatographic sprayer.*—250 ml capacity (Fisher No. 5-719-5, or equivalent).

(f) *Solvent.*—*n*-Butanol-glacial acetic acid-distilled water (12 + 3 + 5).

(g) *Stain.*—Ninhydrin, 0.25% in acetone containing 1% pyridine.

(h) *Monosodium glutamate monohydrate.*—Prepare standard solution containing 0.4–1 g MSG monohydrate (Matheson Coleman & Bell No. GX-145, research grade)/L distilled water.

Sample Preparation

The samples tested were Chinese egg roll, chow mein, fried rice, and egg drop soup. Five to 10 g of each solid food was placed into a 400 ml beaker with 250 ml distilled water at room temperature and a Teflon-coated magnetic bar was added. The beaker was covered with a watchglass and placed on a magnetic stirring motor. The mixture was stirred 2 h at a moderate speed, strained through cheesecloth and filtered through Whatman No. 40 filter paper. The soup was filtered through Whatman No. 40 filter paper and 10–50% dilutions were made of the filtrates.

Determination

Ten µl of each solution (standard, filtrate, or diluted filtrate) was spotted at the origins of a paper chromatogram. A stream of warm air from a portable hair dryer directed at the origins during spotting facilitated the deposition of compact spots. The sheet was then developed overnight by ascending chromatography in *n*-butanol-acetic acid-water solvent. The following morning the sheet was removed from the chromatographic jar and allowed to air-dry. The chromatogram was then sprayed with ninhydrin stain, permitted to dry at room temperature, and, when completely dry, placed in a 75° oven for 15 min.

Results and Conclusions

The purple spots due to glutamate are identified by comparing their distance traveled to that of the standards. The $R_f \times 100$ for glutamate from monosodium glutamate standards was found to be 28. When viewed by transmitted light, the size and

color intensity of sample spots may be compared to size and intensity of the standard spots and an estimation made of monosodium glutamate concentration.

Threonine, by this system, has an R_f value close to that of glutamic acid; however, this substance will interfere only if it is present as the free amino acid. Chinese food investigated by this laboratory contained 0.4–1.5% MSG. Table 1 gives the results of single determinations. We estimated that the glutamate concentration in the material tested was 10–20 times greater than that of the other free amino acids present. Possible interference by free threonine would, therefore, be insignificant.

The sensitivity of the method is more than adequate. One μg MSG from standard solutions can easily be detected.

The efficiency of the aqueous extraction of MSG from the foodstuffs was determined in the following manner. A homogenate of egg roll was prepared, using a Waring Blendor. The homogenate was rendered glutamate-free by repeatedly suspending in distilled water, centrifuging, and decanting the supernatant liquor. MSG (100 mg) was added to a portion of the washed homogenate. No MSG was detected in the "glutamate-free" samples and 100 mg MSG was detected in the fortified samples. For the 4 samples tested, the recovery was 100%, with a standard deviation of 4%.

Twelve samples can be analyzed in approximately 1 hr of total operator time. The method described represents a rapid, inexpensive technique which, we feel, should be considered for use as the method of choice where lack of instrumentation precludes the use of a more sophisticated procedure.

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Table 1. Chinese food examined for added monosodium glutamate

Sample	% Glutamate as Monosodium Salt
Fried rice	0.4 by wt
Fried rice	0.9 by wt
Egg roll	1.5 by wt
Chow mein	1.2 by wt
Egg drop soup	1.0 wt/vol.

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674. *Amino-acids and Peptides. Part V.* Determination of* L-*Glutamic Acid by the Isotope Dilution Method.*

50055

By C. C. Barker, I. W. Hughes, and G. T. Young.

The accuracy of the isotope dilution method for the determination of L-glutamic acid has been examined by using synthetic mixtures of amino-acids. Individual results are within 0·7% of the theoretical, whilst the mean is 99·7% of the theoretical value. The procedure has been used to determine the L-glutamic acid formed on hydrolysis of a sample of gliadin.

Of the general methods available for the analysis of amino-acid mixtures, the "isotope dilution" procedure offers important advantages. Developed by Schoenheimer, Ratner, and Rittenberg (*J. Biol. Chem.*, 1939, **130**, 703) and by Ussing (*Nature*, 1939, **144**, 977), this method involves the addition of a known weight of isotopic amino-acid to the mixture being analysed; a pure sample of this amino-acid is then recovered by any suitable means and the change in isotope concentration is determined, from which the proportion of the amino-acid in the original mixture can readily be calculated. Normally, the isotopic amino-acid contains ^{15}N in the amino-group, in which case the isotopic concentrations are assayed in the mass spectrometer. The difficulties of quantitative isolation are avoided by this method, which should be capable of high accuracy.

Many such determinations of amino-acids in protein hydrolysates have been reported (for reviews see, *e.g.*, Shemin and Foster, *Ann. N.Y. Acad. Sci.*, 1946, **47**, 119; Tristram, *Adv. Protein Chem.*, 1949, **5**, 83). The main object of the work presented here was to examine the accuracy of this method for the determination of L-glutamic acid, by the analysis of known synthetic mixtures.

The accuracy of the isotope dilution method depends primarily on the purity of the added and the isolated samples and on the accuracy of the mass-spectrometric assays. The establishment of the purity of an amino-acid is not easy by conventional means, since a contaminant is likely to be of similar composition or even isomeric. In the past, crystallisation has been repeated until the isotopic concentration is unchanged, and such constants as nitrogen content and specific rotation have been used for confirmation. The difficulties involved are illustrated by the fact that a sample of L-glutamic acid hydrochloride, isolated from the hydrolysate of human serum albumin and recrystallised until the $^{15}N : {}^{14}N$ ratio became constant, was found still to be impure by optical-rotation measurements, containing an appreciable amount of L-cystine dihydrochloride (Shemin, *J. Biol. Chem.*, 1945, **159**, 439). The danger of co-precipit-

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ation of nearly related amino-acids has been emphasised by Keston, Udenfriend, and Cannan (*J. Amer. Chem. Soc.*, 1949, **71**, 249), and there are cases where specific rotations do not differ sufficiently to enable small amounts of impurities to be detected.

We therefore developed the vapour-pressure method for the differential-solubility test of chemical purity (Hughes and Young, *Nature*, 1950, **164**, 503; Hughes, Williams, and Young, *J.*, 1951, 1279), by which contamination of one amino-acid by another, or by its enantiomorph, may be detected in amounts of the order of 0·2%. Experiments with synthetic mixtures enable a limit to be placed on the amount of amino-acid impurity which can be present in a given sample, and errors from this source may be evaluated. Application of the method to the sample of L-[^{15}N]glutamic acid hydrochloride used in this work showed that the sample could not contain more than 0·2% of L-aspartic acid hydrochloride. Although the results of the test are conveniently expressed in this manner, it is likely that part at least of the vapour-pressure difference is due to occluded hydrogen chloride, to which the test will be highly sensitive.

Three synthetic mixtures were prepared and analysed: (*A*) L-glutamic acid hydrochloride, DL-glutamic acid hydrochloride, and L-aspartic acid hydrochloride; (*B*) as above, with the addition of DL-alanine and L-lysine hydrochloride; (*C*) as in (*B*), with the further addition of glycine and L-tyrosine. The relative amounts approximated to those found in insulin. After addition of isotopic L-glutamic acid hydrochloride, basic amino-acids (if present) were precipitated with phosphotungstic acid, and the acidic amino-acids were removed on "Amberlite" IR4—B" anion-exchange resin. L-Glutamic acid was finally isolated as the hydrochloride. Recrystallisation was continued until the differential solubility test gave a difference in pressure corresponding to the presence of less than 0·2% of L-aspartic acid hydrochloride. A portion of the material was then converted into ammonium chloride by the Kjeldahl method and thence into nitrogen by the action of sodium hypobromite. The recrystallisation of the L-glutamic acid hydrochloride was then continued, and samples were converted into nitrogen at each stage.

The mass-spectrometric determinations on the nitrogen samples were carried out at the Atomic Energy Research Establishment, Harwell. The ^{15}N concentrations were calculated from the relative intensities of the peaks at mass numbers 28 and 29 ($^{15}N^{14}N^+$); this calculation assumes equilibrium to be established in the reaction $^{14}N^{14}N + {}^{15}N^{15}N = 2{}^{14}N^{15}N$, and the equilibrium constant $\frac{[^{14}N^{15}N]^2}{[^{14}N^{14}N][^{15}N^{15}N]}$ to be 4·00. The relative intensities at mass numbers 32 (O_2^+) and 40 (A^+) were also recorded, in order to determine contamination by air; appropriate corrections were then applied to the peaks at masses 28 and 29. The relative intensity at mass 44 was used to determine carbon dioxide, and a correction (for CO^+) was similarly

TABLE I.

Synthetic mixture	L-Glutamic acid hydrochloride present, g.	L-[^{15}N]Glutamic acid hydrochloride added, g.	No. of crystns. of isolated sample	L-Glutamic acid hydrochloride found, g.	%	
A	1·1294	0·1208	5	1·1305	100·10	Mean 100·1
			6	1·1311	100·14	
			7	1·1291	99·97	
B	0·4012	0·0488	5	0·3985	99·33	Mean 99·3
			6	0·3977	99·13	
			7	0·3990	99·45	
C	0·9064	0·0923	5	0·9036	99·69	Mean 99·7
			6	0·9022	99·54	
			7	0·9057	99·92	

applied to the peaks at masses 28 and 29. The relative intensity of the peaks at masses 28 and 32 for a sample of air was 5·4 : 1. The relative intensity of the peaks at masses 44 and 28 for a sample of carbon dioxide was 10 : 1.

The results are summarized in Table I. It will be seen that individual assays are within 0·7% of the theoretical, whilst the mean of the three (99·7%) is considerably closer.

Professor A. C. Chibnall kindly made available a sample of gliadin from a batch in which the L-glutamic acid had previously been determined both by the gravimetric method and by the specific decarboxylase procedure (Chibnall, *J. Int. Soc. Leather Trades' Chem.*, 1946, **30**, 11; Gale, *Biochem. J.*, 1945, **39**, 46). We hydrolysed a portion in the standard manner and determined the L-glutamic acid in the hydrolysate by the isotope dilution method. The results are shown in Table II: our mean value (23·7) for the L-glutamic-acid-nitrogen, expressed as a percentage of the total-protein-nitrogen, agrees remarkably closely with the values given by

Chibnall (23·7) and Gale (23·4). These figures do not of course take into account the possibility of destruction or racemisation of the L-glutamic acid during hydrolysis, and attempts are now being made to investigate this aspect of the problem.

TABLE II.

Experiment	Gliadin, g.	L-[^{15}N]Glutamic acid hydrochloride added, g.	No. of crystns. of isolated sample	Atoms-% excess ^{15}N	L-Glutamic acid found, g. per 100 g. of gliadin *	L-Glutamic acid found, N, % of protein-N
i	1·3030	0·0710	4 (*a*)	1·503	44·04	23·70
			(*b*)	1·490		
			5 (*a*)	1·490	44·23	23·81
			(*b*)	1·490		
			6	1·498	43·99	23·67
				Mean	44·1	23·7
ii	0·7744	0·1668	4 (*a*)	4·576	43·92	23·63
			(*b*)	4·574		
			5 (*a*)	4·567	44·22	23·79
			(*b*)	4·539		
			6	4·576	43·90	23·62
				Mean	44·0	23·7

* Calc. on a dry, ash-free basis.

EXPERIMENTAL.

L-[^{15}N]*Glutamic Acid Hydrochloride.*—Reduction of α-ketoglutaric acid by hydrogen and palladium black in the presence of ammonia containing approximately 15 atoms-% excess of ^{15}N, according to Schoenheimer's method (*J. Biol. Chem.*, 1939, **127**, 301), gave DL-[^{15}N]glutamic acid hydrochloride (80—85%). Resolution was effected by the fractional crystallisation of the strychnine salts of the N-benzoyl derivative (Fischer, *Ber.*, 1899, **32**, 2468). Subsequent hydrolysis and repeated recrystallisation gave L-[^{15}N]-glutamic acid hydrochloride [Kjeldahl-N, 9·5%. Differential solubility test (see Part III, *loc. cit.*): 0·1 g. and 1·0 g., respectively, in water (0·1 c.c.) at 45° gave a vapour pressure difference of 5 mm. of oil (d^{10}, 0·80)].

Determination of L-Glutamic Acid Hydrochloride in Synthetic Mixtures.—(*A*) A solution of L-glutamic acid hydrochloride (1·1294 g.), DL-glutamic acid hydrochloride (0·003 g.), L-aspartic acid hydrochloride (0·008 g.), and L-[^{15}N]glutamic acid hydrochloride (0·1208 g.) in water (15 c.c.) was made acid to Congo-red with hydrochloric acid. Amberlite IR—4B (analytical grade) was crushed and washed with water (1 l.), 3% hydrochloric acid (500 c.c.), 3% sodium carbonate solution (500 c.c.), and finally water (4 l.). The treated Amberlite (4 g.) was added to the solution of amino-acids, which was left, with frequent shaking, for 1½ hours, after which the solution was barely acid to Congo-red. The resin was removed by filtration, and the filtrate made acid to Congo-red and retreated with fresh Amberlite (5 g.). The extraction was repeated 4 times using 6, 7, 8, and 8 g. respectively of resin. The combined Amberlite samples were then eluted for 2 hours with water (20 c.c.) kept acid to Congo-red with hydrochloric acid. The resin was then filtered off and eluted a further 4 times. The combined acidic liquors were evaporated to dryness at 50—60° under reduced pressure. The resulting syrupy mass was dissolved in the minimum of water, treated with charcoal, filtered, and saturated at 0° with hydrogen chloride. When the mixture was kept overnight in a refrigerator a crystalline mass separated; this was filtered off, washed, and dried (0·85 g.). The crude L-glutamic acid hydrochloride was recrystallised from 5N-hydrochloric acid in all-glass apparatus. The product was filtered off through a sintered-glass funnel, washed repeatedly, and recrystallised. A small sample from each recrystallisation was transferred to a vacuum-desiccator containing sodium hydroxide pellets and calcium chloride. Each sample was tested for purity in the differential tensimeter, and after the third recrystallisation the vapour-pressure difference for successive samples was nearly constant. Crystallisation was repeated 7 times in all. (All differential-solubility tests on isolated samples were carried out with 0·02 and 0·10 g. respectively of material in 0·01 c.c. of water at 45°.)

No. of crystallisations	4	5	6	7
Yield (based on crude wt.), %	66	50	44	24
V. p. difference (mm. of oil)	4	3	3	3

(*B*) The mixture contained L-glutamic acid hydrochloride (0·4012 g.), DL-glutamic acid hydrochloride (0·005 g.), L-aspartic acid hydrochloride (0·08 g.), L-lysine hydrochloride (0·02 g.), DL-alanine (0·03 g.), and L-[^{15}N]glutamic acid hydrochloride (0·0488 g.). A solution of the mixture in water (15 c.c.) was made acid to Congo-red and heated to 70°. Ether-soluble phosphotungstic acid (2·0 g.) in water (1·5 c.c.) was then added. On the mixture being kept overnight, a heavy white precipitate settled out, which was filtered off. The filtrate was again made acid to Congo-red and treated with 5% phosphotungstic acid solution. After 2—3 hours, a faint precipitate appeared, which was removed by filtration. Further addition of phosphotungstic acid solution gave no precipitate.

The solution was then washed with butanol–ether (1 : 1) (6 × 30 c.c.). The aqueous layer was evaporated to dryness under diminished pressure, water (10—15 c.c.) added, and the solution made

acid to Congo-red. The amino-dicarboxylic acids were isolated with Amberlite IR—4B, as previously described. Isolation and recrystallisation of L-glutamic acid hydrochloride proceeded as before (crude yield, 0·39 g.).

No. of crystallisations	4	5	6	7
Yield (based on crude wt.), %	65	52	45	39
V. p. difference (mm. of oil)	6	4	4	4

(*C*) The mixture consisted of L-glutamic acid hydrochloride (0·9064 g.), DL-glutamic acid hydrochloride (0·003 g.), L-aspartic acid hydrochloride (0·08 g.), L-lysine hydrochloride (0·02 g.), DL-alanine (0·04 g.), glycine (0·02 g.) and L-[^{15}N]glutamic acid hydrochloride (0·0923 g.). L-Glutamic acid hydrochloride (0·755 g.) was isolated as described for mixture (*B*) and recrystallised 7 times.

No. of recrystallisations	4	5	6	7
Yield (based on crude wt.), %	74	62	47	35
V. p. difference (mm. of oil)	4	3	3	3

Preparation of Nitrogen Samples from L-Glutamic Acid Hydrochloride.—Each sample of L-glutamic acid hydrochloride (*ca.* 25 mg.) was converted into ammonia by the modified Kjeldahl procedure and then oxidised to nitrogen with sodium hypobromite according to Rittenberg's method ("Preparation and Measurement of Isotopic Tracers," Edwards, Ann Arbor, Michigan, 1947). We have found it essential to take great care to remove dissolved gases from the solutions before allowing them to react.

Samples of normal nitrogen were similarly prepared from "AnalaR" ammonium chloride. The mass-spectrometric determinations were carried out by Mr. Palmer and his colleagues at the Atomic Energy Research Establishment, Harwell.

The amount of L-glutamic acid hydrochloride (X) in the original mixture was calculated from the formula $X = A[C_0/C) - 1]$, where A is the amount of L-[^{15}N]glutamic acid hydrochloride added and C_0 and C are the atoms-% excess of ^{15}N in the added and the isolated material respectively.

The mass-spectrometer readings for the determination of L-glutamic acid hydrochloride in the synthetic mixtures are recorded in Table III and the calculated results of the analyses in Table I.

TABLE III.

	Relative intensity of peaks.*							
	Observed		Observed		Corrected		Atoms %	Atom-% excess
	mass 28	mass 29	mass 32	mass 44	mass 28	mass 29	^{15}N	^{15}N
Synthetic mixture (A).								
Normal nitrogen	100	0·7342	0·08	1·16	99·452	0·7298	0·3656	—
L-[^{15}N]Glutamic acid hydrochloride	100	35·449	0·74	0·96	95·908	35·419	15·587	15·221
Normal nitrogen	100	0·7316	0·08	0·54	99·514	0·7279	0·3644	—
L-Glutamic acid hydrochloride isolated:								
5th recrystn.	100	3·6417	0·56	1·20	96·856	3·6185	1·8337	1·469
6th ,,	100	3·6834	0·30	0·92	98·288	3·6706	1·8331	1·469
7th ,,	100	3·6659	0·44	0·54	97·570	3·6481	1·8353	1·471
Synthetic mixture (B).								
Normal nitrogen	100	0·7386	0·17	0·86	98·996	0·7310	0·3679	—
L-[^{15}N]Glutamic acid hydrochloride	100	35·710	0·59	1·53	96·661	35·685	15·583	15·215
LGlutamic acid hydrochloride isolated:								
5th recrystn.	100	4·0699	0·36	1·03	97·953	4·0547	2·0278	1·660
6th ,,	100	4·0816	0·33	1·01	98·117	4·0676	2·0308	1·663
7th ,,	100	4·0783	0·30	0·73	98·307	4·0658	2·0261	1·658
Synthetic mixture (C).								
Normal nitrogen	100	0·7344	0·15	1·15	99·075	0·7273	0·3657	—
L-[^{15}N]Glutamic acid hydrochloride	100	34·781	0·71	1·30	96·036	34·752	15·321	14·955
L-Glutamic acid hydrochloride isolated:								
5th recrystn.	100	3·4404	0·80	1·00	95·580	3·4080	1·7516	1·386
6th ,,	100	3·4435	0·80	1·60	95·520	3·4104	1·7539	1·388
7th ,,	100	3·4497	0·70	1·10	96·110	3·4211	1·7487	1·383

* The relative intensity of the peak at mass 40 (A^+) never exceeded 0·01.

Determination of L-Glutamic Acid in Gliadin.—The sample of gliadin had 17·69(9)% Kjeldahl-N, corrected for 9·8% moisture and 0·86% ash.

(i) From gliadin (1·3030 g.), hydrolysed with hydrochloric acid, L-glutamic acid hydrochloride (0·62 g.) was isolated and recrystallised in the usual manner.

No. of recrystallisations	4	5	6
Yield (based on crude wt.), %	65	50	32
V. p. difference (mm. of oil)	3	2	3

(ii) Concentrated hydrochloric acid (3·8 c.c.) was added to gliadin (0·7744 g.) and left at room temperature for 16 hours, complete dissolution occurring. Water (2·79 c.c.) was added to the solution which was then heated for 24 hours under reflux.

L-[^{15}N]Glutamic acid hydrochloride (0·1668 g.) was added to the solution which was again heated for 1½ hours. The solution was then evaporated to dryness at 50—60° under reduced pressure and the residual solid dissolved in water (10 c.c.). L-Glutamic acid hydrochloride (0·37 g.) was isolated as already described and recrystallised 6 times.

No. of recrystallisations	4	5	6
Yield (based on crude wt.), %	71	55	48
V. p. difference (mm. of oil)	5	3	3

The samples of L-glutamic acid hydrochloride were converted into nitrogen in the usual manner. The results of the analyses are recorded in Table II; the added L-[^{15}N]glutamic acid hydrochloride had 14·964 atoms-% excess of ^{15}N. The corrections for air and carbon dioxide were similar in magnitude to those shown in detail in Table III.

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Hypocholesteremic Effect of Glutamic Acid in the Mongolian Gerbil* (34133)

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The hypocholesterolemic effect of glutamic acid in humans fed a chemically defined formula diet has been reported from our laboratory (1–4). These subjects were fed an amino acid formula diet containing the eight essential amino acids required by the adult human subject plus glutamic acid as the sole source of nonessential amino nitrogen. The caloric intake was constant and the dietary lipid maintained at 36% calories with 4% polyunsaturated fatty acids (PUFA) as triglycerides. When the nonessential nitrogen was supplied as ammonium acetate (or citrate), and glycine, no hypocholesterolemic effect was observed.

While the effect of glutamic acid, under the above conditions, is well established, the mechanism of this interesting phenomenon remains to be determined. For the elucidation of the above mechanism, an animal model was sought. It is shown here that under experimental conditions comparable to those used in the human investigations the Mongolian gerbil responds to the high glutamate formula with a fall in serum cholesterol.

Materials and Methods. Mature, male Mongolian gerbils, 40–80 g weight, were obtained from Beaumanor Farms, Cleveland, Ohio. Adult male rats, 200–300 g weight, were obtained from the St. Louis University Colony (Doisy-Wistar Strain). Adult rabbits, 1.5–2.0 kg weight, were obtained from Eldridge Rabbitry, St. Louis, Missouri, while 3-week-old chickens were obtained from Kenroy Hatchery, Berger, Missouri.

All dietary amino acids were purchased from General Biochemicals, Chagrin Falls, Ohio. Vitamin supplements were obtained through the courtesy of Hoffmann-La Roche Inc., Nutley, New Jersey. Cholesterol, chramotographically pure, was purchased from Merck & Co., Inc., Rahway, New Jersey. Rations appropriate for each species tested were obtained from Ralston Purina, St. Louis, Missouri. Serum cholesterol was determined by the method of Abell *et al.* (5).

In early experiments the animals were divided into three groups. Group 1 was maintained on the commercial ration to which lard had been added to bring the total fat content of the diet up to 10% by weight (FCD). Group 2 was fed the control amino acid formula (AAF) while group 3 was maintained on the amino acid–glutamate formula (AAFG). The composition of these diets is reported in Table I.

All animals were fed *ad libitum* for 2 weeks and weights and blood samples obtained at the beginning and at the end of the periods. In later work the experimental design included sequential dietary experiences in various combinations using each animal as its own control.

Results. All animals tolerated the formula diets fairly well after a few days of adjustment. In general they maintained their weight throughout the experimental period. During the formula feeding the gerbils' fur assumed a more glossy appearance; no changes were observed in the rabbits, rats, or chicks. In all animals tested, maintenance on the AAF regimen produced serum cholesterol levels which were not significantly different from those observed on the control ration (FCD).

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TABLE I. Ingredients of the Amino Acid Formula Diet.[a]

Food	g/kg
Lard	100.00
Cornstarch	163.50
Sucrose	420.00
Mineral mix	40.00
Nonessential nitrogen	
Ammonium citrate[b]	110.00
Glycine[b]	110.00
Essential amino acids	56.00
Arginine	2.00
Histidine	4.00
Lysine	6.30
Phenylalanine	8.66
Tryptophan	1.97
Methionine	8.66
Threonine	3.94
Leucine	8.66
Isoleucine	5.51
Valine	6.30
Cholesterol	0.50

[a] Vitamin supplements, much in excess of requirement (6), were as follows (unit/100 g): Vitamin A, 3500 IU; vitamin C, 396 mg; vitamin D, 360 IU; vitamin B_{12}, 5.0 μg; folic acid, 360 μg; thiamine, 12.4 mg; riboflavin, 12.4 mg; nicotinic acid, 84 mg; pantothenate, 17.6 mg; pyridoxine, 3.96 mg; inositol, 30 mg; choline, 100 mg; biotin, 50 μg; vitamin E, 3 mg.

[b] In the AAFG diet ammonium citrate and glycine are replaced by glutamic acid (g/g).

The chicks and gerbils responded to the glutamate formula, however, by significant decreases in the serum cholesterol levels (Table II).

The rabbit did not respond and, the rat paradoxically, showed higher serum cholesterol levels when fed the high glutamate regimen.

In previous clinical experiments (2) human subjects were maintained for successive 4-week periods on the dietary sequence FCD, AAF, AAFG, AAF. To determine if the gerbil would respond in a manner similar to that observed in humans, the experiment shown in Table III was performed. In this experiment each animal was used as its own control. The effect of the glutamate formula on serum cholesterol was even more impressive here: a 53% decrease in serum cholesterol levels was observed upon the first dietary change from AAF to AAFG. During the second AAF feeding, on the fourth week, the serum cholesterol increased significantly even though it did not reach the levels observed during the previous AAF regimen (second week). During the fifth week (second AAFG) the characteristic response of serum cholesterol to glutamate feeding was again shown with an additional 45% drop from the previous AAF (fourth week). These results clearly demonstrate a consistent repetitive hypocholesterolemic effect of glutamic acid in the gerbil when compared to levels observed when glycine and ammonium acetate are fed as a source of nonessential nitrogen in the formula diet.

Discussion. Of all animals tested in this study, the gerbil and chick appear to be the only two in which the hypocholesteremic effect of glutamate formula diets was observed. Because of the greater magnitude of the changes, the gerbil appears to be the experimental animal of choice.

The curious hypercholesteremic effect of the AAFG feeding in the rat was not studied further, hence it remains unexplained. No effect whatsoever was demonstrated in the adult male rabbit.

In humans the hypocholesteremic effect of glutamate is not accompanied by overt changes in plasma free amino acid concentration, nor in nitrogen balance; there is, however, an increase in the ratio of nonessential to essential amino acids in the plasma. It appears that the fall in plasma cholesterol which occurs in human subjects fed the glutamate formula is accompanied by a reduction in cholesterol biosynthesis by the liver (8). It is hoped the gerbil will aid in the elucidation of the mechanism of this interesting phenomenon. This hope is supported by the finding that the gerbil responds to changes in dietary regimen in a like manner to the human being.

Summary. Several animals have been tested in search of a model to study the mechanism of the hypocholesteremic effect of glutamic acid formula diets, previously demonstrated

TABLE II. Serum Cholesterol Values ± SEM in Animals Maintained for 2 Weeks on the Designated Diet.

Animal	Dietary regimen		
	FCD[a]	AAF	AAFG
Chick	145 ± 6.7 (21)	167 ± 10.7 (12)	124 ± 6.9 (12)[b]
Rabbit	59 ± 10.5 (5)	--	79 ± 10.1 (5)
Rat	77 ± 7.2 (4)	84 ± 3.9 (12)	102 ± 3.7 (10)[c]
Gerbil	128 ± 11.2 (8)	138 ± 14.6 (12)	80 ± 4.2 (12)[d]

[a] Commercial rations (FCD), after addition of lard to bring the crude fat content up to 10%, contained: (a) for gerbils and rats: crude protein > 23.0%, crude fiber > 6.0%; (b) for rabbits and chicks: crude protein > 15%, crude fiber > 18%.

[b] AAFG serum cholesterol significantly ($p < .02$) lower than AAF.

[c] AAFG serum cholesterol significantly ($p = .005$) higher than AAF.

[d] AAFG serum cholesterol significantly ($p = .002$) lower than AAF; all other differences were insignificant at $p = .05$.

Number of animals in each experiment given in parentheses.

TABLE III. Serum Cholesterol Values ± SEM in Gerbils Fed the Designated Diets, for 7-Day Periods.

	Dietary regimen[a]					
	FCD	AAF	AAFG	AAF	AAFG	FCD
Period no.	1	2	3	4	5	6
Mean serum cholesterol (mg/100 ml)	155 ±12.9 (14)	169 ±11.1 (14)	80 ±8.4 (14)	115 ±11.2 (14)	63 ±6.2 (10)	101 ±9.6 (4)
Significance of change		$p = .35$	$p < .0005$	$p < .01$	$p < .005$	$p < .01$

[a] Number of animals in each experiment given in parentheses.

in human beings. Upon feeding the glutamate formula diet to the Mongolian gerbil, the characteristic decline in serum cholesterol has been demonstrated. These data support the view that the Mongolian gerbil is an excellent animal model for the study of the mechanism by which dietary glutamic acid, under our experimental conditions, lowers serum cholesterol in human beings.

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STUDIES OF GLUTAMIC ACID METABOLISM IN THE CENTRAL NERVOUS SYSTEM

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INTRODUCTION

EVIDENCE accumulated in recent years increasingly points to the significant role of glutamic acid and its metabolic derivatives in the metabolism of the nervous system in normal and pathological conditions. The relationship between glutamic acid metabolism and convulsive states has attracted particular attention[1, 2]. Work now in progress in our laboratory[3] suggests a connection between the metabolism of glutamic acid, glutamine and γ-aminobutyric acid, and the development of cortical electroencephalographic spikes resulting from experimentally produced lesions in the cat.

Experiments of previous workers[4, 5] suggest that in extreme stress such as hypoglycemia the brain can utilize glutamic acid and that in such cases glutamic acid can be taken up from the blood by the brain. It has been shown, however,[6] that in normal animals the blood brain barrier prevents elevated blood glutamic acid concentration from effecting a net increase of the amino acid in the brain although a net increase of glutamine could be demonstrated after the administration of the amide. The possibility still remained that the glutamic acid which did penetrate the blood brain barrier was rapidly metabolized and therefore no longer detectable. Furthermore, the available data did not show whether the lack of net uptake was also indicative of an absence of exchange between blood and brain glutamic acid. The importance of distinguishing between net uptake and exchange in brain was emphasized by the finding of a rapid exchange of ^{14}C lysine or leucine between plasma and brain[7] although no net increase of these amino acids in the brain could be demonstrated[8].

Results recently reported by ROBERTS, *et al.*, show that intracerebrally injected glutamic acid is converted rapidly to glutamine and γ-aminobutyric acid[9].

In order to study the *in vivo* metabolism of glutamic acid in the brain, the exchange of ^{14}C labeled glutamic acid between blood plasma and brain as well as the conversion of glutamic acid to glutamine, γ-aminobutyric acid, and glutathione in brain and other organs were investigated[10].

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EXPERIMENTAL

For the separation of the glutamic acid, and its metabolic products, certain features of a method recently developed[10, 11] for the micro determination of these compounds were utilized.

For the calculation of specific activity (counts/min/μM), the glutamic acid, glutamine, γ-aminobutyric acid, and glutathione concentrations were determined by the above method. Aliquots of the various fractions were then plated on stainless steel planchets and counted in a gas flow counter. All values were corrected for the measured retention of blood in the organs.[12]

RESULTS

Table 1, representative of several experiments, shows the distributions of total radioactivity in whole rat organs five minutes after the injection via the tail vein of uniformly labeled ^{14}C glutamic acid. The total radioactivity was much higher in the liver, kidney, and muscle than in the blood plasma, which shows that much of the plasma glutamic acid has entered the tissues from the blood. The brain glutamic acid was also significantly radioactive. Considerable counts were found in the glutamine and glutathione of liver and brain and in γ-aminobutyric acid of brain. Similar experiments were repeated with mice and essentially the same results were obtained.

TABLE 1

Total radioactivity in whole rat organs

	Glutamic Acid	Glutamine	GSH
Plasma*	6,400	4,700	
Brain	860	140	72
Liver	40,000	17,000	8,200
Kidney	14,000	1,600	4,000
Muscle*	30,000	12,000	4,700

* Estimated total weight.

0.2 ml glutamic acid ^{14}C (uniformly labeled containing 24 γ glutamic acid and 1 μC) was injected into adult Sprague-Dawley rats. The animals were sacrificed after five minutes.

It was calculated that of the total counts injected about 14 per cent were present in glutamic acid, 5 per cent in glutamine, and 2·5 per cent in glutathione. Thus 22 per cent of the radioactivity can be accounted for in these three substances distributed in approximately 40 per cent of the body weight. It is estimated from these figures and from the counts present in other fractions of the separation procedure that within the experimental period of 5 min about 50 per cent of the glutamic acid had undergone metabolism to substances other than those analysed.

The results of short term experiments in mice are presented in Table 2. In these experiments mice were injected via the tail vein with uniformly labeled ^{14}C glutamic acid. It can be seen that within minutes after the administration of the ^{14}C glutamic acid there was significant labeling in all the organs. In each organ the specific activity of the glutamic acid is higher than that of glutamine, an indication that much of the glutamic acid must have entered the organs as such. In almost all the experiments the specific activity of the plasma glutamine was higher than its radioactivity in the other organs, including the liver. The importance of this finding will be discussed later.

TABLE 2

Specific activity of compounds after injection of ^{14}C glutamic acid into mice

Duration of experiment (min)	Organ	Specific activity of compounds count/min/μ Mol			
		Glutamic acid	Glutamine	γ-aminobutyric acid	Glutathione
2	Plasma	29,000	3,600		
	Brain	50	35	17	16
	Liver	2,000	1,300		94
	Red blood cells	1,900	220		65
	Kidney	700	390		340
5	Plasma	14,000	3,000		
	Brain	93	57	34	50
	Liver	2,400	2,000		200
10	Plasma	8,000	3,000		
	Brain	920	230	60	
	Liver	9,200	11,000		

Each time point is the average of two experiments. Four adult mice were used for each experiment. 0·1 ml uniformly labeled ^{14}C glutamic acid was injected. Each animal received 0·5 μ C and 7 γ glutamic acid corresponding to 30% of the plasma glutamic acid content.

In brain and liver the specific activities of glutamic acid and glutamine were frequently close. The animals very rapidly converted glutamic acid to glutamine with a resultant rapid rise in specific activity of plasma glutamine. It cannot be decided on the basis of these data whether brain glutamine originated from brain glutamic acid or blood glutamine. It was thought that the evidence substantiating the ingress of glutamic acid into the brain might be accentuated if the specific activity of the glutamine could be diluted by the injection of unlabeled glutamine along with the radioactive glutamic acid and vice versa.

The results of such experiments are presented in Table 3. In the first experiment relatively large amounts of glutamine (approx. 40 times that usually present in the blood) were injected along with the radioactive glutamic acid. In the second experiment non-labeled glutamic acid was injected along with the radioactive glutamine.

TABLE 3

Specific activities of glutamic acid and glutamine after their injection into mice

Experiment	Compound injected	Organ	Count/min/μ Mol	
			Glutamic acid	Glutamine
1	C^{14} glutamic acid C^{12} glutamine added	Plasma	130,000	700
		Brain	77	40
		Liver	1,600	1,900
2	C^{14} glutamine C^{12} glutamic acid added	Plasma	8,300	11,000
		Brain	97	380
		Liver	3,000	5,900

Four mice were pooled in each experiment. Experiment 1: 0·2 ml containing 4 mg glutamine and 8 γ (0·5 μC) glutamic acid was injected over a period of three min and each animal was killed immediately afterward. Experiment 2: 0·2 ml containing 1 mg glutamic acid and 70 γ (0·5 μC) glutamine was injected rapidly and the animal killed after 5 min. In both experiments the compounds were injected via the tail vein.

These experiments again indicate the ability of the liver and brain to rapidly interconvert glutamic acid and its amide. The result of the first experiment suggests that part of the brain glutamine was derived from the brain glutamic acid and not from blood glutamine, since otherwise the specific activity of brain glutamine should have been considerably lower. This question, however, is not answered unequivocally as part of brain glutamine probably is derived from plasma. The second experiment in Table 3 shows the rapid entrance of plasma glutamine into brain. When the specific activity of the plasma glutamic acid is greater than that of the plasma glutamine, this also becomes true for the brain, and when the ratio of specific activities in the plasma is reversed then the specific activity of the glutamine in the brain is also higher than that of the glutamic acid. This again supports the probability that plasma glutamic acid and glutamine enter the organ without extensive interconversion. The absorption of glutamic acid and glutamine from the gut without interconversion has been observed previously[13]. Five to ten times as much glutamine as glutamic acid passes through the brain in the plasma in a given period of time. Therefore, any elevation of the brain glutamic acid specific activity over that of the glutamine specific activity becomes that much more significant.

If the specific activity of an amino acid in blood plasma and in an organ is measured as a function of time, the flux of this substance from the plasma into the organ and

its half-life time can be calculated. While half-life times or turnover rates of proteins can be measured fairly accurately, the flux of a free amino acid from plasma into an organ can be estimated only approximately as the changes in plasma are extremely rapid. The fact that in two to five minutes more than 90 per cent of the plasma glutamic acid disappeared from the blood gives an idea of the speed of these changes. An additional difficulty in measuring the flux rates of glutamic acid is its very high metabolic activity; it is rapidly converted to other products, some of which may be reconverted to the acid.

The approximate flux values for glutamic acid in brain are compared with values obtained for other amino acids in Table 4. Let us again emphasize that these values only serve to show the order of magnitude of flux of the amino acids into the organs. Such calculations give values of 0·15–4·0 γ/g of brain/min for glutamic acid, which are in the range of those previously found for leucine and lysine[12, 14].

TABLE 4

Turnover of free amino acids in brain

		Flux	½ life (Min)	γ Substance/g fresh tissue	
				Plasma	Brain
Lysine	young	1·1–1·5	21–29	49	51
	adult	0·7–5·2	5–43	50	38
Leucine	adult	0·1–3·0	3–67	17	9·6
Glutamic acid	adult	0·15–4·0	200–5,000	15	1,600

Flux = γ amino acid/g fresh tissue/min exchanged.

As pointed out above, in most of the experiments the specific activity of plasma glutamine was higher than that in any of the other organs and frequently greater than the specific activity of the organ glutamic acid. The plasma glutamine may be derived from organs other than those analyzed, which were brain, liver, kidney, muscle, and red blood cells. To test this hypothesis attempts were made to eliminate the liver and other organs from the circulation[15].

The abdominal aorta and inferior vena cava of rats under anesthesia were tied below the diaphragm, and radioactive glutamic acid was injected via the subclavian vein (Table 5). The organs below the dotted line were those with constricted blood flow. Comparing the counts in the muscle of the fore leg with that in the hind leg, it is evident that perhaps 5 per cent of the blood continued to flow in the lower part of the body. The plasma specific activities again were high; however, the organ of next highest specific activity was the lung, where the glutamine specific activity closely approached that of the plasma. This could mean that much of the plasma glutamine comes from the lung. However, it could be a reflection of the

membranous nature of the lung and the lack of a blood-lung barrier for glutamine, with the amide in the plasma, rapidly exchanging with that in the lung.

TABLE 5

Specific activity in rat organs with restricted blood flow

	Organ	Counts/μM/min.		
		Glutamic acid	Glutamine	γaba
Unrestricted circulation	Plasma	520,000	38,000	
	R. B. C.	27,000	8,200	
	Lung	68,000	34,000	
	Heart	7,800	5,300	
	Muscle (front legs)	3,800	2,000	
	Brain	300	550	36
Restricted circulation	Liver	1,800	790	
	Kidney	17	190	
	Muscle (hind legs)	100	54	

The inferior vena cava and the abdominal aorta were tied off in rats and 0·1 ml ^{14}C glutamic acid (5μC and 1μMol) was injected in the subclavian vein, and the animals sacrificed after 5 min

In other short term experiments (Table 6), radioactive glutamic acid or glutamine was injected via the tail vein into normal unanesthetized rats[15]. We see that when

TABLE 6

Specific activity of glutamine in rats in vivo

Time of experiment (min)	Substance injected	Organ	count/min/μ Mol of glutamine
5	^{14}C glutamic acid	Plasma	1,200
		Lung	1,300
5	^{14}C glutamine	Plasma	5,300
		Lung	4,100
2	^{14}C glutamic acid	Plasma	430
		Lung	190

Unanesthetized rats were injected via the tail vein with ^{14}C glutamic acid (0·1 ml 80 γ and 5 μC) or ^{14}C glutamine (0·2 ml 140 γ and 1 μC) respectively.

^{14}C glutamic acid is injected, in 5 min the specific activity of the glutamine in the lung and in the plasma are quite close. However, in 2 min the plasma glutamine

specific activity is higher than that of the lung. When ^{14}C glutamine is injected, in 5 min its specific activity in the plasma and lung approaches equality. We therefore conclude that in lung we are dealing mainly with rapid exchange of glutamine rather than with its synthesis.

It can be concluded on the basis of the data presented that the plasma glutamine with high specific activity was synthesized in one of the organs analyzed: brain, liver, or kidney; otherwise, one organ should have been found with a glutamine of higher specific activity than the plasma glutamine.

The finding of high specific activity of plasma glutamine may be explained by a cellular compartmentalization of its synthesis. Since the cell is not a homogeneous unit but composed of several compartments, it is likely that separate pools of substrates coexist, each metabolized at its own rate. It appears possible that glutamine is synthetized from ^{14}C glutamic acid which has not mixed with the organ glutamic acid and that the newly formed amide is returned to the blood again before being equilibrated with the organ glutamine.

It is also of note that in the brains of animals with restricted circulation (Table 5) the glutamine specific activity is increased over that of glutamic acid, an observation which becomes understandable on the basis of compartmentalization of glutamine synthesis. It is interesting in this connection that FLOCK, *et al.*[16], in studying the effect of total hepatectomy on the changes in free amino acids of the brain and muscle of dogs, found that immediately after removal of the liver the brain glutamine begins to rise and the increased amide content of the brain is not proportional to the increased glutamine content of the blood. Our data supports the conclusion that under conditions in which the liver is removed from the circulation there is an increased rate of conversion of glutamic acid to the amide in the organ compartment of glutamine synthesis.

We have no definite data as to the compartment of glutamine synthesis, it may be the capillary walls, or a specialized cell type in liver, brain, or kidney. In view of the fact that glutamine synthetase and transferase are found chiefly in the microsomal fractions of the brain and liver[17, 18] it could be visualized that the endoplasmic reticulum is the cellular compartment in which glutamine is synthesized from the administered and not highly diluted glutamic acid. Subsequently, without being mixed with the amide in other compartments of the tissue, part of the newly synthesized glutamine returns to the plasma and has a higher specific activity than the average organ value. We are in the process of further investigating this possibility with brain and liver perfusion experiments.

It cannot be decided whether the glutamine of high specific activity is formed in an ATP dependent or a transfer reaction.

SUMMARY

A very rapid exchange of plasma glutamic acid with the free glutamic acid of the brain as well as other organs has been demonstrated in intact animals. The indications

are that glutamic acid can enter the organs without conversion to glutamine and vice versa.

Within 2 min after intravenous administration of ^{14}C glutamic acid or ^{14}C glutamine, the following reactions have been demonstrated; (a) decarboxylation of glutamic acid to γ-aminobutyric acid in brain, (b) an interconversion of glutamic acid and glutamine, and (c) incorporation of glutamic acid into glutathione in brain and liver.

Within this time period the specific activity of brain γ-aminobutyric acid rose up to 40 per cent and that of glutamine up to 60%–70% of the specific activity of glutamic acid found in the organ. When glutamic acid is administered intravenously, the specific activity in short-time experiments of plasma glutamine is usually higher than that of organ glutamic acid or glutamine. This finding suggests that the synthesis of glutamine is compartmentalized.

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DISCUSSION

A. LAJTHA: Dr Bessman's remarks concerning the possible interference of protein turnover with the quantitative measurement of the turnover of free amino acids will be true in organs where the contribution of protein bound amino acids to the free amino acid pool is significant. In such cases, the true flux of free amino acids in the organ would be higher than that measured, as part of the newly entered amino acids is incorporated into proteins. In brain, we measured the turnover rate of proteins as well as that of the free amino acid pool. These measurements show that incorporation into, or release of the amino acid from the proteins would not in these short term experiments influence flux values. Admittedly, most of our present values are only approximations because of all the limitations discussed above.

W. M. SPERRY: I was impressed by the high specific activity in the plasma at 19 hr. when the specific activities in the tissues were relatively very low. I should appreciate further comment on this point.

D. B. TOWER: I would like to reinforce what Dr Berl has said about 'compartmentalization' of glutamine. In preliminary experiments, we have estimated much of the glutamine in brain to be in a different locus than the glutamic acid and γ-aminobutyric acid. The latter two in cerebral cortex appear to be essentially neuronal and mitochondrial in position whereas glutamine appears to be non-neuronal and non-mitochondrial in position.

H. WEIL-MALHERBE: I noticed that your figures for the specific acivity of glutamic acid in brain amount to about 3 per cent or less of the specific activity in plasma. This concentration could be accounted for by the blood content of brain. I wonder if you have taken this point into account.

S. BERL: The contribution of the radioactivity in the blood contained in the organs to the specific activity of the organ glutamic acid and glutamine was considered. This was estimated and all values were accordingly corrected. However, this correction was not significant, since the concentration of glutamic acid and glutamine in the organ, particularly the brain, is so much greater than in the blood. Despite the differences in specific activities, the quantitative contribution of the blood to the total radioactivity of the brain is small.

To the Editor: There seems to be another possible component of the Chinese-restaurant syndrome: for years I have noticed an intense throbbing temporal headache within about 15 minutes of the first dish in certain Chinese restaurants, and have spoken to several other people who have the same complaint. (It disappears within a few hours.) We have named it our "Chinese headache" and merely accepted it as one of the side dishes that go along with the meal. Perhaps it too is related to a possible hypernatremia, resulting in a vascular headache.

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Monosodium Glutamate induces Convulsive Disorders in Rats

THE physiological and pharmacological effects of L-glutamate have received much attention since its implication in the etiology of the "Chinese restaurant syndrome"[1-3]. Although an excess of L-glutamic acid (GA) as the monosodium salt (MSG) was known to cause retinopathy in experimental animals[4-7], brain lesions have been noticed only recently[8-10]. There seems to be no agreement about its pharmacological and neurophysiological effects in humans[11] and experimental animals[12].

While investigating glucose metabolism[13-15], we used L-glutamate (as MSG) as a gluconeogenic precursor *in vivo* (unpublished), and observed that rats became somnolent after intraperitoneal administration of carrier doses. In a study of the effect of L-glutamate on the *in vivo* incorporation of amino-acids into cerebral proteins (unpublished), several animals experienced severe tonic-clonic convulsions, usually between 30 and 120 min after intraperitoneal injection of a large dose of MSG (2 mmol/100 g body weight). Some convulsions terminated in death. We have now made a detailed study of the incidence of seizures following treatment with MSG. As well as taking an electroencephalogram (EEG) during the convulsions, we studied the influence of pyridoxine on the incidence of these convulsive disorders, for pyridoxine is directly involved in the metabolism of GA and gamma-amino-butyric acid (GABA).

We used weanling Sprague–Dawley male rats maintained on either laboratory chow (Teklad), or pyridoxine-control or deficient diets of the following composition: vitamin-free casein, 22%; sucrose, 69%; corn oil, 4%; salt mixture (Northern Regional Research Laboratories), No. 446, 4%; vitamin-fortification mixture (pyridoxine-free), 1%. This basal diet supplemented with 30 mg of pyridoxine HCl/kg served as the pyridoxine-control diet. Controls weighed 380–550 g (10–20 weeks old) and the deficient animals 80–120 g (9–12 weeks old). MSG (2.5 mmol/ml.) was injected intraperitoneally (2.0 mmol/100 g body weight). Pyridoxine HCl (5.0 mg per 100 g body weight) was administered intraperitoneally to some animals 30 min before MSG treatment. For EEG recording, animals were prepared under pentobarbital and chlorprothixene anaesthesia with chronically implanted stainless steel electrodes and then allowed to recover for 1 week before recordings were taken.

Table 1 summarizes the incidence of the various symptoms observed during 2 h after injection of MSG. Marked somnolence was observed in 99% of the animals within 5–20 min. After this (1 h after injection), 52% of the animals salivated copiously, and 31% then displayed what we have described as spastic tremors, varying in intensity from mild to severe myoclonic jerking sometimes followed by vigorous running about the cage and stereotyped biting. This behaviour occurred significantly more often ($\chi^2 = 5.25$; d.f. = 1; $P < 0.05$) in rats on the pyridoxine-control diet pretreated with pyridoxine than in animals on the same diet pretreated with 0.9% NaCl. Seizures, which occurred 30–120 min after MSG injection, were invariably preceded by spastic tremors, and terminated in death in some cases, although the animals usually recovered within 2–3 min and resumed the spastic tremors until the next seizure. The largest proportion of seizures was observed in the animals fed pyridoxine-control diet and pretreated with pyridoxine; the lowest proportion was found in the pyridoxine-deficient and laboratory chow animals, both pretreated with pyridoxine.

To ascertain whether the effects of MSG could be attributed

Table 1 Incidence of Somnolence, Hypersalivation, Spastic Tremors, Seizures and Death in MSG*-treated Rats

Diet	Pretreatment	Somnolence		Hypersalivation		Spastic tremors		Seizures		Death†	
		N	%	*N*	%	*N*	%	*N*	%	*N*	%
Laboratory chow (21)	Pyridoxine HCl‡	20	95	6	29	5	24	1	5	2	10
Laboratory chow (22)	0.9% NaCl	22	100	16	73	7	32	5	23	6	27
Pyridoxine-control (25)	Pyridoxine HCl	25	100	15	60	15	60	7	28	9	36
Pyridoxine-control (25)	0.9% NaCl	25	100	17	68	6	24	5	20	8	32
Pyridoxine-deficient (15)	Pyridoxine HCl	15	100	4	27	2	13	1	7	1	7
Pyridoxine-deficient (11)	0.9% NaCl	11	100	4	36	2	18	1	9	3	27
Total 119		118	99	62	52	37	31	20	17	29	24

Figures in parentheses indicate the number of animals.
* 2 mmol/100 g body weight, intraperitoneal.
† During 24 h after injection.
‡ 5 mg/100 g body weight.

to the administration of excess sodium *per se* and/or to the tonicity of the solution injected, two groups (eight and four rats respectively) were injected with either NaCl or sodium DL-lactate (2.5 mmol/ml., 2 mmol/100 g body weight). None of the animals developed any symptoms characteristic of MSG treatment.

Of the eleven MSG-treated animals from which EEG recordings were taken, five displayed tonic–clonic convulsions during the 2 h recording session. The seizures were characterized by high amplitude spikes typical of this form of convulsion.

Thus MSG in a dose smaller than that used by Olney[8] in adult mice, and by Adamo and Ratner[12] in infant rats, can induce tonic–clonic seizures in adult rats. Administration of pyridoxine to animals which already had an excess of this vitamin (30 mg/kg diet) significantly increased the occurrence of spastic tremors. It should be noted that we used intraperitoneal administration rather than the subcutaneous method of the previous authors.

The types of seizure seen in the rat are remarkably similar to those noted by Wiechert *et al.*[16–18] in dogs and rats after intracisternal injection of L-glutamate (but not other amino-acids), and pyridoxal-5′-phosphate (PLP). Wiechert *et al.*[16–18] and Elliot and van Gelder[19] have suggested that the balance between GA and GABA but not the absolute amount of GABA *per se*[20] is a decisive factor in the control of cerebral neuronal activity. Unphysiological shifts in this balance have been implicated in the onset of seizures. Our findings suggest that intraperitoneally administered L-glutamate in the dose used may cross the blood-brain barrier, which would affect the steady state levels of GA and GABA, thus inducing seizures. The effect of pyridoxine, on the other hand, may be a consequence of differential stimulation of the two enzyme systems—GA decarboxylase and GABA aminotransferase—the latter having a higher affinity for PLP[21]. The resulting change in the relative concentrations of GA and GABA may determine the seizure activity. Other possibilities such as a disturbance in the electrolyte balance do exist and a detailed neurochemical study of L-glutamate-induced convulsions is necessary.

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MONOSODIUM GLUTAMATE

To the Editor: Two hundred and twenty million pounds of monosodium glutamate (MSG) were sold in the world in 1966. One might assume from its practically ubiquitous presence in prepared foods that MSG has been thoroughly tested and found harmless. This is not so. MSG was accepted because of its natural character and long history of use. For this reason, it is categorized along with salt, pepper, sugar and baking powder as a GRAS (Generally Recognized as Safe) substance by the Food and Drug Administration.

In 1957, one year before MSG was made GRAS, Lucas and Newhouse[1] reported a toxic effect of MSG on the ganglion cells of the retina of neonatal mice. This report was followed by studies confirming the fact that the neonatal mouse is peculiarly susceptible to toxic effects of MSG. Recent articles by Olney[2,3] summarize the previous data and describe retinal lesions, in addition to demonstrating that hypothalamic lesions can be produced with MSG.

A distinctive response to a common agent may remain hidden for a long time simply because the agent is so common and the response threshold differs among individuals. The Chinese-restaurant syndrome demonstrates this.[4] Only direct pharmacologic and toxicologic studies or very extensive and careful public-health and epidemiologic surveys can identify untoward responses to such an agent. In the future, MSG may be very important in the development of palatable but inexpensive foods for the world's population. Since MSG is not a nutritional necessity and is not added to the diet for any reason except palatability, there is no medical rationale for opposing a change in its regulatory status. Before an explosion in the use of MSG occurs because of expanding world food needs, we think it would be appropriate to remove MSG from the GRAS list and establish safe levels of MSG intake during pregnancy and infancy.

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An Electron Microscopic Study of the Modification by Monosodium Glutamate of the Retinas of Normal and "Rodless" Mice [1]

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ABSTRACT Sodium-L-glutamate was administered to post-natal Swiss albino and agouti C3H/HeJ "rodless" mice. In both cases there was at two months a marked reduction in myelinated axons of the optic nerve from about 25,000 to less than 225 axons but Swiss mice had more ganglion cell somas than axons. Glutamate-treated eyes seemed smaller and the lenses were 65% of the weight of control lenses. The inner nuclear layer of Swiss-glutamate retinas was reduced from 5–7 nuclei deep to 1–2. In treated Swiss mice the receptor concentration appears unchanged and electron microscopy revealed that both spheroidal and pedicular receptor terminals were contacted by presumed neurites of second order neurons. There was a small, relative increase in terminals of bipolar neurons in the markedly reduced inner plexiform layer. Relative to these findings a description is given of normal synaptic arrangements. In glutamate-treated "rodless" mice at two months the retina contained two rows of nuclei. Imbricated processes of glial cells of Müller faced the pigment epithelium and the retina contained scattered ganglion cells, bipolar cells, and other neurons less readily characterized. At two months most glutamate-treated mice, rod containing or "rodless," had a direct pupillary response and a detectable but obviously weaker consensual response. Preliminary examinations of animals one year after glutamate treatment showed receptor deterioration in albino animals and further retinal deterioration in both groups. The two surviving C3H-glutamate animals possessed milky cataractous lenses, however full but sluggish direct iris constrictions could be elicited.

In 1957 Lucas and Newhouse showed that monosodium glutamate administered suckling mice caused extensive destruction of the ganglion cell and inner nuclear layers of the retinas of these animals but spared the receptors. Potts, Shirrell, and Kingsbury ('60) subsequently found only the "a" wave of the electroretinogram (ERG) in their glutamate treated mice, a finding consistent with current hypotheses relating the "a" wave of the electroretinogram to receptor activity. Since ganglion cell activity does not contribute to the electroretinogram the loss of the "b" wave must reflect changes in the inner nuclear layer. The effect of more healthy degeneration on the ERG of glutamate treated rats has been described (Potts, '65).

The residual retinal histology and physiology as evidenced in the above reports raises a number of interesting questions and possible opportunities for the student of neuroanatomy and physiology. A retina with normal concentrations of receptors but a paucity of other neurons, what are the persisting synaptic arrangements? Would the simplified retina be useful in clarifying the synaptic connections of this complex organ? Are the normal concentrations of receptors in treated animals evidence that receptors may survive in the absence of synaptic connectives or have the remaining neurons of the inner nuclear layer adapted to contact more receptors than usual? Is the inner nuclear layer of the mouse retina populated largely by a cell which does not normally contact the receptors and is it this cell species which has diminished in sufficient numbers to account for the sharply reduced inner nuclear layer of these retinas?

To try to answer these questions the retinas of normal and glutamate treated animals were examined by electron mi-

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croscopy. To clarify further certain questions bearing on the factors influencing neuron survival, the glutamate experiments were extended to the C3H/HeJ strain of mouse which is homozygous for a recessive gene resulting in total receptor dystrophy.

MATERIAL AND METHODS

Six litters of Swiss albino mice (35 surviving experimental animals) and four litters of C3H/HeJ mice (10 surviving experimental animals) were used in these observations as well as control litters of the same races. The light microscope sufficed to show whether the glutamate effect occurred, as it invariably did, and electron microscopic examination was usually confined to one eye of an animal. As per the schedule of Potts et al. ('60), Swiss albino mice were given subcutaneous injections of monosodium glutamate for 18 days beginning on the second post-natal day but in later experiments it was observed that ten days of injection gave the full effect and this was employed with later groups of animals. Control injections of water or saline were given but proved to have no discernible effect. Lukas and Newhouse ('57) have explored compounds related to glutamate for their ability to induce this effect and the current investigation was not designed to explore further the mechanism of the glutamate effect.

Because in all animals the synaptic arrangements may differ in different retinal regions, an effort was made to make comparisons in similar retinal areas. Therefore, at a possible sacrifice of quality of fixation, the retina was fixed *in situ*. Some animal perfusions were tried but these did not give uniform fixation. In the final procedure adopted, the mice were decapitated, and the eyes were evulsed and placed in fixative. The cornea was punctured and then dissected away with iridectomy scissors and a defect cut in the sclera to indicate the horizontal axis. The lens was then removed and the remainder of the eye allowed to fix. A variety of fixatives were explored, but best results were obtained by one hour's fixation in cold 2.5% glutaraldehyde in Earles saline containing 1 ml of 0.067 M K_2HPO_4–KH_2PO_4 (pH 7.4) per 100 ml and 450 mg glucose per 100 ml followed without washing in buffer [illegible] overnight fixation in 2% osmium tetroxi[illegible] in the same saline. The eyes were de[illegible]drated in ethanol and embedded in epo[illegible] resin (Durcupan). The embedded e[illegible] were cleaved along the horizontal axis a[illegible] two regions were taken and mounted [illegible] examination. One, termed "central," [illegible] immediately temporal to the nerve he[illegible] The other, termed "peripheral," was [illegible] extreme periphery of the neural reti[illegible] Comparison of random fields at a stand[illegible] magnification of 8000 × in both the[illegible] areas in normal or experimental eyes [illegible] estimates of the intrinsic variability in [illegible] frequency of those cell processes be[illegible] studied. Sections were mounted on 1 [illegible] mm single hole discs over Formvar f[illegible] and stained with lead and uranium sa[illegible] Sections were studied with an Elmis[illegible] 1A electron microscope.

Pupillary activity was crudely obser[illegible] by leading light from a 6v microscope la[illegible] to 1 cm in front of the eye by a fiber op[illegible] bundle while the eye was observed wi[illegible] dissecting microscope. To test for a [illegible] sensual eye response, one eye was sti[illegible]lated as before and the other eye was [illegible]served using a red filter (Corning 24[illegible]) equipped 6v microscope lamp (AO) wh[illegible] was focused on the eye at a distance [illegible] eight inches from the eye. The effect [illegible] heat was ruled out in preliminary exp[illegible]ments by passing the light through one [illegible] of a 10% copper sulfate bath without [illegible]fecting the responsivity. Animals w[illegible] taped to a rubber hose stuffed with co[illegible] during these examinations and were [illegible] anaesthetized.

RESULTS

The two day post-natal mouse had [illegible] largely undifferentiated retina with [illegible] ganglion cells demarked and these w[illegible] putting forth dendrites and axons.

Two months following the glutam[illegible] treatment it was noted on removing [illegible] eyes of treated animals that they appe[illegible] smaller than those of controls from [illegible] same or other litters. No effort was m[illegible] to quantitate this observation but the [illegible] pairs were saved and weighed. The le[illegible] of experimental animals averaged 65[illegible] the weight of lenses of control an[illegible] with no overlap in weights of lenses [illegible] treated and untreated groups.

ghts: controls 0.014 ± 0.001 gm; ex-mentals 0.009 ± 0.002 gm).

Figures 1 and 2 show paired light mi-graphs demonstrating the effect of the ment on the retina, confirming ear-reports. The two-month-old animals wed a normal receptor concentration treatment but in the inner nuclear the normal 5–7 tiers of nuclei were 1–2 and only occasional ganglion cell as were now evident. Cross-sections the optic nerve of the normal two month se (fig. 3) revealed about 25,000 mye-ed nerve fibers and electron micros-of the nerve gave no evidence of myelinated fibers; the rare, isolated myelinated cross-sections seen being ely accounted for as representing cut within nodes of Ranvier. Ex-ation of the normal retina permitted ady identification of fascicles of the myelinated axons (fig. 5) which only their sheathing on entering the optic Using this morphological guide, an ction of the optic nerve of the two-d mouse, prior to glutamate treat-revealed many non-myelinated in the nerve (fig. 6), however, their made it difficult to obtain a pre-numerical estimate since obliquely ned fibers with sparse neurotubules be confused with slightly fibrous processes. Following the glutamate ment from 0 to 225 myelinated axons seen (fig. 4) in the optic nerves of month animals. Since small volumes ghly concentrated solutions are in-subcutaneously part of this varia-may be due to leakage. Since the er of surviving axons was substan-less than the number of optic nerve prior to treatment it demonstrates the glutamate treament has destroyed axons.

the retinas from glutamate treated als there was an obvious reduction in number of ganglion cell somas sur-at two months. Horizontal sections h two full normal retinas revealed t microscopy some 200–250 gan-cell somas whereas in two glutamate Swiss animals some 30–40 were Flat retinal preparations were not but it was highly probable that there more somas than myelinated optic nerve fibers in experimental animals since the ratio of myelinated fibers in the normal optic nerve to those in the experimental optic nerve is much greater than the ratio of somas in sections of the two retinas. The ganglion cell somas of experimental animals were clearly smaller than those of control animals. This most likely is an atrophy but could represent in part an enhanced survival of a smaller ganglion cell variety. One must also consider the possibility that these represent an intra-retinal ganglion cell as Gallego and Cruz ('65) have claimed to demonstrate in other species.

In examining the inner plexiform layer one is concerned with the interaction of ganglion cells, bipolar neurons, and amacrine cells. The ganglion cells are readily identified since they occur as a separate and innermost neural layer and in the mouse constitute a single line of cells (figs. 1, 7) above the layer of their axons, the optic fiber layer. This axonal layer is absent in glutamate-treated animals (fig. 8). The ganglion cells possess the largest somas of the retinal neurons with large nuclei with prominent nucleoli, prominent Golgi regions, a ribosome rich (Nissl) cytoplasm and frequent sub-membranal cisterns. Near the nerve head the adjoining ganglion cell somas were often contiguous (fig. 11) without intervening glia and a single cell at times possessed broad contiguities with as many as six adjoining cells. Ganglion cell axons were likewise not typically separated by glial processes. Surviving ganglion cell somas of glutamate-treated animals are similar in appearance to the normal ganglion cells except for size (fig. 8).

Stout apical dendrites contained microtubular elements similar to those in the axons. The dendrites extended outward toward the inner nuclear layer and branched. Ribosomes were not found in the finest branchings. At least some and perhaps all ganglion cell somas of glutamate-treated animals also send forth dendrites.

On the apex of the ganglion cells there were often found in contiguity the bag endings of the bipolar neurons (fig. 7), but these were also seen free of ganglion cell contact in the inner plexiform layer

(fig. 17). The bipolar neurons possessed a close morphological kinship to the receptors. Their nuclei lie in the inner nuclear layer and they send a stout, unbranched process into the inner plexiform layer which there terminates in a large, highly irregular bag filled with vesicles of 300–400 Å. These bags are clearly related to the receptor spherules and pedicles. The vesicle containing portion of the bag was somewhat branched or lobulated (fig. 17). In the mouse, as noted in other species (Missotten, '61; Fine, '62; Dowling and Boycott, '65), bipolar neurons possess at irregular intervals along the inner walls of the terminal bag curious plates arranged perpendicularly to the membrane. These plates or synaptic lamellae are lunate in shape and in cross-section appear as ribbons which, following appropriate staining, have a pentalaminate appearance (figs. 12, 13). The plates are surrounded by a halo of vesicles which stand off the plates as if a gel intervened. Between a small region of the basal curvature of the lunate plate and the cell membrane the arciform density, U-shaped in cross-section, is sometimes seen. Since nothing is known of the physiology of this apparatus it cannot be said whether cell contacts contiguous to the region containing this density differ in function from cell contacts in other lamella regions. This organelle is highly similar, except in size, to those in receptor bases as described by others (Ladman, '58; Lanzavecchia, '60; Cohen, '63). If the plane of section was satisfactory the synaptic lamella was included in a plane that typically passed between two cell processes terminating on the externum of the bag ending and the contacting membranes usually possessed an enhanced density when combined uranium and lead staining were employed.

Dowling and Boycott ('65) have recently coined the useful term "dyad" for the paired external neurites opposite the synaptic lamellae of bipolar bags, following Missotten's ('64) use of "triad" for a slightly more complex system of external contacts at synaptic lamellae in pedicles of human cones. Although the bipolar bag must primarily be a presynaptic structure no vesicle clusters were seen anywhere against the inside of its cell membrane. All the above bipolar features are essentially similar to those of the visual receptors from their nuclei to their terminations. In addition to the "dyad" contacts bipolar bags were also contacted at other sites by processes which contained a cluster of vesicles in them and whose contact site is marked by a density (figs. 7, 17). These contacting processes had a typical "presynaptic" appearance according to the working hypotheses of most students of the fine structure of the central nervous system (Gray, '59). Moreover, as noted by Dowling and Boycott in primates ('65) one of the external dyad processes sometimes possessed a typical presynaptic contact on the bipolar bag thus suggesting possible bidirectional transmission or "feedback" arrangements (figs. 8, 15, 17). A reciprocal synaptic arrangement has recently been described in the olfactory bulb by Rall, Shepherd, Reese and Brightman ('66). Typical presumptive presynaptic contacts of unknown origin were sometimes seen on the apices of the somas of ganglion cells and on their dendritic extensions (fig. 10) but the contacts of the vesicle filled bipolar bags and ganglion cell somas seemed to be simple contiguities with no cell membrane specialization (fig. 9). Dowling and Boycott ('65) have recently claimed that such bipolar bags and ganglion cell contacts in primate retinas may possess tight junctions but after an intensive search I have only seen one possible instance of this in the mouse and I have failed to observe them in peripheral and central regions of human and macaque retinas. It might be noted that the very same sections in all these species routinely exhibit the tight junctions at the inner faces of the cells of the pigment epithelium so that the ability to preserve such junctions would not seem in doubt.

Amacrine cells have proven difficult to identify unequivocally in the mouse but the innermost (vitread) cell of the inner nuclear layer often had its nucleus surrounded by cytoplasm on all sides and the cytoplasm was rich in ribosomes (fig. 1[illegible]). This concurs with Dowling and Boycott's identification ('65). Processes from these cells swept inwardly and laterally and differed from known bipolar axons in being less dense and often possessing ribosomes

While these cells were the most likely candidates for amacrine cells, the presence of somas of amacrine cells in the midst of the inner nuclear tier was also likely but somewhat more difficult to establish since the distribution of cytoplasm differed in this compacted area. A working hypothesis suggested by Dowling and Boycott ('65) is that dyads usually represent a paired termination on the bipolar bag of a ganglion cell dendrite and an amacrine neurite, the latter alone possessing synaptic vesicles. While one often finds instances in the normal mouse where only one of the terminals possesses synaptic vesicles (fig. 12) and is the presumed amacrine neurite, it is not rare that one finds both of the terminals with vesicles (figs. 14, 15), a situation similar to that in rod spherules in a number of species where both submembrane processes usually possess vesicles. In fact in "dyad" pairs in the mouse instances of one, both, or rarely, neither (fig. 14) containing vesicles may be found. On the other hand, whether possessing vesicles or not, the two dyad processes usually differed in appearance, suggesting a different origin.

All the presumed synaptic arrangements seen in normal mice may be found in the inner plexiform layer of the glutamate-treated animals (fig. 8). There is a small but definite relative increase in the frequency of sections of bipolar bags both in central and peripheral retina suggesting that bipolar cell number is relatively less severely reduced than that of the amacrine and ganglion cells; or less likely, that more sections were being encountered because the bags had become larger and more branched or irregular.

The outer cells in the inner nuclear layer, to judge from other retinas, could be expected to include both horizontal cells and bipolar neurons although the latter are not found deeper in this layer. In the mouse all cells in this region possessed a cytoplasm that is rich in mitochondria and ribosomes and sent processes into the inner plexiform layer and toward the receptors (fig. 18). A reasonable working hypothesis is that with respect to the receptors, the bipolar processes are functionally post-synaptic elements, i.e., dendrites — processes which are ordinarily expected to be free of synaptic vesicles. If one considers an extension to mice of Polyak's ('41) description of horizontal cells in the primate retina and assumes horizontal cells to possess neuronal character, they would then link pedicles to spherules and pedicles. Thus their axonal terminals on rods would be telodendrites and might possess synatpic vesicles whereas their terminals on cones might either be vesicle-free dendrites or vesicle-containing telodendrites.

Turning to the mouse, a previous study of the receptors in this retina (Cohen, '60) showed that two receptor classes could be discerned on the basis of receptor terminals — i.e., a class ending in spherules like those of human rods and one ending in pedicles like those of human cones although but one class of outer segment was identified (figs. 19, 20, 21). These observations were confirmed. No significant changes in spherule and pedicle frequency were noted in comparisons of central and peripheral retina.

The spherules were spheroidal sacs containing numerous bare vesicles and some coated vesicles. Terminating neurites were deeply embedded in the spherule and were always contiguous to spherule membrane. Most deeply within the spherule one usually observed neurite terminations with a conspicuous although variable number of vesicles, mostly bare but occasionally coated. These were associated with synaptic lamellae, one or two, in the contiguous spherule cytoplasm. When the section plane through the lamella included the arciform density, it typically passed between two vesicle bearing processes but sometimes the processes lacked vesicles. Less deeply within the spherule were terminations of processes which were relatively clear in that they largely lacked vesicles although rare vesicular profiles were noted. While always contiguous to some internal surface of the spherule membrane, these clear terminations were typically not adjacent to lamellae. Where the clear processes entered the sperules, the adjacent spherule membrane often had enhanced density. The external spherule surface had process terminations containing synaptic vesicles (fig. 21). These exhibited enhanced membrane densities as

did points where sperules and pedicles were visibly contiguous and probably represent similar junctions. Sjöstrand ('58) showed a similar situation in the guinea pig. It did not prove possible by serial sectioning to trace the neurites within spherules to an identifiable cell of the inner nuclear layer, because the weaving of the neurites caused them to be lost in the plexiform area. In any event such tracing would have had limited value because the cells of the inner nuclear layers were not readily categorized.

In sections the pedicles possessed numerous basal contacts containing vesicles (fig. 20). These were on occasion traced to neurites containing ribosomes, an organelle not seen in receptor terminals. On other occasions serial sectioning showed apparent external contacts to be curled under portions of the same pedicle, the appearance resulting because the plane of the pedicle base was not perpendicular to the section plane. The pedicle synaptic lamellae were associated with a pair of neurites, which, in contradistinction to the typical situation in spherules, tended to be clear or relatively free of vesicles (fig. 20). Only occasional triad arrangements (Missotten, '64) were evident. Lateral pedicle to pedicle contiguity was occasionally observed.

In glutamate-treated animals the frequency of receptor terminals possessing neurite contacts with second order neurons approached those of control animals (figs. 22, 23). On occasion one discerned a spherule with a clear area possibly suggesting loss of neurites but these were few. Slight differences in the nature of contacting neurites were suggested but not statistically established. Thus fewer vesicle containing endings were seen to terminate on the pedicles and some spherules seemed to lack vesicle containing processes. Apart from these suggestive alterations the area appeared surprisingly normal.

To see whether there would be less neuronal survival in the inner nuclear layer in a retina in which the chances of centripetal as well as centrifugal stimulation was reduced, glutamate was administered to post-natal mice of the C3H/HeJ strain. In this "rodless" strain the receptors actually form during the first post-natal week but then degenerate. At two months only one row of receptor nuclei (but no inner or outer segments) and a vestige of the outer plexiform layer remain (fig. 24). Subsequently even these disappear. An electroretinogram was only obtained during the early period before receptor degeneration (Noell, '58, '65). The remainder of the retina and optic nerve were, however, quite normal in appearance by electron microscopy (Karli, '65; Cohen, unpublished observations). Another surprising but long known feature of "rodless" mice, including the animals employed here, but also albino "rodless" strains, is the persistence of a pupil response, both direct and consensual (Brückner, '51; review by Karli, '52). The iris also shows usual responses to constricting or dilating drugs (Brückner, '51).

Following glutamate treatment, the effect of this compound added to the dystrophic process yielding the comparison shown in figure 25. The retina was but two cells thick with a mixture of glia cells of Müller, ganglion cells, bipolar neurons and probably other neurons of the inner nuclear layer with a residuum of the inner plexiform layer (figs. 27, 28). The residuum of receptor nuclei and outer plexiform layer usually seen in two month C3H controls was absent. The optic nerve typically had less than 225 myelinated axons. The pigment epithelium appeared somewhat thinner but otherwise intact (figs. 25, 26). It faced a retinal surface of imbricated processes of Müller cells (fig. 26).

Remarkably enough, despite the reduction in optic nerve axons in glutamate treated animals, at two months both treated Swiss albino and treated "rodless" animals had a vigorous direct pupil response and a detectable but less marked consensual response. An occasional two month "rodless"-glutamate animal gave no pupil response and the pupil was markedly dilated. This became more common in older animals. Unfortunately one two month Swiss mouse with no axons evident in its optic nerve had been examined before residual pupillary activity had been noted. At one year after glutamate treatment, six albino animals showed recep-

deterioration with some retinal areas being devoid of receptors. Four of these showed a moderate to strong direct pupil response and weak consensual responses. The other two animals showed either slight or no direct pupil response and no consensual response. The two surviving C3H-glutamate mice both possessed milk-white cataractous lenses in both eyes and a full but sluggish direct pupil response. No consensual responses were elicitable. Their retinas suggested slight further deteriorations. Preliminary examinations of the eyes of normal albino mice have revealed no unusual specializations.

The possibility of a pseudo-consensual response due to leakage of light from one eye to the other was explored. Using the heads of freshly decapitated normal Swiss albino mice and pigmented "rodless" mice where in both cases the pupil is markedly dilated, light was brought to one eye and the other observed. In a darkened room the contralateral albino eyes but not the pigmented eyes showed a faint but distinct glow. Accordingly, living non-glutamate treated Swiss albino and pigmented "rodless" animals were unilaterally enucleated. After 48 hours a glass ball was placed in the empty orbit after checking to see if the orbit was free of clotted blood which could block the transmission of light. Technical success was only achieved with two albino animals and one pigmented animal. In repeated observations of the unanaesthetized animals these exhibited strong direct reflexes but no pupil response was seen when the eyeless orbit was illuminated. In the case of the albinos the observation of iris movements is sufficiently difficult in red light so that very small movements might be overlooked.

DISCUSSION

With the exceptions of the spared receptors the effect of glutamate appears to represent randomized destruction in that none of everything persists. The retina in the mouse differentiates postnatally; thus opportunities for adaptive remodeling should be greatest since the glutamate is being applied at this time. An effect of glutamate on retinal vasculature is possible since growth of the lens, still vascularized in the post-natal period by the hyaloid artery, a branch of the central retinal artery, is obviously retarded. However, this could be an indirect consequence of the retinal damage (see Coulombre and Coulombre, '66). The receptor sparing might represent an undamaged choroid blood supply or some intrinsic resistance of these cells. The relative bipolar sparing might have a similar origin.

The fact that no single class of neuron was eliminated in its entirety precluded an easy route to learning something new about the neuronal cytoarchitecture of the retina. Attempted detailed statistical analyses of types of processes were abandoned because in this small eye it was difficult to standardize location except grossly, and in a modified eye it is hard to decide what area corresponds to a given area in a normal eye. The thinner experimental retinas seemed somewhat better fixed than the controls. This could influence vesicle concentration which is one of the obvious things one might use to characterize cell processes. In other species a fortuitous distinctive character of some class of neurites would facilitate a statistical approach to retinal neural architecture. Because of the limitations of serial sectioning in tracing weaving processes in neuropil areas and in tracing processes travelling considerable distances, for most retinas a combined Golgi and electron microscopic serial section analysis would seem to offer the greatest promise (Stell, '65; Blackstad, '65).

A feature of special interest in the mouse is the presence of two receptor classes in this nocturnal animal. Is the pediculated receptor a photopic receptor? A recent report states that no duplex activity was detected physiologically in the mouse retina (Hellner, '66). Of general interest is the receptor-like morphology of the bipolar neuron. In addition to the evidence cited in this report, one may note that in many species retinas possess Landolt's Clubs (Landolt, 1871). These are processes from bipolar neurons that enter the ventricle of the retina between the receptor inner segments and Müller cell microvilli. The receptor-like aspects of the bipolar neurons suggest that it might

be worth considering that they possess similar electrophysiological characteristics.

The finding that almost all receptors are entered by neurites despite a drastic reduction in the inner nuclear layer suggests an adaptation by residual inner nuclear layer neurons to encompass more receptors. The validity of this surmise depends on how broad and overlapping bipolar and horizontal cell contacts with receptors may be in the normal retina and unfortunately this has not been established by Golgi methods, early efforts in this direction having failed. However, superior functional plasticity in the face of damage to the perinatal nervous system as compared to older nervous systems is a classic neurological observation and suggests that adaptation is a distinct possibility. Because of the possible importance of the above observations for the understanding of the factors influencing neural organization, further efforts to obtain successful Golgi preparations are clearly indicated.

The case of the C3H (rodless) mice shows that in the mice the conventional receptors are not necessary to obtain vigorous direct and consensual responses. This compels the conclusion that photoreceptive molecules are located in at least a second site in this eye. The existence of the consensual response says that information from one eye reaches the other eye and suggests involvement of the retina and optic nerve.

The fact that the glutamate-treated mice usually showed a strong direct but weaker consensual response suggests that the pigment site(s) in the stimulated eye is (are) essentially intact but that there is more difficulty in conveying the presence of light to the other eye than to the pupil of the stimulated eye.

In considering these phenomena one has to take into account possible intrinsic iris irritability as shown by Brown-Sequard (1859) for irides of certain fish and amphibians. Brückner ('51) obtained an iris response in the isolated mouse iris or eye with optic nerve sectioned but discounted the response as it possessed a long latency and required intense stimulation. It is possible, however, that mammalian irides have more fastidious requirements for survival and funct when removed from their blood supp Karli ('54, '63), also pointing out that iris response (latency 30 sec–1 min) der Brückner's conditions markedly fered from the quasi-normal thresh latency, and speed of the *in vivo* respo of the "rodless" mouse iris, has presen evidence for the need for an intact ret and optic nerve for the quasi-normal sponse. Indeed, Karli ('63) has presen evidence that his "rodless" mice can taught to respond to an intense vis signal, that the peak wavelength of r dopsin adsorption is also the peak wa length for this learning, and that op nerve section abolishes the response. I not clear whether optic nerve section in this small orbit was carried out with damage to the general blood supply of eye. Karli suggests ('65) that rhodop in the pigment epithelium (none was tected in extracts in a procedure w limited sensitivity) is bleached and t electrical activity of these cells (No '58, '65, did not detect any electrical tivity) stimulates the bipolar neurons.

In the case of both rodless and rodl glutamate treated mice in this study bricated Müller cell processes interve between any neurons and the cells of pigment epithelium so that any stimu could not be direct. It might be poin out that the cells of the pigment epit lium are linked laterally by occluded junctions as part of their terminal b (Cohen, '65) and that such junctions other tissues have been said to repres paths of low resistance to ion curre (Dewey and Barr, '64). Since a chain cells with such junctions extends all way into the iris (Cohen, unpublished servations) one could consider a m direct path for iris stimulation. Howe not only is the distance considerable e in this small mammal but no electr activity to light was demonstrable in "rodless" retina (Noell, '58, '65). may always raise questions of sensiti and speculate as to what would be adequate stimulus. Karli's work ('63) this report (consensual response) sug at least a partial involvement of the re and the questions of whether the re is, in fact, involved and of a possible

... of light sensitivity in bipolar or gan-...n cells may have to be considered. A ...ent report describing experiments on ... suggests that the circulation may ...ribute to a consensual response under ...erimental conditions by bringing neu-...umor released in one iris to the other ...pp and Paulson, '66). In this case ... second iris may have been rendered ...ersensitive by the experimental proce-...es. However, to have this apply in ...less" mice without intervention of the ...a, neurohumor would have to be re-...d by an intrinsically sensitive iris. ... does not seem likely but should ...theless be considered.

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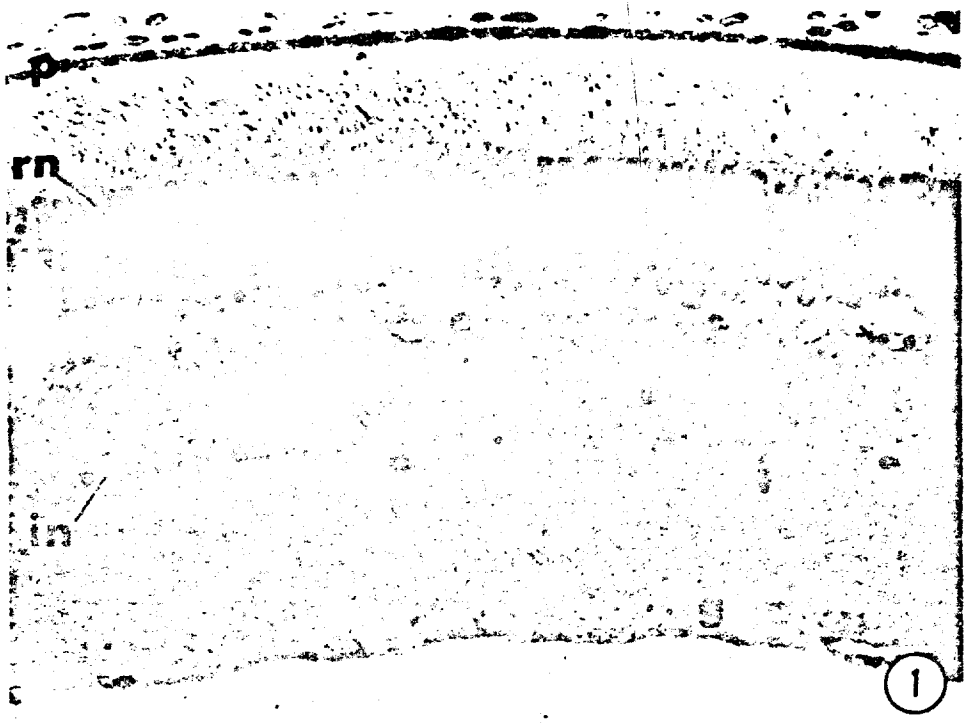


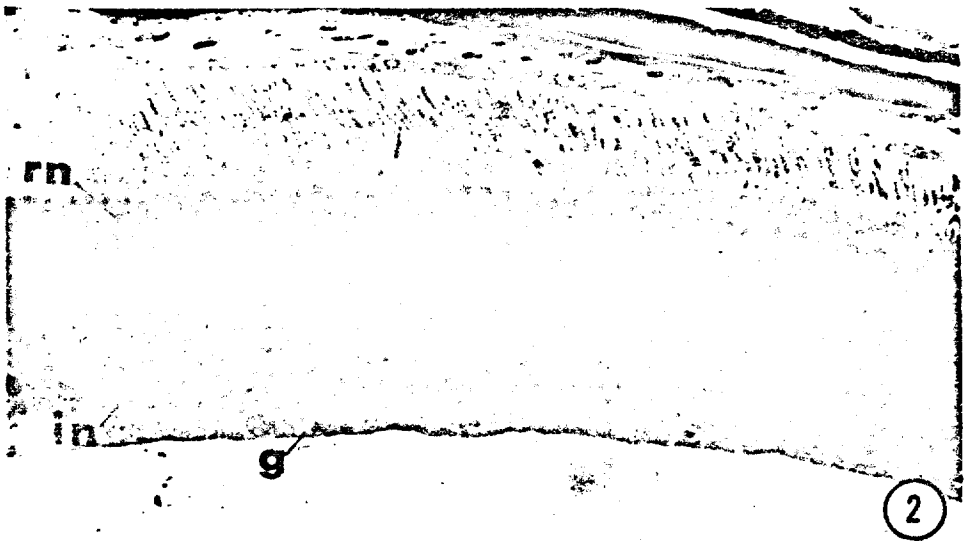
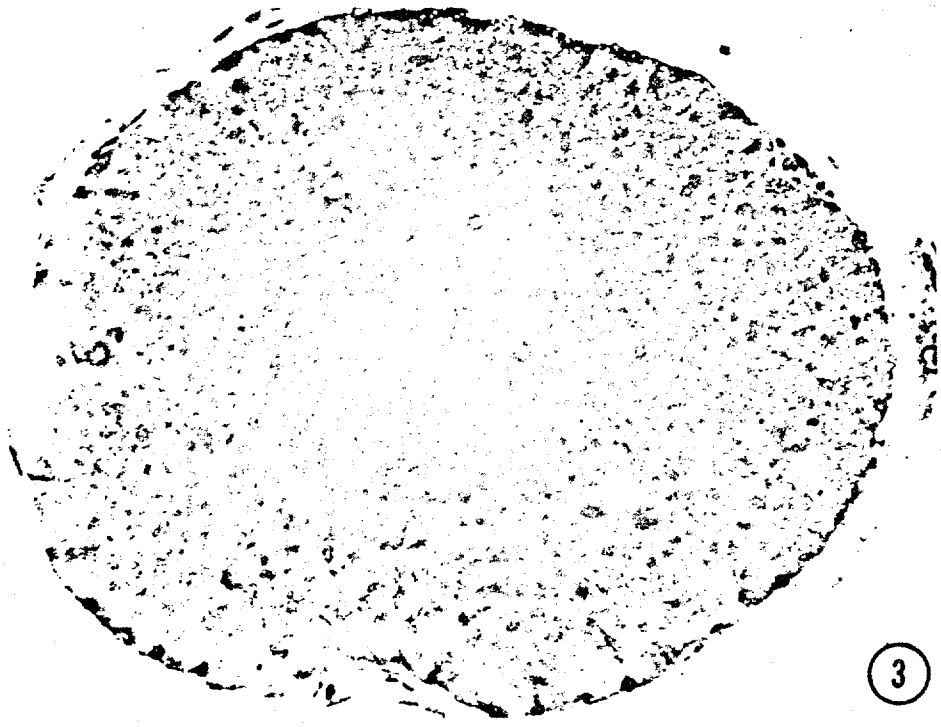


PLATE 1

EXPLANATION OF FIGURES

1 A light micrograph of the retina of a normal two-month-old Swiss mouse. The levels indicated are those of the pigment epithelium (p), the receptor nuclei (rn), the inner nuclear layer (in), and the ganglion cell layer (g). The outer plexiform layer lies between the receptors and the inner nuclear layer; the inner plexiform layer lies between the ganglion cells and the inner nuclear layer. × 490.

2 A light micrograph of the retina of a two-month-old Swiss mouse which had been treated with glutamate. The labels are as in figure 1. × 490.



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4

PLATE 2

EXPLANATION OF FIGURES

3 A light micrograph of a section of the optic nerve of a normal two month Swiss mouse. × 370.

4 A light micrograph of a section of the optic nerve of a glutamate-treated two month Swiss mouse. The optic nerves of glutamate-treated "rodless" mice of this age are indistinguishable. × 550.

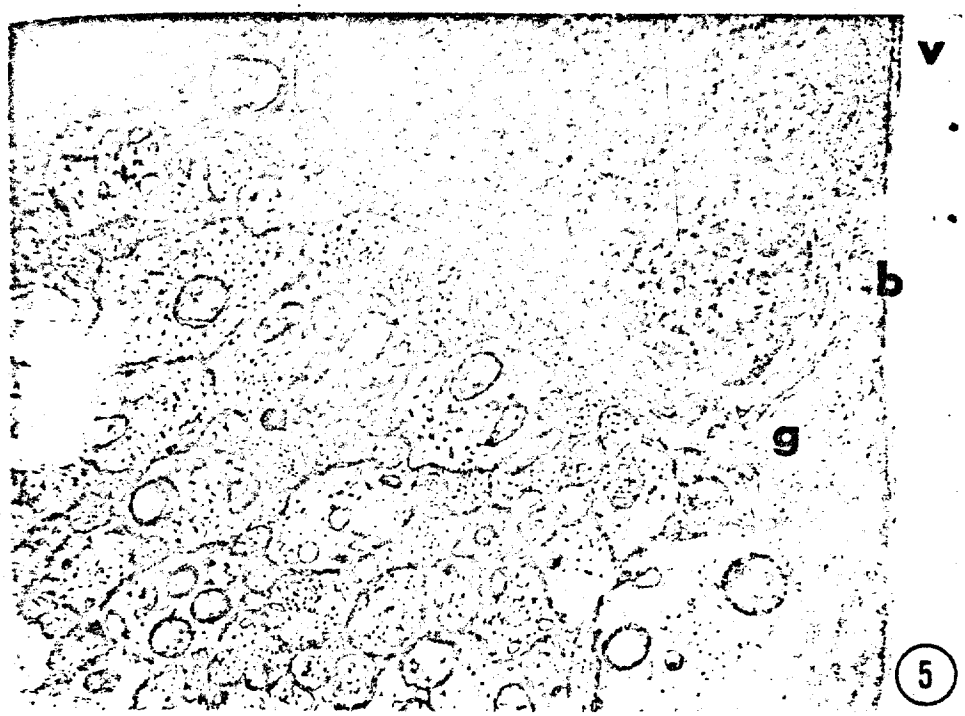


PLATE 3

EXPLANATION OF FIGURES

5 An electron micrograph of the inner surface of the retina of a two month Swiss mouse. Facing the vitreous chamber (v) are a basement membrane (b), processes of glial cells of Müller (g), and a portion of a large fascicle of axons of retinal ganglion celss (a). × 21,000.

6 An electron micrograph of a section of the optic nerve of a two-day-old mouse. The principal cell processes shown are non-myelinated axons (a) and those of fibrous astrocytes (f). × 32,300.

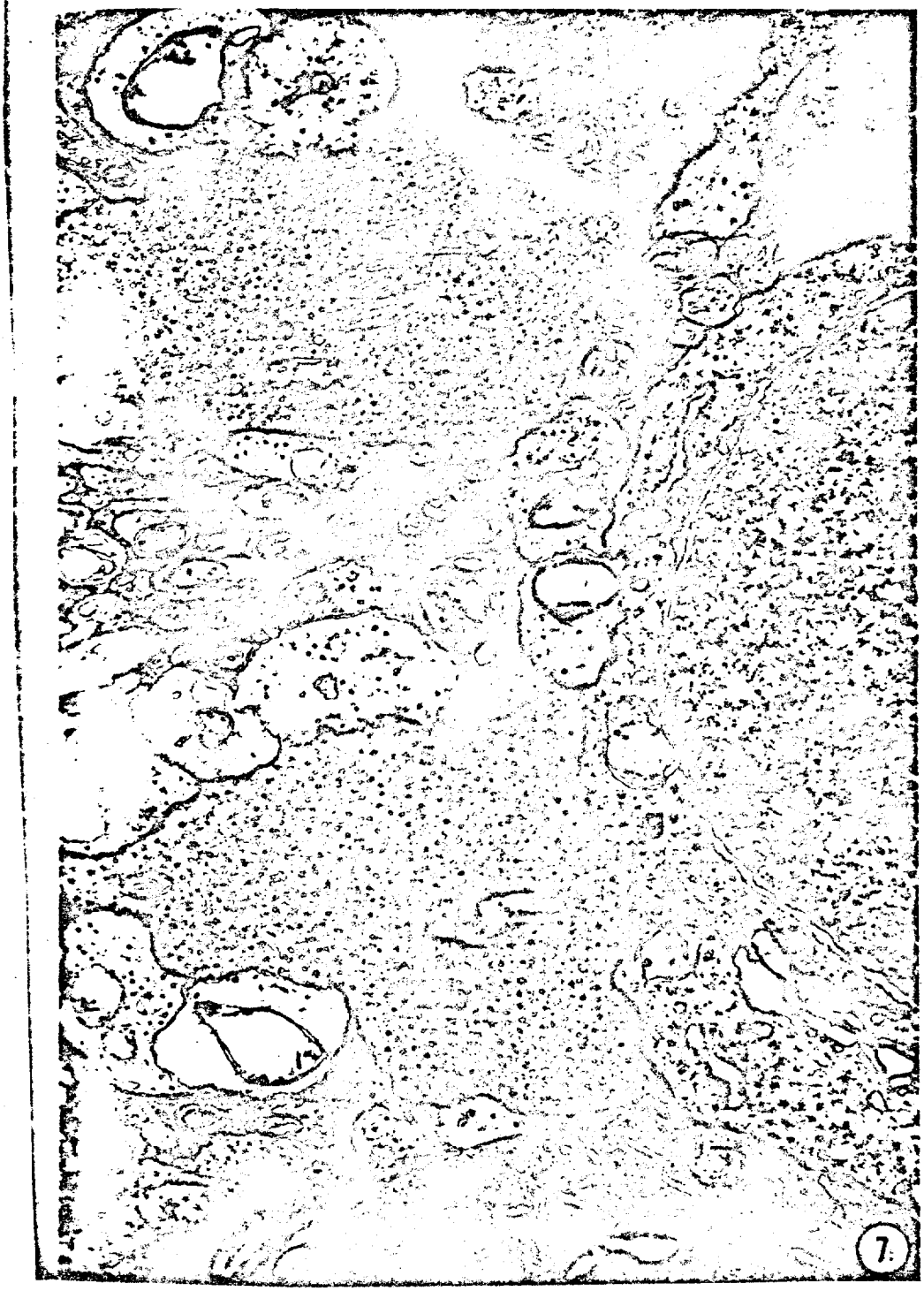


PLATE 4

EXPLANATION OF FIGURE

7 An electron micrograph of the apical surfaces of ganglion cells (g) of a control retina. In contiguity with both cells is a portion of a bipolar bag (bb) and a second bag is also seen. Both exhibit dyad contacts opposite synaptic lamellae (sl). Also note vesicle-containing contacts on the bipolar bags. × 20,400.

8

PLATE 5

EXPLANATION OF FIGURE

8 An electron micrograph of the retinal surface of a glutamate-treated mouse. Facing the vitreous chamber (v) are processes of glial cells of Müller (m), artifactually somewhat dehydrated. No layer of ganglion cell axons is evident. A large ganglion cell (g) has contiguity with dyad processes opposite synaptic lamellae (sl). One of the dyad processes (arrow) also has a conventional synaptic contact on the bipolar bag. × 12,800.

PLATE 6

EXPLANATION OF FIGURES

9 An electron micrograph from a control animal illustrating the contiguity of a bipolar ending (bb) and the apical surface of a ganglion cell (g). Note the absence of any membrane specialization at the junction. × 31,000.

10 An electron micrograph from a control animal of a neurite contiguous with the apex of a ganglion cell (g) near its main dendrite. Note the presumptive synapse of conventional configuration (arrow). × 32,000.

11 An electron micrograph of a control retina exhibiting contiguity of ganglion cell membranes. × 32,000.

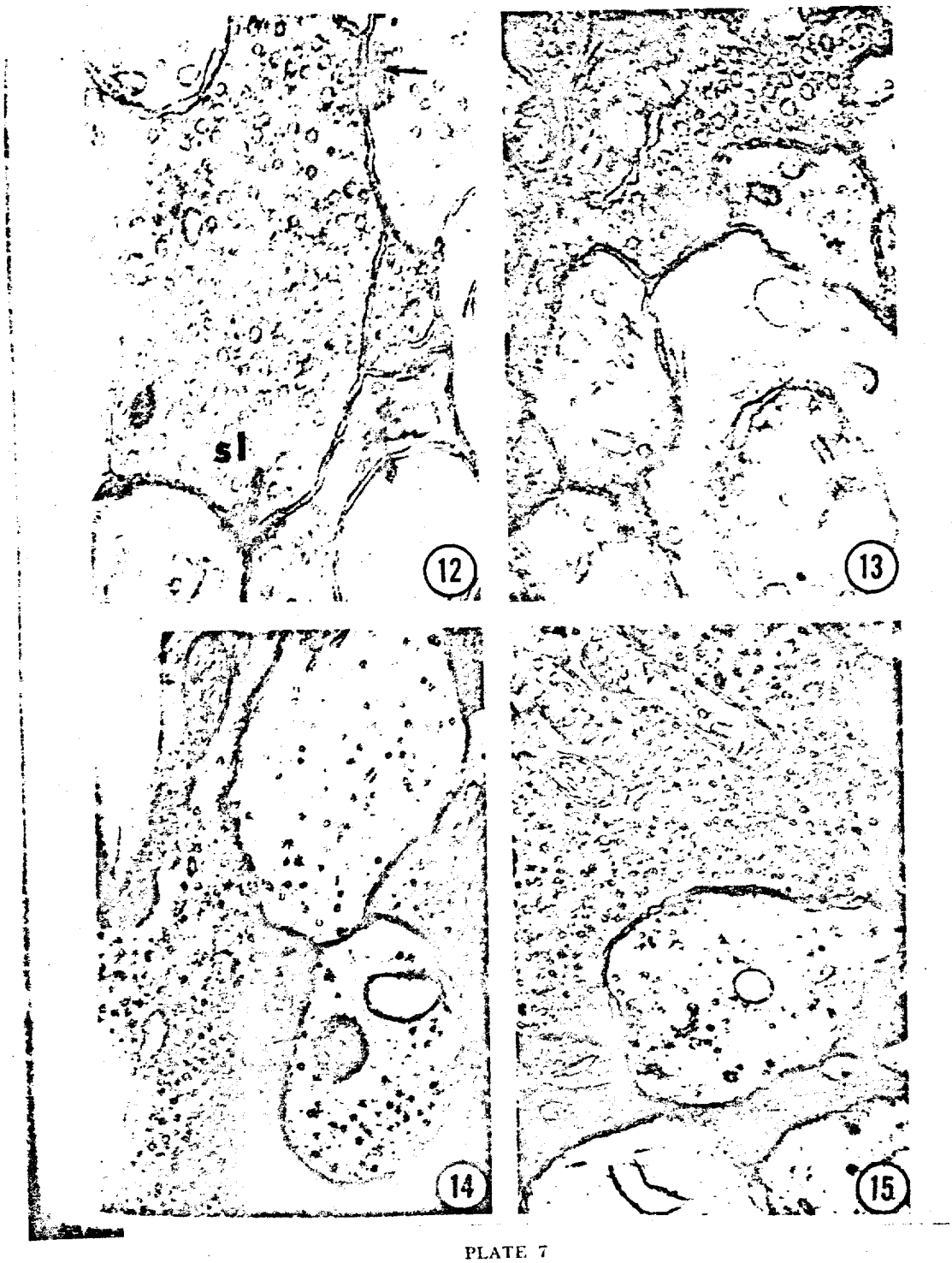


PLATE 7

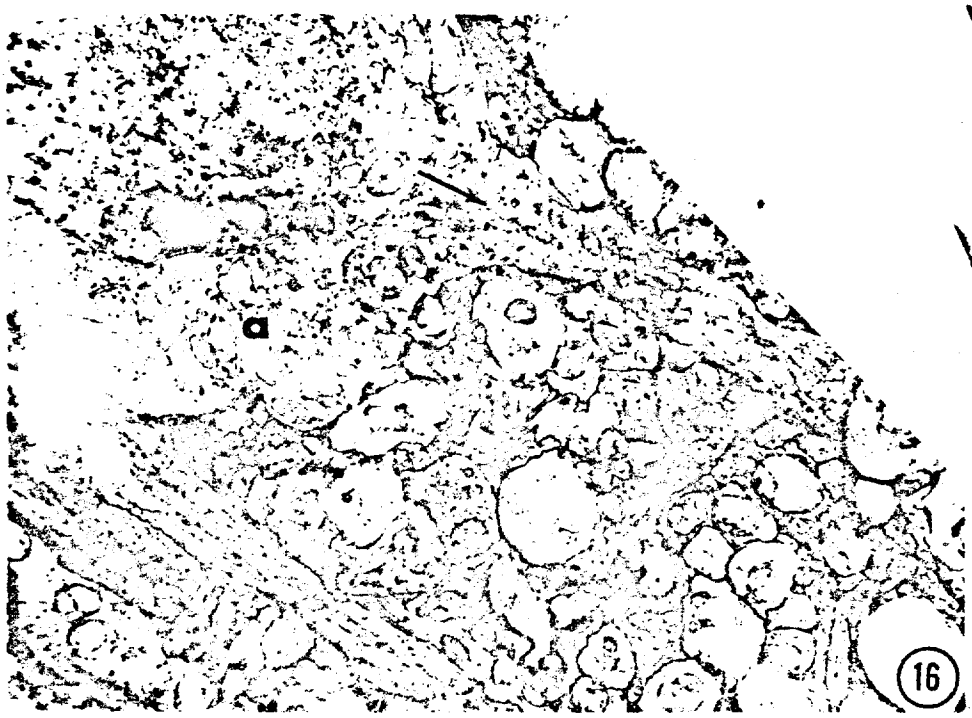
EXPLANATION OF FIGURES

12 A common type of dyad contact in the inner plexiform layer from a control retina. The photograph exhibits the pentalaminate structure of the synaptic lamella (sl), the density of the opposed membranes of the dyad contacts, and vesicles in one of these contacts. A presumptive synapse of a conventional type is also seen (arrow). × 60.000.

13 An infrequent variety of dyad contact from a control retina showing only sparse vesicular profiles in either dyad process. × 62.000.

14 A dyad contact in the inner plexiform layer of a control retina showing vesicles in both contiguous neurites. While vesicles in both dyad contacts were often seen, it was unusual to have both neurites so similar in appearance. × 33.600.

15 A dyad contact in the inner plexiform area of a glutamate-treated Swiss mouse showing dyad contacts differing in appearance but both containing vesicular profiles. × 33.000.



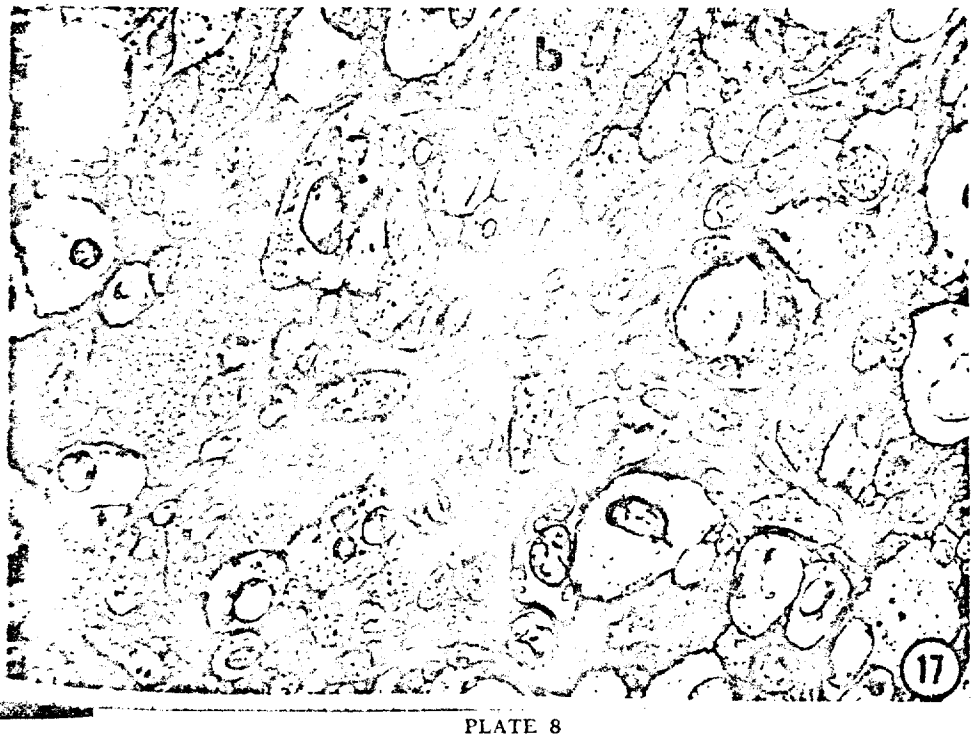


PLATE 8

EXPLANATION OF FIGURES

16 An electron micrograph of the inner aspect of the inner nuclear layer of a control retina showing a presumptive amacrine neuron (a) sending a process (arrow) into the inner plexiform layer. × 10,600.

17 An electron micrograph of a section of a bipolar bag in the inner plexiform layer of a control retina showing two enlarged bag areas (b, b′) connected by a narrower process. This illustrates the irregularity of the bipolar terminus. Also note vesicle containing endings on the bags, with the contacts exhibiting membrane density. × 28,500.

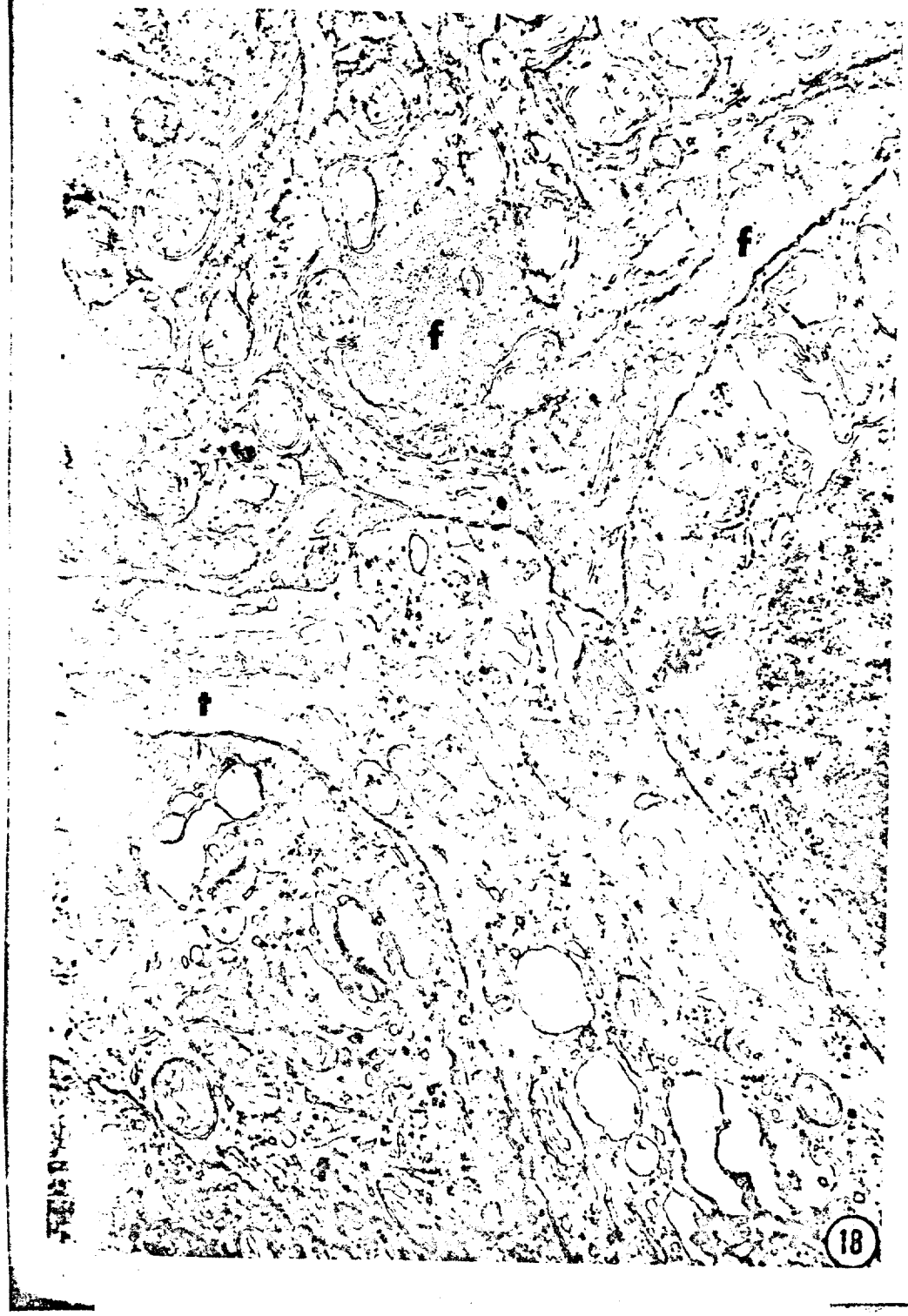


PLATE 9

EXPLANATION OF FIGURE

18 An electron micrograph of the outer aspect of the inner nuclear layer from the retina of a glutamate-treated Swiss mouse illustrating the general cytoplasmic similarity of the neurons in this region. The cytoplasm is rich in ribosomes and mitochondria and often shows tubular elements (t). Fibril containing cytoplasm (f) of glial cells of Müller is also seen. × 21,600.

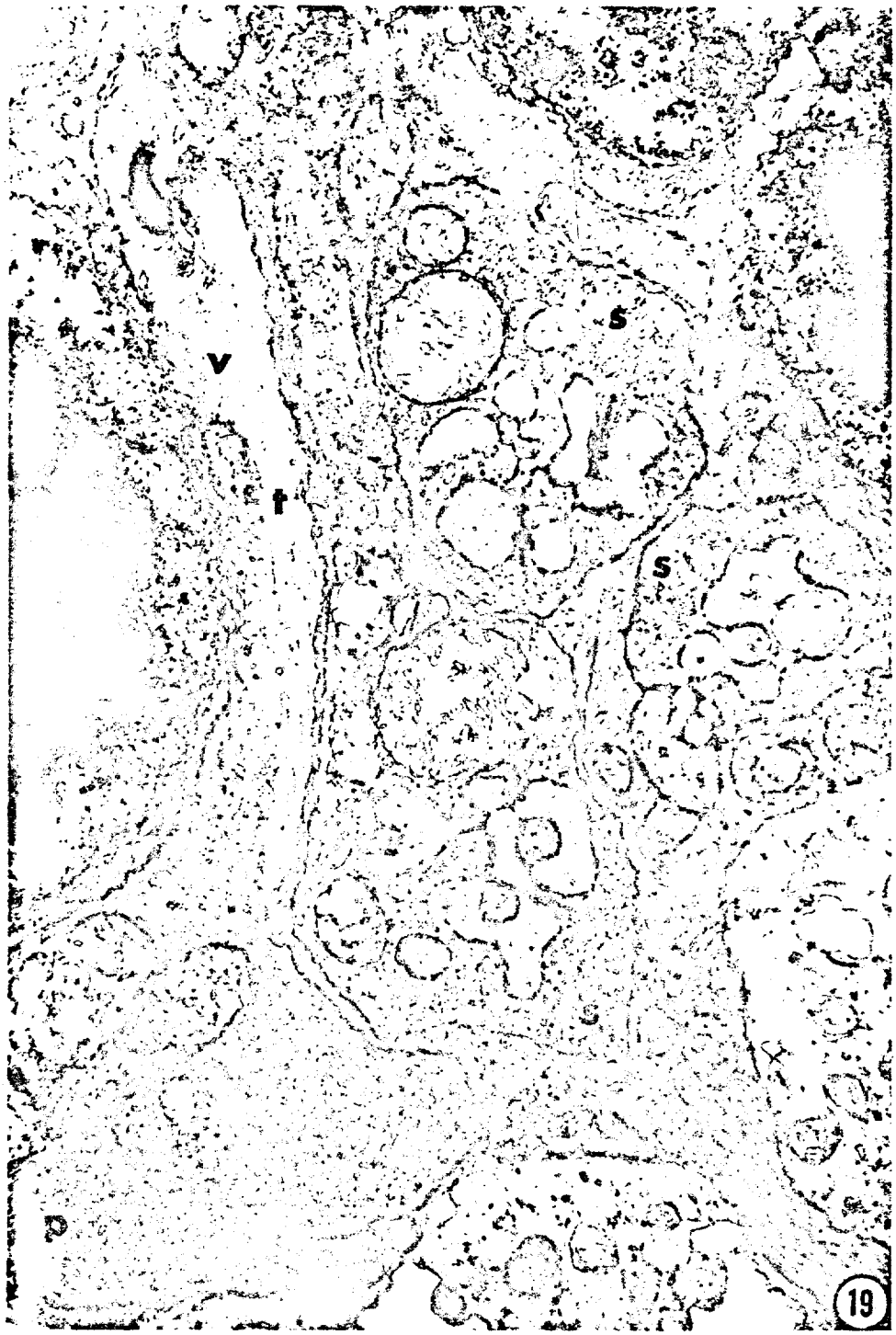


PLATE 10

EXPLANATION OF FIGURE

19 An electron micrograph of a control retina illustrating receptor spherules (s) and a portion of a pedicle (p). Note the neurotubules (t) and vesicles (v) in the pedicle stalk or axon. × 26,200.

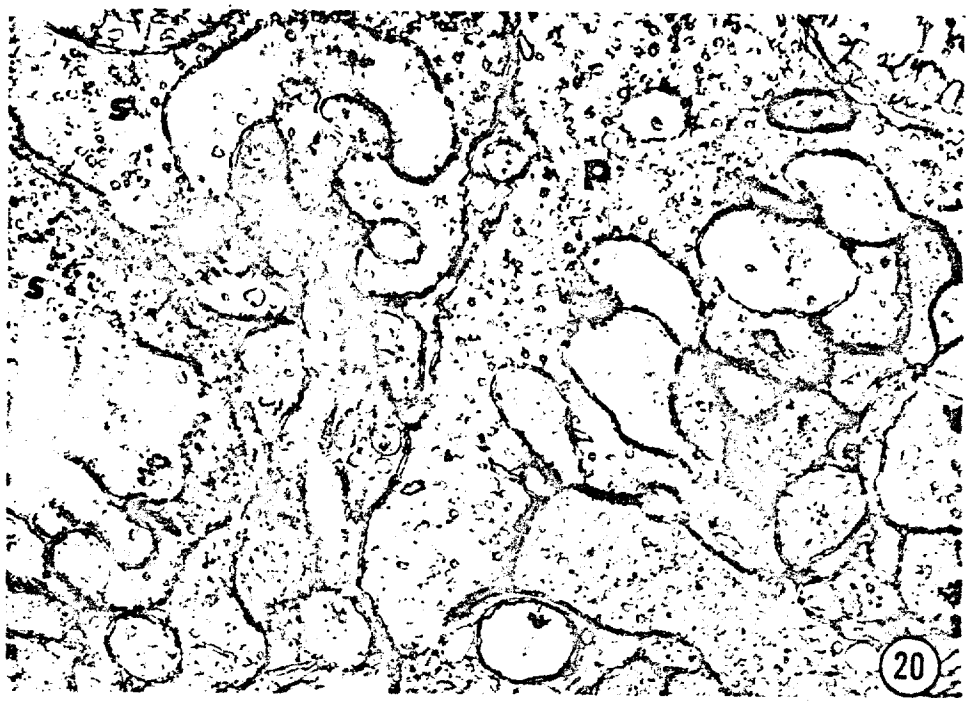


PLATE 11

EXPLANATION OF FIGURES

20 An electron micrograph of a control retina showing spherules (s) and a portion of a pedicle (p). The micrograph illustrates the typical situation that endings associated with synaptic lamellae in pedicles tend to lack vesicles while those associated with lamellae in spherules tend to possess them. Basal contacts on pedicles are rather numerous and possess vesicles and the contacting membrane are dense. × 34,500.

21 An electron micrograph of a control retina illustrating additional spherule features. At the arrow is an external contact containing vesicles. This type of contact was occasionally traced to processes from pedicles. Note the U-shaped arciform densities between the synaptic lamellae (sl) and adjacent cell membrane. × 33,400.

PLATE 12

EXPLANATION OF FIGURES

22 An electron micrograph of a glutamate-treated retina to illustrate the typical appearance of spherules. × 22,200.

23 An electron micrograph of glutamate-treated retina to illustrate the typical appearance of a pedicle. There was a suggestion of somewhat fewer vesicle containing contacts on the pedicle base. × 42,700.

24

25

PLATE 13

EXPLANATION OF FIGURES

24 A light micrograph of the retina of C3H/HeJ mouse at two months of age. The pigment epithelium (p) abuts against the external limiting membrane. One remaining row of receptor nuclei (rn) is evident and below this is a residue of the outer plexiform layer. The inner nuclear (in) and ganglion cell (g) layers appear entirely normal. × 550.

25 A light micrograph of a retina of a C3H HeJ mouse treated with glutamate. The pigment epithelium (p) faces a retina with two rows of nuclei and an inner plexiform layer. × 540.

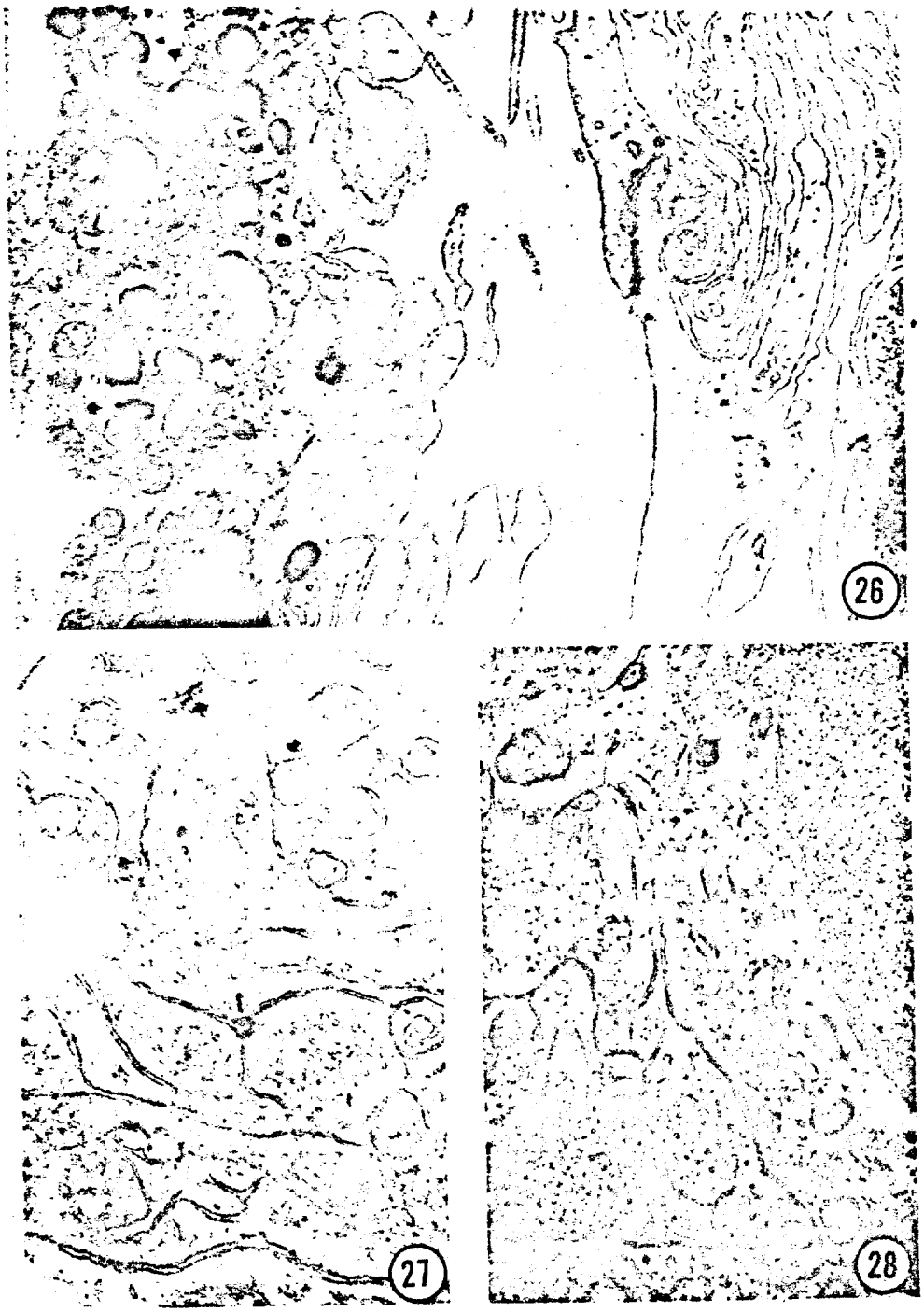


PLATE 14

EXPLANATION OF FIGURES

26 An electron micrograph of the apposition of the pigment epithelium and retina in a glutamate-treated C3H/HeJ mouse, showing the residual outer retinal surface consisting of imbricated processes of glial cells of Müller. × 15,500.

27 An electron micrograph of the retina of a glutamate-treated C3H/HeJ mouse. A dyad synapse is evidence of the survival of some bipolar neurons as well as other neurons. × 32,000.

28 An electron micrograph of the retina of a glutamate-treated C3H HeJ mouse. The cell soma illustrated (g) appears to be that of a ganglion cell as also evidenced by the sub-membranal cistern (c) thus far only observed in the mouse retina in ganglion cells. × 19,800.

Technical Data Sheet

FOOD CHEMICALS

1476 1476 c134

FC SERIES
TDS No. 3
(Revised August 1, 1969)

CSC/MSG®
Monosodium Glutamate

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COMMERCIAL SOLVENTS CORPORATION
245 PARK AVENUE, NEW YORK, N.Y. 10017

General Background

Although glutamic acid is widely distributed in foods, the activity of the monosodium salt as a flavor enhancer was unknown until 1909 when Ikeda[1] referred to it in a patent. Later, in 1912, he reported that a seaweed used in Japanese cookery yielded glutamic acid on hydrolysis and, when neutralized with soda, it possessed an agreeable flavor. About this time, ammonium glutamate was found in the Oriental soya sauce prepared by enzymatic hydrolysis of proteins, and it gradually became evident to investigators that glutamic acid salts were linked with a flavor boosting effect.

In 1910, active research was begun in Japan for methods of production of monosodium glutamate (MSG) and by the early 1920's it was in commercial production in both Japan and China. By the late 1920's, production had begun in the United States. MSG is now produced in quantity by several processes and it is widely used throughout the U.S. It has been found especially valuable for maintaining and enhancing the flavor of frozen and canned processed foods. The rapid growth of this segment of the food industry means continued demand for MSG.

Initial production of MSG in the U. S. was based on hydrolysis of protein by mineral acids, but in recent years this process has been largely replaced by fermentation processes. Commercial Solvents Corporation has recently perfected a new process for obtaining MSG by fermentation and with it a new, unique recovery process.

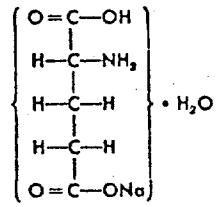
Fermentation is an old art with CSC. The company was founded in 1919 to make industrial chemicals by the fermentation of corn and its Rossville alcohols have long been familiar to manufacturers of extracts and vinegar. In 1936 the discovery of riboflavin (Vitamin B_2) in fermentation residues led to the development of a special process for mass production of this essential nutrient. In 1943 CSC became the first large scale commercial producer of penicillin and this antibiotic was followed by bacitracin and cycloserine. During the Korean War, a blood plasma extender—dextran—was produced by fermentation. With a background of over 40 years in fermentation chemistry, CSC recently expanded its production facilities to supply the ever growing demand for monosodium glutamate.

What is MSG?

Proteins are formed from various chemical building blocks known as amino acids. More than a score of these have been found in proteins where they are linked together in diverse ways. One of these amino acids is glutamic—a relatively simple compound easily utilized by the body. It is very widespread in nature, but usually in combined form. Glutamic acid possesses two acid groups and one alkaline one—the amino—which effectively neutralizes one acid group. The remaining acid group can be neutralized to give monosodium glutamate, and in fact must be neutralized to enhance flavor. MSG loses its flavor enhancing properties in acid solution below pH 4.

It is possible to neutralize both acid groups with sodium hydroxide in which case the product is known as disodium glutamate. However, neither this product nor free glutamic acid has flavor enhancing properties. MSG itself when pure has little flavor of its own; it is usually described as slightly salty and sweet.

Chemically, MSG has the following structure:



It should be noted that glutamic acid (and MSG) can exist in two structural forms which are mirror images of each other and are known to chemists as "stereo-isomers." Only one has flavor enhancing properties, however. Users of CSC's product need not be concerned with this fact, because our fermentation process yields only the flavor-active form. Thus there is no possibility of receiving a low-activity shipment when CSC is the supplier.

CSC/MSG crystals are carefully screened so as to provide Large Crystals, Medium Crystals, and Small Crystals for the variety of applications in the food industry. These crystals of monosodium L-glutamate monohydrate or aqueous solutions thereof are stable at ordinary temperatures. The dry material loses water of crystallization when heated above 100°C.

How does MSG Enhance Flavor?

Flavor is a subjective quality and is not measurable outside the human body. The exact biochemical or physiological mechanism by which we detect flavors is largely unknown.

Taken by itself, pure MSG is slightly sweet and slightly salty but nearly tasteless. Most people report[2] a feeling of stimulation of the tongue and

oral tissues which suggests that it may act directly on the taste buds. However, experiments conducted by Pilgrim et al[10] failed to show such an effect. Thus its action is still poorly defined. Its ability to prevent deterioration of both color and flavor suggests a preservative action separate from flavor enhancement. Some investigators have ascribed this to an anti-oxidant effect, but Neill and Page[9] reported that their data indicated pro-oxidant activity while preserving palatability. At any rate it does promote salivation and numerous taste panels of trained and untrained individuals have proved repeatedly and conclusively that MSG has a significant flavor enhancement in proteinaceous as well as certain other foods.

In addition to outright enhancement of flavor, MSG promotes a blending of the seasoning used with food so that no one flavor predominates. It also suppresses some unpleasant notes sometimes found in foods, notably metallic flavor in spinach, tinny flavor in shelf-aged tomato juice, fishiness in lima beans, and earthiness in potatoes.

As a general rule, all protein foods benefit from MSG, with the possible exception of eggs. Manufacturers of condensed and dehydrated soup were quick to recognize the value of MSG and have long been among the largest users. Other foods where it is useful include meats, poultry, seafood, and many vegetables. Sharp cheese, such as cheddar, benefits from it. Fruits and fruit juices, candy, baked goods, milk and butter are but little affected by MSG, so its use is not helpful. In acidic foods and beverages (such as fruits) MSG loses its flavor enhancement properties below a pH of 4.

Processed and Frozen Foods

The development of processed frozen food has been one of the great technological achievements of recent years. While consumer acceptance is due in part to convenience, quality at reasonable cost is especially important for repeated purchases. MSG has proved a real boon in maintaining quality of flavor in such preparations. The amount used - which is small per package unit - can be carefully controlled so that the costs are low and the improvements large.

In general, MSG can be said to show three advantages in pre-cooked frozen foods:

1. Prevents development of off-flavors during storage.
2. Retains color and freshness of products treated.
3. Enhances original natural flavor.

MSG is classified as "Generally Recognized as Safe" (GRAS) by the Food and Drug Administration and may be used freely in accord with good manufacturing practice[11] in canned, frozen, and processed foods provided it is included in the ingredient statement. MSG is cleared by the Meat Inspection Division of USDA to flavor various meat products, in amounts sufficient for the purpose. Ask your CSC sales representative for additional information or suggested formulations.

Canned or Frozen Vegetables

Many vegetables in their natural state contain glutamic acid but, since it is highly soluble in water, it is easily leached during cooking.[1] Processing of vegetables unavoidably extracts water-soluble components during washing, blanching, cooking, and cooling operations. Such losses have been reported[2] to be as much as one-half the amounts originally present. Fortification with added vitamins is common procedure in many processed foods, and it is only logical to fortify flavor components similarly. Replacement of MSG during the canning or freezing process is therefore recommended to preserve flavor.

While processors of frozen vegetables have been in the forefront in the use of MSG in their products, the same beneficial effects may be obtained when CSC/MSG is incorporated in canned vegetables. The economic cost of CSC/MSG should encourage its use by all vegetable packers.

In many foods CSC/MSG is used at levels of 0.1-0.2% by weight, but more than this amount is not harmful and may be advantageous. The following table shows the amount of CSC/MSG which should be added per 100 lb. of vegetable.

Asparagus	3.25 oz.
Beets	2.5
Broccoli	2.5
Brussels sprouts	2.5
Carrots	4.0
Cauliflower	2.5
Corn - creamed style	2.5
Corn - whole kernel	3.5
Green beans	3.25
Lima beans	3.0
Mixed vegetables	3.25
Mushrooms	3.5
Peas	4.0
Potatoes	2.0
Sauerkraut	2.0
Spinach	3.5
Squash	2.5
Tomatoes	2.5
Tomato juice	3.5
Wax beans	2.5

MSG confers another important quality when used in frozen foods: It protects color. Color is well-known to possess psychological values in

foods (many housewives plan their meals around a color scheme), and pale, off-color foods will often be rejected. It is especially noteworthy, therefore, that use of MSG protects the brilliant green color of peas, broccoli, green beans, and spinach[9] and the natural color of meats, seafood, and poultry.

Fresh, Canned, and Frozen Meats

FRESH MEAT

Fresh cuts of beef, pork, veal, and lamb are greatly improved by the use of MSG. This is especially true of the poorer quality cuts which often do not possess as good a flavor as the choice cuts. Use of CSC/MSG by the meat packer on these cuts of meat will insure consumer acceptance. It should be dusted on the meat at the rate of 2.5 oz. per 100 lb.

STEAKS, CHOPS, AND ROASTS

It is a common experience that quality meat cuts deteriorate in flavor when frozen and stored for any length of time. CSC/MSG dusted on the meat at a rate of 2.5 oz. per 100 lb. before freezing will largely prevent this loss of flavor. In order to maintain customer acceptance, it is very important for the packer of frozen meat to protect his product by consistent use of MSG. Cube steaks dusted with CSC/MSG after being cubed are kept in better condition during frozen storage. They are reported to be juicier and more tender than untreated steaks.[9]

GROUND MEATS

Hamburger, pork sausage, and veal patties all benefit from CSC/MSG and the longer the storage the more important it is to use MSG.

HAMBURGER - Panel tests on hamburger containing 2.5 oz. MSG per 100 lb. showed in only one-month storage at 0°F that MSG is important for protection of flavor, for the treated hamburger was superior in flavor to the control. During the succeeding months the flavor of the untreated hamburger declined seriously, while that containing MSG was much superior.[9] CSC/MSG is very easy to add to hamburger; it may be dusted on the meat before grinding or added after the first grind if subsequent grinding and mixing are to be done.

CANNED HAMBURGER - Taste experts reported strong preference for hamburger canned with MSG just as similar panels have voted for fresh hamburger with MSG. CSC/MSG should be added to the hamburger at the time of the grinding process at a minimum level of 2.5 oz. per 100 lb.

FROZEN PORK SAUSAGE - CSC/MSG is an essential ingredient in pork sausage. Untreated sausage develops a flat flavor within 3 months and by 7 months it will have lost its natural color, become rancid and in general be in poor condition. However, the addition to the seasoning mix of 2.5 oz. CSC/MSG per 100 lb. of pork greatly reduces loss of color and protects the flavor. At 5 months storage the flavor was still good while the untreated sample was poor and already showing rancidity.[9] Tests on both cooked and raw, seasoned and unseasoned ground pork have been reported by several other investigators.[9,5] They also found superior results with MSG as compared with controls or when compared with antioxidants such as butylated hydroxyanisole.

SAUSAGE - The flavor improvement obtained with MSG on fresh ground pork sausage is similar to that observed with frozen sausage. On one taste panel 87% preference for the MSG-treated sausage was reported. MSG has been described as improving the blend of flavors, reducing saltiness, and augmenting sweetness. Since a variety of spices are usually used in sausages, the MSG has the effect of blending them so that no one spice predominates. MSG levels as high as 6.5 oz. per 100 lb. are recommended.

LIVER SAUSAGE AND BOLOGNA - These processed meats also benefit from the inclusion of CSC/MSG at a concentration of 2.5 oz. per 100 lb.

VEAL PATTY - The Quartermaster Food and Container Institute conducted a study - which is now regarded as something of a classic - on precooked ground veal patties for satisfactory storage qualities. They employed only 0.8 oz. MSG per 100 lb. with storage temperatures of 0°F. The untreated veal declined in flavor, and had a dry appearance with "old" flavor. Veal treated with MSG held up much better and remained moist in flavor, with no stale or off-flavors.

HAM

Whether frozen or not, hams treated with 2.5 oz. of CSC/MSG per 100 lb. have superior flavor. This was conclusively demonstrated by investigators who compared hams from the same carcass. The MSG was applied in solution in the pumping pickle. Treated ham held at 0° F for as long as 14 months was still in satisfactory condition, while the untreated ham was showing a deterioration in flavor and color at 9 months and was rancid at 14 months.[9]

Taste experts reported reduced saltiness, augmented sweet and smoke taste as well as gener-

ally improved flavor. The ham should be so treated that at least 2.5 oz. of CSC/MSG (0.15%) will be present per 100 lb. of ham. For short-cured (1-3 days in the cooler after pumping) hams, similar results can be obtained with 0.1% MSG in the finished product. The following table shows the amount of CSC/MSG to use per gallon of pumping pickle to obtain various levels of MSG in the ham.

Pumping Schedule	Level of CSC/MSG in Ham 0.1%	0.15%	0.2%
5%	2.8 oz.	4.3 oz.	5.8 oz.
10%	1.4 oz.	2.2 oz.	2.8 oz.
20%	0.7 oz.	1.0 oz.	1.4 oz.

BACON

In tests on paired bacons at 0°F, MSG was found to increase the storage life considerably.[9] It was added to the salt mixture at a rate of 1.17 lb. per 24.41 lb. of salt cure mixture, and this was used for curing 615 lb. of pork bellies. At the end of 7 months, the untreated bacon was off-flavor and slightly rancid, while that containing MSG was still good. Taste experts have reported that bacon cured with MSG has a smoother, better flavor; augmented sweetness and smoke; and improved mildness of flavor.

The quantities as given in the above table for ham are also applicable for curing bacon. In addition, bacon can be dry-cured using 1 lb. of CSC/MSG per 35-40 lb. of salt.

FRESH AND FROZEN POULTRY AND POULTRY DISHES

MSG is highly recommended to processors of poultry to prevent deterioration of flavor during processing and transportation to market. Young broilers in particular have a delicate flavor and the use of CSC/MSG will not only protect this flavor from deterioration but also will greatly enhance it at the table.

MSG will not penetrate the skin of chicken so it is preferably dusted into the cavity. If poultry is to be held in a cold room, the MSG should be applied while the bird is still warm and before cooling. However, if cooling is to be done by an ice slush bath the MSG should be added after removal from the bath, although as an alternate suggestion the MSG can be dissolved in the cooling bath where it will be rapidly absorbed by the poultry.

Frozen chicken and turkey containing MSG is greatly superior in flavor after storage compared to untreated birds. MSG does not penetrate the skin of poultry so they should be treated on the inside of the cavity by hand-dusting or under pressure from an air line. Broilers and chicken parts can be treated by dusting the parts after the birds are cut up if desired. Absorption of glutamate through the cavity, however, is comparatively rapid so that it can be applied to the cavity before the birds are cut up. The recommended level is 2.5 oz. CSC/MSG per 100 lb. of chicken or turkey.

MSG is essential for holding the flavor of turkey and chicken pies, and chicken a la king for any length of time at low temperature storage. CSC/MSG should be added to the mix at the same time as the salt at a level of 2.5 oz. of MSG per 100 lb. of mix.

CANNED POULTRY DISHES

Canned chicken and turkey as well as prepared foods made from them, such as fricassee, and turkey or chicken a la king, possess much better flavor with MSG than similar preparations without it. A number of different levels have been tested, some as high as 12 oz. per 100 lb. of boned chicken.

Two to three ounces of MSG per 100 lb. of chicken or turkey is probably sufficient. Slightly more should be employed for prepared chicken dishes, such as fricassee or chicken a la king.

SEAFOOD

Seafood has a very delicate flavor, so any development of off-flavor of fresh, frozen or canned seafood or prepared seafood dishes is very objectionable. Ascorbic acid has been used in the past to preserve the flavor of seafood, but many experts rate MSG superior to ascorbic acid. MSG has therefore come into widespread use.

At 0.2-0.25%, MSG is reported[15] to have a definite beneficial effect on the odor and taste of canned tuna during storage for one year at 75°F.

CSC/MSG may be applied directly to the fish as a powder or spray. Both sides should be treated, and if the fish is to be frozen, application should be made 10-15 minutes beforehand. The following table shows the recommended amounts:

Fish, fresh, or for canning or freezing	2.5 oz. per 100 lb.
Fillets and steaks	2.5
Shellfish (canned or frozen), shrimp, scallops, oysters, crab, lobster	2.5
Sardines in tomato paste	3.5
in oil or mustard sauce	2.0
Salmon, fresh, or for canning or freezing	3.0
Tuna	2.0
Tuna pies, frozen	2.5
Fish pastes, canned	4.0
Breaded seafood, frozen (add the MSG to the wet wash)	2.5
Clam juice cocktails or broth	3.0
Fish and crab cakes	3.0
Seafood bisque	3.5
Seafood salads	5.0

Canned Meat Dishes

CORNED BEEF HASH

Several different investigators have studied the effect of various levels of MSG in canned corned beef hash. Taste panels repeatedly show overwhelming preference for hash containing 2.5 oz. of MSG per 100 lb. Experts have described its effect as suppressing onion, salt, starchiness and tinny flavors. It also contributes to the over-all blended flavor effect of the spices and pepper. It should be added at the time the hash is mixed.

MEAT AND GRAVY

Studies at the Quartermaster Food and Container Institute[3] showed that canned meat and gravy, namely, pork and beef respectively, containing MSG was the overwhelming choice of a panel of taste experts. A level of 4 oz. MSG per 100 lb. was employed in these tests. In products such as this CSC/MSG should be added by the canner at the same time as the salt and for convenience it may be mixed with the salt in the proper proportion.

BEEF STEW

Use of MSG in canned beef stew was reported to reduce "tin-can" taste and, in the words of the experts, "to increase sweet and meat notes." It should be added with the salt before cooking at a rate of 2.5 oz. per 100 lb.

TAMALES AND ENCHILADAS

In a large taste panel test on canned tamales 70% preference was shown for the product containing MSG. The concentration used was 2.5 oz. per 100 lb. in the gravy and 4.0 oz. per 100 lb. of corn. Similarly, a large preference was shown for MSG-treated canned enchiladas containing 2.5 oz. MSG per 100 lb.

DEVILED HAM AND DEVILED TONGUE

Canned deviled ham was much preferred by a test panel when it contained 3.0 oz. of MSG per 100 lb. Similarly, 8.0 oz. of MSG per 100 lb. in canned deviled tongue was preferred to the untreated material.

LIVER SPREAD

Liver spread containing 3.5 oz. of MSG per 100 lb. was found to have improved flavor. The MSG reduced the liver taste and generally resulted in a less crude taste.

MEAT BALLS

Meat balls containing 3.5 oz. of MSG per 100 lb. were greatly preferred by a large consumer panel compared with untreated samples. The MSG should be added by the processor to the meat ball mix before cooking.

Miscellaneous Foods

DEHYDRATED AND CANNED SOUPS

The commercial success of dehydrated soups is due largely to the flavor-enhancing properties of MSG. Fairly high concentrations of CSC/MSG are used in these formulations. The actual amount will vary somewhat depending on the type of soup and the seasonings used. For example, one recipe for dehydrated chicken soup uses over 12 lb. of MSG per 100 lb. of mix.

Dehydrated vegetable soups usually require somewhat less MSG for flavor enhancement than do those containing meat. Formulations for a number of specific types of dehydrated soups containing CSC/MSG are available upon request.

Canned soups and chowders also benefit greatly from the use of CSC/MSG. Most concentrated canned soups require at least 1.5 oz. per 10 gallons, while ready-to-serve canned soups require about twice that much. Freshly prepared soups also are greatly improved in flavor by use of CSC/MSG.

FREEZE-DRIED RATION FOODS

Precooked dishes which are freeze-dried after formulation have been developed[16] as tasty, palatable rations for the Armed Forces. Products such as beef hash or spaghetti incorporate 0.3% MSG in the seasoning mix, and chicken stew or chili con carne use 0.1% in the seasoning.

SALAD DRESSINGS

CSC/MSG in salad dressings brings out the piquant flavors of the seasonings and heightens the palatability of the salads. Its tendency to suppress off-flavors is especially valuable because highest quality greens are sometimes unavailable for salads.

Mayonnaise usually is prepared as an emulsion of eggs, oil, vinegar, and salt. CSC/MSG is best added at the same time as the salt, and to the extent of about 0.15% of the weight of the finished mayonnaise. No change in the other ingredients or processing conditions is necessitated, and the keeping quality of the product is not impaired because there is no change in pH. However, there is a noticeable smoothing of the acid or astringent tones in dishes prepared from mayonnaise containing CSC/MSG.

CSC/MSG should be added to French and similar types of dressing at the rate of 1 oz. per 5 gallons of finished dressing. Packages of dry mixes for salad dressings should use sufficient CSC/MSG to provide for this same concentration in the final mix.

ROASTED NUTS

The Small Crystals of CSC/MSG are particularly recommended for enhancing the flavor of dry-roasted peanuts and other nuts. Because of the strong adherence of the Small Crystals to the surface of food particles, CSC/MSG is less likely to be jostled loose during handling and shipping and settle to the bottom of the container.

The wet nuts should be coated completely with a mixture composed of:

CSC/MSG Small Crystals	1 part
Corn starch (low density)	3 parts
Flour salt	6 parts
Spice as preferred	

After drying in forced hot air at 425°F, roasting is carried out at 425°F in still air with intermittent tumbling until the nuts have attained the desired color.

POPCORN AND POTATO CHIPS

Cocktail snacks made with CSC/MSG are more flavorful and stimulating. MSG should also be incorporated in cheese dips and spreads. Typical usage is:

Popcorn	1 lb. per 25 lb. salt
Potato chips	1 lb. per 10 lb. salt
Crackers, all kinds	1.5 oz. per 100 lb.
Pickles	1 oz. per 100 lb.

CHEESE DISHES

All canned or frozen foods using cheese require CSC/MSG for full flavor. The amounts to use will vary considerably, but the following can be used for a guide.

Cheese sauce (such as Welsh rarebit)	1.5 oz. per 20 gal.
Macaroni and cheese	1.0 oz. per 40 lb.
Potatoes au gratin	2-3 oz. per 100 lb.
Grated cheese, Romano or Parmesan	1-2 oz. per 100 lb.

Physical Properties

Molecular weight $C_5H_8NO_4Na{\cdot}H_2O$	187.13
Sodium content, calc.	12.3% by wt.
Solubility in water at 77°F	42.5% by wt.
at 142°F	>50% by wt.
pH of 5% aqueous solution	approx. 7.0

Non-hygroscopic and free-flowing white crystals:
- Large Crystals, predominantly 30-80 mesh
- Medium Crystals, predominantly 60-200 mesh
- Small Crystals, majority through 200 mesh

CSC Specifications

Purity, min.	99% by wt.
Moisture, max.	0.2% by wt.
Chlorides, as NaCl, max.	0.3% by wt.

Shipping Containers

	Net weight, lb.
Polyethylene-lined fiber drums	100
Four-ply multiwall paper bags with moisture barrier	50

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The information and data contained herein are believed to be correct. However, we do not warrant either expressly or by implication the accuracy thereof. In presenting uses for this product, no attempt has been made to investigate or discuss any patent situations which may be involved.

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8-69

Separation of Acidic Amino-acids by means of a Synthetic Anion Exchange Resin

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Synthetic ion-exchange resins have recently been widely used for the separation of basic and acidic from neutral compounds. Thus Cannan (1944), by repeated applications of Amberlite IR4, separated glutamic and aspartic acids quantitatively from protein hydrolysates. Earlier, Freudenberg, Walch & Molter (1942) had used a column of Wolfatite M for the separation of the dicarboxylic amino-acids, and Cleaver, Hardy & Cassidy (1945) investigated the chromatographic separation of amino-acids by organic exchange resins. Recently, Tiselius, Drake & Hagdahl (1947) have employed synthetic ion exchange resins chromatographically for the quantitative separation of amino-acids into groups. The aim of the present investigation was to extend such methods to cover the separation of the acidic amino-acids from one another (at pH 2·5) after their isolation from the rest of the protein hydrolysate (at pH 3–4). Although completely quantitative recoveries were not obtained, complete separations have been achieved. The separation depends on differences in net charge between the acidic amino-acids at a given pH (Fig. 1). Thus, glutamic acid with pK_1 2·16 and pK_2 4·32 (Neuberger, 1936) has a net charge of −0·31 at pH 4; aspartic acid with pK_1 1·88 and pK_2 3·65 (Miyamoto & Schmidt, 1931) has a net charge of −0·69 at pH 4; whilst cysteic acid with pK_1 1·88 and pK_2 1·30 (Andrews & Schmidt, 1927)

has a net charge of −0·99 at this pH. It would therefore be expected that a column of Amberlite IR4 maintained uniformly at pH 4, because of its positive charge, will preferentially retain cysteic, aspartic and glutamic acids in this order. Theoretically, it would appear that the optimum pH for the separation of glutamic from aspartic acid is about 4, since at this pH the largest difference in net charge occurs, but experimentally it was found that for satisfactory separation a pH of 2·5 was necessary. At this pH glutamic and aspartic acids normally carry a net positive charge of +0·29 and +0·13 respectively. Nevertheless, at this pH they are retained on Amberlite IR4 columns and can be separated by elution in relatively small volumes; at higher pH values, although separation will take place, relatively large volumes are required. This suggests that the pK values are depressed when these amino-acids are adsorbed on Amberlite IR4. Similarly, cysteic acid is partially retained at pH 1·5, although its net charge at this pH should be almost zero.

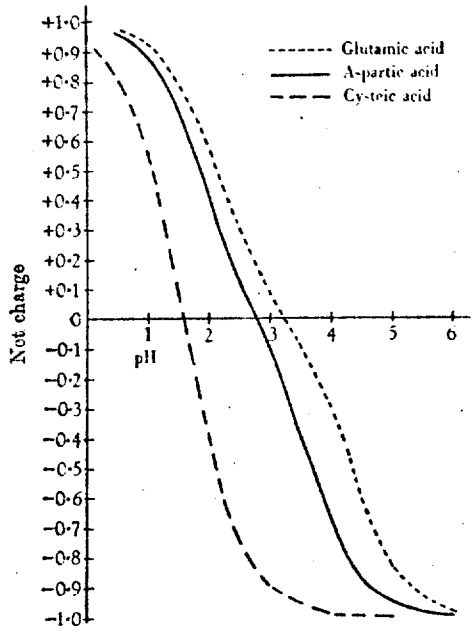

Fig. 1. Net charge of glutamic, aspartic and cysteic acids at different pH values, calculated from the dissociation constants given in the text.

To apply these principles, care has been taken to bring the Amberlite to the required pH and to maintain it uniformly in that part of the column where separations are taking place. In order to establish equilibrium conditions, the resin was finely powdered and the width of the column kept small in relation to its height so that low rates of flow were maintained. In the first stage, no difficulty was found in effecting the separation of the acidic from the basic and neutral amino-acids in a protein hydrolysate at pH 3–4. As is shown by its titration curve (Fig. 2), at this rather acid pH, the percentage charge on the resin is sufficiently high for it to compete successfully with lysine and arginine for the acidic amino-acids. The departure from the expected sigmoid shape of the curve below pH 1 may be due to decomposition of the resin. This is also suggested by the fact that the nitrogen (Kjeldahl) washed from a column by acid is about ten times as high for strong acid (pH 0–1) as for weak acid (pH 3–4) and is independent of the history (previous washings) of the column. By the above means and by the application of the chromatographic principle, considerable economy of resin was achieved. Separations have been followed by partition chromatography on paper (Consden, Gordon & Martin, 1944) of samples from each eluate; this method has provided a convenient means for identifying the amino-acids present in each fraction, without using more than negligible quantities.

The copper titration method of Pope & Stevens (1939) was chosen as a convenient means of estimating the acidic amino-acids after separation by the resin and was adapted to deal with micro quantities. Unfortunately, it was not found possible to obtain fully quantitative recoveries of glutamic and aspartic acids after their separation from synthetic mixtures. The recovery of glutamic acid was about 91 %, that for aspartic about 96 %. A possible cause for low recoveries may be formaldehyde which appears to be continually liberated even after the washing cycle employed by Cannan (1944). Attempted removal of formaldehyde failed to improve the results. Estimation of amino N or total N in the eluates by the Van Slyke manometric procedure or Kjeldahl method was tried and abandoned owing to the high blanks given by eluates washed off the column after the removal of amino-acids. Cysteic acid, as isolated from hydrolysates by means of Amberlite, is always accompanied by pigment having a similar acid strength. As similar pigment material from hydrolysates of wool not containing cysteic acid gives significant titrations in the Pope & Stevens (1939) procedure, estimations of cysteic acid would presumably give fictitiously high results.

As the synthetic ion exchange resins can absorb relatively large amounts of amino-acids (Englis & Fiess, 1944; Cleaver *et al.* 1945), they are suitable for isolations. Thus, cysteic acid has been isolated from a hydrolysate of chlorinated wool. This material was almost fully optically active, whereas that obtained by Lissizin (1928) from hair oxidized by alkaline permanganate was inactive.

METHODS

*Preparation of the Amberlite IR4.** The resin is alternately ground wet in a ball mill and sieved with a jet of water, −90 +200 grading being used for the columns. It is washed free from NH_3, treated with HCl until the supernatant liquor is permanently acid to thymol blue (pH <1) and then washed with water. Alternate treatments with acid and water are repeated a number of times.

Preparation of columns. (i) The resin is stirred and washed by decantation with water until the supernatant liquid is less than 0·001 N with respect to alkali. The slurry is transferred to a glass tube of 0·5 cm. internal diameter, forming a column 20 cm. in height (containing about 2 g. air-dry resin), and washed overnight with water at a rate of approx. 15 ml./hr. This does not significantly raise the pH of the eluate, the acid concentration of which should be checked by titration before the next stage. (ii) The second column (height 30 cm.) is prepared from about 3 g. Amberlite and the pH is adjusted accurately to 2·5 by equilibration with dilute acid or water until the acidity of the supernatant fluid is slightly above 0·003 N. The material is then transferred to a 0·5 cm. diameter tube and washed overnight with 0·003 N-HCl. The acidity of the eluate is finally checked by titration. The columns should not be allowed to run dry and remain so for any length of time; otherwise extensive cracking may occur.

Procedure for estimation of glutamic and aspartic acids. The hydrolysate prepared with HCl and containing up to 15 mg. acidic amino-acids is repeatedly evaporated *in vacuo* by rotating manually in a warm water bath, allowed to stand overnight *in vacuo* over KOH, and quantitatively transferred in a small volume to the first column. Water is then passed down the column; the neutral and basic amino-acids are eluted in the first 25 ml. The next 5 ml. should be free from amino-acids. To test the eluates, a portion of the first 25 ml. and the whole of the second 5 ml. fraction are separately concentrated *in vacuo* to very small bulk and samples of each tested by partition chromatography on a strip of Whatman no. 4 paper using phenol-NH_3, developing for 5 hr. or more, with suitable control (cf. Consden *et al.* 1944). After development and treatment with ninhydrin, basic and neutral, but no acidic amino-acids, are found in the first eluate, whilst the second eluate should contain no amino-acids. If cystine is present, the eluate sample is oxidized by treatment with excess Br_2 water before chromatography; the cysteic acid thus produced occupies a position on the chromatogram not overlapping the positions of aspartic and glutamic acids (Consden, Gordon & Martin, 1946) if these are present.

The next stage consists in transferring the material remaining in the column to the second column. This may be done in either of two ways:

Method 1. The column is eluted with N-HCl causing the pigment to run down as a sharp band. When this has run out, the washing is stopped and the eluate repeatedly evaporated *in vacuo* and allowed to stand overnight *in vacuo* over KOH to remove excess HCl. It is then quantitatively transferred to the second column which is eluted with 0·003 N-HCl, 10 ml. eluates being collected. Each is concentrated *in vacuo* to a volume of 1–2 ml. and a sample (4 μl.) tested as described above.

* Obtained from the Resinous Products and Chemical Co., Pa.

Method 2. The column is washed with 3 ml. N-HCl, causing the pigment band to travel about half way down. (The eluate should be free from amino-acids as revealed by chromatography.) The column is then fixed to the top of the second column. Elution is continued with water, but the acid already added is sufficient to cause the pigment band to continue to travel down and eventually to pass into the lower column. When the pigment has travelled a few cm. into the second column, the top column is removed and elution of the lower column continued with 0·003 N-HCl. If no pigment is present, elution may be followed by the change in shade of the resin which is orange when strongly acid. Eluates are collected and tested as in method 1.

Method 1 has been found to behave more consistently than method 2; in proteins where the ratio of glutamic to aspartic acid is of the order 2 : 1, the glutamic acid is eluted from the second column in the first 20 ml., and aspartic acid in the next 30 ml., whereas in method 2 the stages at which glutamic and then aspartic acids begin to appear are rather less certain. Method 2 is quicker than method 1 but requires more attention.

Each eluate is evaporated as above *in vacuo* to dryness, and a known volume (5–10 ml.) of copper phosphate-buffer suspension (Pope & Stevens, 1939) is added to the crystalline residue. Titrations (20–50 μl. 0·1 N-$Na_2S_2O_3$) are carried out on 1 ml. portions after filtering and addition of the other reagents. Neutralization before addition of the copper phosphate is unnecessary.

Procedure for the isolation of cysteic acid. In order to assess the amount of protein from which a workable quantity of cysteic acid may be isolated, its amount in a hydrolysate is roughly estimated by partition chromatography on paper. A known volume (5–10 μl.) is applied to Whatman no. 4 paper and known amounts (0·5–20 μg.) of cysteic acid, each at the same volume as that of the hydrolysate, applied by the side of the hydrolysate spot. After development of the chromatogram in phenol-NH_3-coal gas for 17 hr., the paper is dried, coloured with ninhydrin and the depth of colour of the cysteic acid spot in the hydrolysate compared with those of the controls. The isolation may be conducted either (*a*) by eluting the column with N-HCl after separation of the dicarboxylic amino-acids by method 1 or 2, or (*b*) at pH 1·5. If (*a*) has been used, partition chromatography reveals that the whole of the cysteic acid runs out in the region of the pigment band, at which stage there is a sharp change in pH from 2·5 to <1. No complete separation of the pigment from cysteic acid was achieved by using the resin and eluting solution at a lower pH; however, at pH 1·5 all the amino-acids except cysteic acid are rapidly eluted, thus making possible its isolation on only one column. Thus, on a column, length 30 cm., diameter 0·5 cm., eluted with 0·03 N-HCl, the neutral, basic and dicarboxylic amino-acids accompanied by some pigment, run out in the first 25 ml. followed soon after by cysteic acid, the elution of which can be speeded up by N-HCl.

RESULTS

Recovery of glutamic and aspartic acids from a mixture of amino-acids

An amino-acid mixture simulating a wool hydrolysate (Astbury, 1942) was prepared containing 0·0517 mg. aspartic acid N (4·12 % of total N) and

Table 1. *Recoveries of glutamic and aspartic acids from a synthetic mixture*

		Experiment							
		(i)		(ii)		(iii)		(iv)	
	Amount (mg. N)	Found	Recovery (%)	Found	Recovery (%)	Found	Recovery (%)	Found	Recovery (%)
Glutamic acid	0·8416	0·767	91·3	0·773	92·0	0·776	92·3	0·747	88·8
Aspartic acid	0·4126	0·394	95·2	0·390	94·2	0·404	97·6	0·397	96·0

0·1052 mg. glutamic acid N (8·40 % of total N) ml. This solution (8 ml.), after evaporation *in vacuo*, was subjected to the foregoing procedure, using method 2. Recoveries of glutamic and aspartic acids are recorded in Table 1.

In experiments on glutamic and aspartic acids alone, similar recoveries were obtained. The odour of formaldehyde could usually be detected in the eluates from the columns after concentration; treatment of the eluates with alkali, HCl, H_2O_2, or dimedone failed to improve the results and treatment with strong NH_3 gave high results. Addition of formaldehyde to glutamic and aspartic acid solutions led to low titrations.

Isolation of cysteic acid

Degraded protein produced by the action of chlorine on wool (Consden *et al.* 1946), containing 156 mg. N and which by the paper chromatographic method described above was expected to contain approx. 4 mg. cysteic acid N, was hydrolyzed and prepared as already described for application to the resin. This material was transferred to a column (30 cm. high; 1·5 cm. diam.) which had been equilibrated to pH 1·5 and was then eluted with 0·03 N-HCl, the rate of flow through the column being about 60 ml./hr. Six eluates of 20 ml. each were collected and tested as described above. The neutral amino-acids plus glutamic and aspartic acids were eluted in the first three fractions. The fourth fraction contained much cysteic acid and subsequent fractions successively less. The column was eluted with a further 500 ml. 0·03 N-HCl and, as small amounts of cysteic acid were still being washed out, it was finally eluted with N-HCl which removed all the remaining cysteic acid and yellow material from the resin. The united fractions containing cysteic acid were concentrated *in vacuo* to small volume and the solution, after standing at 0°, deposited brownish crystals, which after filtration were decolorized by boiling with animal charcoal. The mixture was filtered, evaporated *in vacuo* to dryness and recrystallized three times from aqueous ethanol. The pale yellow powder (15 mg.) had $[\alpha]_D^{18°} + 7·1°$ (*l.* 2; *c* = 0·3) in water. Clarke & Inouye (1931) give $[\alpha]_D^{25·5°} + 9·4°$ for L-cysteic acid. Its equivalent by titration with $Ba(OH)_2$ was 185; calc. for $C_3H_7O_5N.H_2O$, 187; mixed partition chromatograms using phenol-NH_3-coal gas show no difference from authentic L-cysteic acid. Analysis of the copper salt gave Cu 25·78; calc. for $C_3H_7O_6NSCu$, Cu, 25·57 %.

Titration of Amberlite IR 4

The resin, which had been treated as described at the beginning of the experimental section, was brought to *c.* pH 10 by treatment with dilute NaOH. It was then thoroughly washed with water and left to dry at room temperature. A sample of the resin (202·8 mg.) on drying to constant weight at 105° lost 30·4 mg. Samples of air-dry resin (200 mg.) were mixed with varying quantities of HCl and each made up to a final volume of 20 ml. and allowed to equilibrate overnight. The pH of the filtrate was determined (glass electrode) and acidity by titration against alkali. The absorption of acid by Amberlite IR 4 at different pH values is shown in Fig. 2.

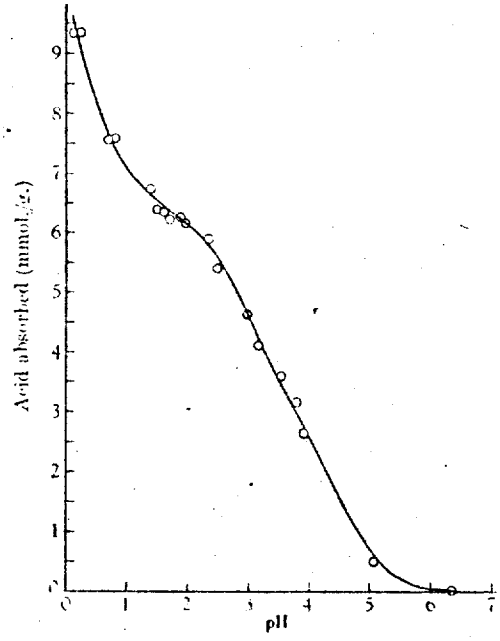

Fig. 2. Titration curve of Amberlite IR 4.

SUMMARY

1. A simple chromatographic method is presented for the separation of glutamic from aspartic acid, and their estimation, after their separation together from a protein hydrolysate, employing the synthetic anion exchange resin, Amberlite IR 4.

2. Optically active cysteic acid was isolated from a hydrolysate of chlorinated wool.

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FATAL ANAPHYLACTIC SHOCK FOLLOWING THE ADMINISTRATION OF A PROTEIN DIGEST (AMIGEN)*

A Case Report

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IN A REVIEW of the literature through 1947, we were able to find only four deaths due to the injection of protein hydrolysate. The first case, as reported by Hopps and Campbell,[1] was that of a patient operated upon for carcinoma of the cystic duct. The cystic duct and gall bladder were removed, the common duct ligated, and the hepatic artery ligated and severed. Postoperatively, there was a steady downhill course with a precipitant drop in the serum proteins. Two days later, 200 c.c. of Amigen were injected intravenously, followed by a severe chill and death one hour later. Post-mortem examination revealed extensive liver damage as would be expected.

Curreri and associates[2] report the second fatality. A supposedly healthy individual suffering only from an inguinal hernia was given Amigen during a metabolic study on three successive days. On the third day, after receiving 150 c.c., the patient developed chills, headache, vomiting, and a fever of 106° Fahrenheit. He expired 40 hours later. The autopsy showed generalized congestion of all organs, cerebral edema, hyperplasia of the splenic pulp cords, and extensive liver damage.

Elman[3] mentions a fatal case reported to him, in which, following the intravenous administration of 15 to 20 c.c. of Amigen, there was rapid death with circulatory collapse and respiratory distress. Autopsy failed to reveal any other mechanism and death was attributed to an allergic reaction to Amigen.

Ficarra[4] mentions a fatality which he attributes to Amigen administration, but he gives no essential data or circumstances, so the case cannot be evaluated.

Harris and co-workers[5] report a nonfatal case of apparent sensitivity to Amigen which occurred in a 17-year-old suicidal girl who had ingested a large amount of barbiturate. On the fourth day, a shocklike condition developed after the administration of 100 c.c. of Amigen. Recovery followed the administration of large amounts of nikethamide. It was their conclusion that Amigen may give rise to a serious allergic foreign protein reaction.

The first two case reports are not suggestive of allergic shock. Hopps and Campbell[1] are of the opinion that severely damaged livers are unable to conjugate amino acids at sufficiently rapid rates to escape acidosis and that this may be the mechanism causing death. They were unable to demonstrate antibody formation to Amigen, using rabbits. Ficarra[4] believes that sensitivity

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to a foreign protein is the underlying cause of the various reactions which may occur and advocates routine intracutaneous testing before administration. Madden and associates[6] performed a series of experiments with various amino acid solutions and casein digests and found no serious reactions. The common reactions of nausea, vomiting, and flushing were apparently related to the amount of glutamic acid present in the solution and not to any allergic manifestation.

The case history to be reported here represents, in our opinion, a true hypersensitivity reaction. This developed immediately after the administration of not more than 4 c.c. of protein hydrolysate (Amigen) and death occurred with the classical signs and symptoms of anaphylaxis within sixty minutes. By means of the Prausnitz-Küstner passive transfer test, an immediate urticarial skin reaction to Amigen was demonstrated similar to that found in atopic disorders.

CASE REPORT

C. M., a 44-year old white man, was admitted to the hospital on July 22, 1948 with a chronic duodenal ulcer and pyloric obstruction. This condition had been present with quiescent and recrudescent periods for the past four years.

The past history, as related by the patient, was irrelevant; however, the family stated that the patient had attacks of asthma, apparently precipitated by dust. No food allergy or idiosyncrasy could be elicited. No familial history of food allergy was obtained. There was no history of previous administration of protein digests.

Physical examination revealed a well-developed, moderately well-nourished white male of stated age in no acute distress. Blood pressure was 130/80; pulse, 90; temperature, 98.6° Fahrenheit. Pain and tenderness in the right epigastrium were the only significant findings.

Laboratory tests were essentially normal, as follows: R.B.C., 4,750,000; Hb., 15 Gm.; W.B.C., 13,900; neutrophiles, 71 per cent; lymphocytes, 28 per cent; monocytes, 1 per cent; Kahn reaction, negative; urinalysis, negative; total serum proteins, 6.25 Gm. per cent; albumin, 4.34 Gm.; globulin, 1.91 Gm.; A-G ratio, 2.25; fasting gastric acidity, 40 degrees free hydrochloric acid.

X ray findings in this hospital were equivocal regarding the presence of a duodenal deformity.

The patient was placed on a medical peptic ulcer regimen of diet, antiacids, and antispasmodics, with relief of all symptoms except nocturnal pain, which was relieved only by gastric aspirations. This program was followed for a period of six weeks when gastric surgery was decided upon. On September 8, 1949, an operation was performed using endotracheal cyclopropane anesthesia. A chronic anterior duodenal ulcer was found. A Polya type of subtotal gastric resection, with a posterior gastroenterostomy, was done. During the course of the operation 500 c.c. of citrated whole blood was given, followed later in the day by another transfusion of 500 c.c. of blood. The operative and postoperative courses were entirely uneventful, there being at no time any evidence of shock, circulatory or respiratory difficulty.

On the following morning, the patient was ambulatory and appeared to be in excellent condition. At 10:00 A.M. Amigen solution, 5 per cent in 5 per cent glucose, was started intravenously. Immediately, the patient became apprehensive and complained of difficulty in breathing. The infusion was stopped after about 4 c.c. had entered the vein. At 10:02 A.M., 0.5 c.c. of adrenalin hydrochloride (1:1,000) was given intramuscularly. The dyspnea became more marked and 100 per cent oxygen was administered through a mask. Another 0.5 c.c. adrenalin was given intravenously at 10:05 A.M. The respiratory difficulty became progressively more marked, and at 10:10 the patient was showing signs of upper respiratory

obstruction with marked inspiratory efforts, flaring of lower edge of the ribs, and suprasternal retraction. A laryngoscope was inserted for the purpose of placing an endotracheal tube, and the uvula, epiglottis, pharyngeal wall, and vocal cords were found to be extensively swollen and edematous. The glottis was closed and it was impossible to insert a tube. At this time, the patient was extremely cyanotic and respirations ceased. Artificial respiration was started and at 10:15 a tracheotomy was performed and 100 per cent oxygen given through the tracheotomy tube. No pulse could be discerned and 0.5 c.c. of adrenalin was given intracardially, after which heart action was resumed. The cyanosis gradually disappeared. Coramine, 2 c.c. intramuscularly, failed to restore respiratory function and artificial respiration was started. At this time, some spasmodic rigidity of the extremities became evident and sodium amytal, gr. 3¾ intravenously, promptly inhibited these motions. Good respiratory exchange continued for thirty minutes after the tracheotomy. It then progressively diminished and numerous moist rales developed in the lungs. Following the onset of pulmonary edema, the patient rapidly became worse and died at 11:00 A.M., one hour after parenteral administration of Amigen.

Permission for autopsy was obtained and the following pertinent findings were noted:

Brain: Multiple sections of the brain revealed cerebral congestion with prominent engorged vessels. Microscopically the small vessels were distended as were those in the pituitary gland.

Throat and Neck: The pharyngeal, laryngeal, and tracheal mucosae were pale and edematous. The vocal cords were boggy, wet, and swollen. Peritracheal and carinal lymph nodes were grossly edematous. Sections of the vocal cords showed recent hemorrhage with considerable edema and congestion of the submucosa and infiltration by large mononuclear cells, plasma cells, neutrophiles, lymphocytes, and a few eosinophils. The tracheal submucosa presented a similar picture.

Lungs: The right lung weighed 700 grams and the left lung, 610 grams. Although crepitant, considerable congestion was present, especially at the bases. Sectioning revealed wet, red-gray surfaces with dark bloody areas at the bases. The bronchial tree contained frothy red fluid. Microscopic studies revealed distended alveoli with rupture in some areas, while edema was a constant finding. The vessels were generally contracted. The bronchi and bronchioli showed contracted outlines and the lumens contained mucus, fibrin, debris, and varying amounts of blood.

Liver: The liver weighed 1,560 grams and sectioning revealed a congested, red-brown surface. Microscopic sections showed marked engorgement of the central veins and general dilatation of the sinusoids. No evidence of hepatic cell damage was present.

Spleen: The spleen weighed 130 grams. On sectioning, the pulp was firm and the trabeculations were prominent, but the corpuscles were not easily visualized. Microscopically the splenic pulp was congested and the sinuses were dilated and engorged.

Kidneys: Grossly no changes were detectable in the kidneys, but microscopic examination revealed considerable congestion of the parenchyma. The vessels were markedly dilated and engorged, especially the glomerular capillaries. The tubules were swollen and granular with their lumens containing eosinophilic albuminous material.

Gastrointestinal tract: There was edema of the submucosa of the esophagus; otherwise, aside from the recent gastric resection and gastrojejunostomy, no significant changes were found in the remainder of the gastrointestinal tract.

Lymph Nodes: Sections of cervical, peritracheal, and mediastinal lymph nodes showed edema, congestion, and early reactive hyperplasia.

Laboratory Tests.—

Prausnitz-Küstner Passive Transfer Test: The Prausnitz-Küstner test was performed using blood of the patient obtained by auricular puncture immediately after death. This blood was permitted to clot, the serum was separated and kept for twenty-four hours in the refrigerator. Then 0.1 c.c. of this serum was injected intracutaneously in the left forearm

of four normal test persons. Each person received also an intracutaneous injection of 0.1 c.c. of a normal serum in the right forearm. There was the usual slight swelling after the injection which disappeared in a very short time. The sensitized areas were encircled with ink marks. Twenty-four hours later the sensitized areas could be distinguished from normal skin by the ink marks only. At this time undiluted Amigen solution from the original flask was injected intracutaneously into the two areas and in a nonsensitized area on the right forearm. Three of the four test persons received 0.05 c.c. Amigen in each site, the fourth test person received injections of 0.1 c.c. at the described areas. Immediately after the injection of Amigen, the areas previously sensitized with the patient's serum began to itch and developed redness and swelling. This occurred in all test persons, with slightly greater severity in the person receiving the larger injection of Amigen. No significant reaction was observed at any of the two control sites of Amigen injection in any of the test persons. Only slight burning from the injection proper was noted and this disappeared within a few minutes. The reaction at the site of sensitization with the patient's serum increased in severity during the following four to six hours, then subsided gradually, disappearing completely after sixteen to twenty-four hours. In all test persons the reaction consisted of itching, swelling, and redness involving areas of approximately 7 × 10 centimeters. In addition, in two test persons wheals and pseudopods developed, persisting for about eight hours. In the test person receiving the 0.1 c.c. dose of Amigen, pain and a feeling of pressure extended into the elbow and upper arm.

Studies of Blood Donors: The patient had received two blood transfusions in the twenty-four hours prior to the administration of Amigen and to his death. The donors were available for study. One had had a severe attack of hives eighteen months before but was negative to all the usual skin tests at this time. Neither donor had ever received a blood transfusion or Amigen injection. Serum obtained from these donors gave negative passive transfer tests with Amigen. Furthermore the donors were not sensitive to the intradermal injection of Amigen.

Further attempts to determine possible antigenic properties of the Amigen used in our patient were carried on as follows:

Guinea Pig Inoculations: Four guinea pigs were given intracardiac injections of 0.5 c.c. of Amigen solution from the original flask. Ten days later two of these animals were reinjected with 0.2 c.c. of the same Amigen solution. These injections were tolerated without any reaction. The animals survived and are well at present. The two remaining animals were used on the eighteenth day after sensitization when they received intracardially 0.2 c.c. of the original Amigen solution. No anaphylactic reaction developed after these injections. The animals survived and are well.

Similar but more extensive experiments with the same lot of Amigen were carried out in the Research Laboratories of the manufacturers,* who permit us to include their observations in this report. The results confirmed our own findings. Forty test animals and fifteen control animals were used with the following results:

1. Ten guinea pigs were injected with the Amigen solution and after a three-week period were reinjected with Amigen. All responses were negative.

2. Ten guinea pigs were injected with the Amigen solution and after a three-week period were reinjected with skim milk; all responses were negative. Five control animals, sensitized and reinjected with skim milk, responded positively.

3. Twenty guinea pigs were injected with the Amigen solution and after a three-week period were injected with pancreas extract. All responses were negative. Ten control animals sensitized and reinjected with pancreas extract responded positively.

From these results it can be concluded that the involved lot of Amigen was "non-anaphylactic" to guinea pigs.

*Mead Johnson & Company, Evansville, Ind.

COMMENT

The autopsy findings are compatible with those described as occurring in anaphylactic shock. Vance and Strassman[5] state that death usually occurs due to asphyxia resulting from bronchial spasm. Autopsy findings are: inflated lungs, contraction of the bronchial musculature, and submucosal edema with infiltration by eosinophiles. Other organs such as brain and liver often show an intense congestion.

The positive Prausnitz-Küstner passive transfer test, the exclusion of a specific reagin from the transfused blood, and the failure to demonstrate in test persons and in animal experiments allergenic properties in the Amigen would indicate that the patient's serum contained a specific reagin probably dependent on a constitutional abnormality. Such an abnormality will have to be classified as an atopic sensitivity.

Lund and Hunt[7] believe that the passive transfer reaction is a reliable test for the diagnosis of hypersensitivity to a specific allergin. The diagnostic criteria of allergic shock as given by them are fulfilled in our case. (1) "The injected material was not toxic for a normal person." (2) "The symptoms produced were characteristic of an allergic state." (3) "The autopsy disclosed no other explanation of death." (4) "A specific sensitizing agent corresponding with the material injected was found in the blood serum by the technic of passive transfer."

One can only speculate as to the character of the atopic sensitivity demonstrated. If the patient had been allergic against his own pancreatic trypsin, as has been suggested, then his blood may have carried a hapten for Amigen. Thus an anaphylactic reaction might have been precipitated with a non-allergenic protein digest.

Hypersensitivity reactions to protein digests are not frequent and seldom very severe. The incidence of fatal reactions is extremely rare and is probably no higher than the risk attending intravenous administration of many other substances usually considered harmless. Yet, an experience like the one reported should call for caution and careful evaluation of the benefits and the dangers from the use of such a substance. It seems to be advisable to carry out in all patients sensitivity tests prior to the administration of protein digests. This precaution should be observed especially if an interval of time has occurred since a previous administration.

SUMMARY

1. A case of fatal allergic shock following Amigen administration is reported.

2. Passive transfer tests with the patient's serum were positive. The solution of protein hydrolysate (Amigen) used in this patient was not allergenic when tested in guinea pigs and normal persons. It is assumed therefore that the death of the patient was due to an atopic reaction.

3. It is suggested that all individuals to receive protein digests parenterally should be tested previously for sensitivity to the digest.

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Monosodium L-glutamate—Inhibition of glucose uptake in brain as a basis for toxicity*

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RECENT reports have implicated monosodium L-glutamate as the causative agent in a variety of pathological and physiological lesions. Oral or parenteral administration of glutamate in amounts in excess of 3 g may be associated with symptoms of burning, facial pressure, chest pain and headache collectively known as the Chinese restaurant syndrome.[1-3] Parenteral administration of glutamate to neonatal mammals has been reported to cause degenerative changes in the inner layer of the retina[4-7] and in the hypothalamus,[8,9] the latter effect probably accounting for the skeletal stunting, obesity and sterility observed.[8,9] There has been controversy over some of these reports,[10,11] but one practical result has been the discontinuation of the practice of adding glutamate to commercial baby foods.

Our own interest in this compound stems from its action as an antagonist of the antimitotic alkaloid, vinblastine.[12,13] There is a competitive relationship between vinblastine and glutamate for entry into human leukocytes,[14] despite their great disparity in chemical structure, and the alkaloid also inhibits

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the transport of other amino acids and uridine.[15] Because of these findings, it occurred to us that glutamate also might inhibit the transport of other natural metabolites as did vinblastine, and that this could be a basis for its toxicity. Among the metabolites tried was glucose. In our human leukocyte system, either at rest or during phagocytosis, glutamate failed to affect the uptake of glucose. However, the critical dependence of cerebral function upon glucose led us to study the effect of parenteral glutamate on glucose uptake by the brain. The present data from adult mice indicate that a significant if brief depression of the uptake of this sugar does indeed occur.

Adult Swiss mice (CD-1; 30 g) received intraperitoneal injections of monosodium L-glutamate (300 mg/kg; 9 mg in a 0·3-ml injection volume) or isotonic saline as a control, followed after a 15-min interval by glucose-2-^{14}C (0·62 mc m-mole; 1 μc) given by the same route. At intervals thereafter, the mice were killed, their brains removed into ice-cold saline, and blood samples were collected from the hearts. Brains were washed free of blood, homogenized with perchloric acid (0·5 M), heated at 80° for 20 min, cooled and centrifuged. Radioactivity in the supernatant was measured with a Packard Tri-Carb liquid scintillation counter using a toluene-ethanol based fluor. The precipitate

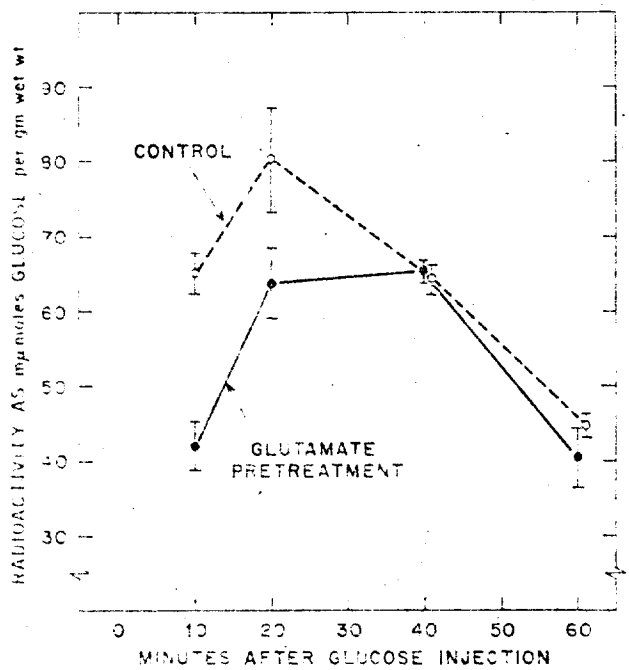

FIG. 1. Effect of glutamate on the levels of radioactivity in brain derived from glucose-^{14}C. Glutamate (9 mg) was injected 15 min before glucose-2-^{14}C which was given at time zero. Procedures were as described in the text. Standard errors of the means (six animals per point) are indicated.

was suspended in NaOH solution (0·2 N) and counted similarly. Data for the acid-soluble fraction (Fig. 1) showed that at 10 and 20 min after injection of glucose, the ^{14}C levels in the brain, expressed as glucose equivalents, were significantly lower in the glutamate-treated mice ($P < 0·005$ at 10 min, $< 0·01$ at 20 min, by the t-test). By 40 min, however, there was no difference between the groups. The acid-insoluble residue contained between 13·3 and 17·4 per cent of the total brain radioactivity, and its behavior closely paralleled that of the soluble fraction. Thin-layer chromatography of the neutralized 10-min acid extracts (six mice per group) on Silica gel H (Merck) with n-butanol–formic acid–water (77:10:13) as solvent showed that 64·2 ± 0·7 per cent of the control and 59·7 ± 1·4 per cent of the treated ^{14}C migrated as unchanged glucose. This small reduction, although significant ($P = 0·02$), is unlikely to explain a 35·5 per cent decrease in brain radioactivity; indeed such a reduction in the glucose component might be expected if the amount of glucose entering the brain declines without changes in its rate of utilization for metabolic processes.

The blood samples (0·02 ml) were plated out with NaOH solution on stainless steel planchettes, dried at room temperature, and counted in a Nuclear Chicago gas-flow counter without a window. There was no difference between the blood levels of ^{14}C in control and glutamate-treated mice (Fig. 2); the bulk of this radioactivity migrated as glucose in the early samples.

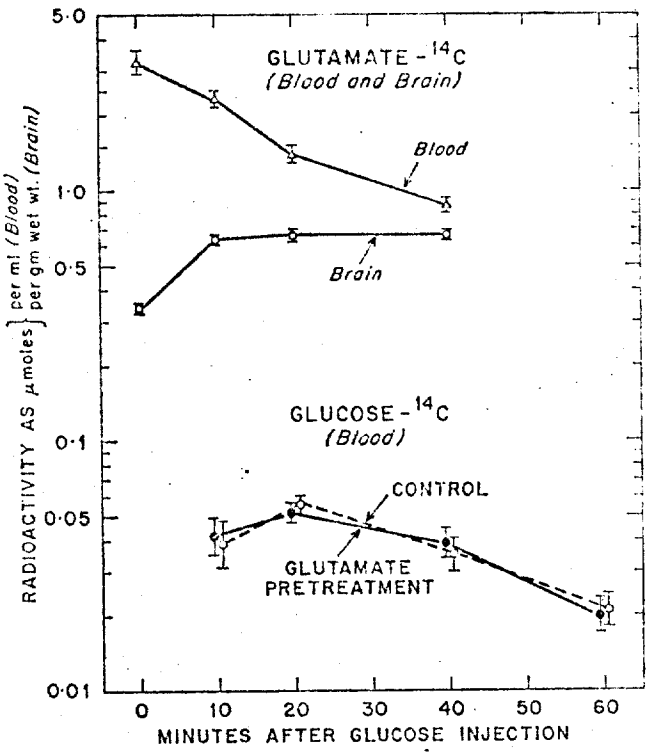

FIG. 2. Radioactivity derived from glutamate-^{14}C (blood and brain) or from glucose-^{14}C (blood). Glutamate, either labeled or unlabeled (9 mg), was given 15 min before zero time, when glucose was injected. Levels were determined as described in the text. Standard errors of the means (six animals per point are indicated.

In order to determine the levels of glutamate in the blood needed to achieve an effect on glucose uptake, intraperitoneal injections of glutamate-3,4-^{14}C (9 mg and 1 μc/mouse) were given and the blood and brain levels of radioactivity measured as for glucose-2-^{14}C. The results (Fig. 2) indicate that the excess blood levels of glutamate over those occurring normally—for mouse plasma reported as 33·3 μg/ml or 0·27 μmole/ml[16]—fell from an initial 3·26 to 1·42 μmoles/ml during the time that an effect on glucose transport was evident.

Thus, large doses of glutamate in adult mice can reduce the uptake of glucose into the whole brain by up to 35·5 per cent. That the overall effect is dose-dependent was shown by another experiment in which twice the amount of glutamate (600 mg/kg) produced an inhibition of 64·1 per cent. Neither glycine nor sucrose (300 mg/kg) affected the entry of glucose into the brain to any significant extent when compared with saline.

One might speculate that, since some areas of the brain are likely to be more sensitive to glutamate than others, the differences in glucose uptake in critical tissues may be even greater. There is evidence for the existence of centers within the lateral and ventromedial regions of the hypothalamus that control feeding behavior,[17] and which appear to take up labeled glucose more readily than surrounding areas.[18] In addition, gold thioglucose exerts a selective toxic effect on the hypothalamus that is not shown by the other related agents such as gold thiomalate, and apparently depends on the ability

of the thioglucose moiety to facilitate transport of gold into the sensitive areas.[19,20] The ventral hypothalamus is especially affected, including both lateral areas and such ventromedial structures as the ventromedial nucleus, the arcuate nucleus, the supraoptic nucleus and the median eminence.[15] Damage from monosodium glutamate is prominent in the region of the arcuate nucleus, the pre-optic nucleus and the median eminence.[8] Aspartic acid and cysteine, but no other amino acid, produce similar lesions.[21]

In previous studies, 0·5–4 g/kg of glutamate produced brain damage or developmental abnormalities[7,8] and similar amounts given daily over 10–18 days resulted in retinal lesions;[4–6] these dosages are far larger than those used in this study. The amounts of glutamate needed to induce the Chinese restaurant syndrome in man varied widely, but were about 3 g in the most sensitive individuals. Some subjects had thresholds of 7–12 g, corresponding to 100–170 mg/kg. These doses approach those needed to affect glucose uptake and, in addition, man, whose plasma glutamate level is little more than one-fifth that of the mouse,[16] may be more sensitive to this amino acid. Interference with glucose uptake is thus one plausible basis for the toxicity of monosodium L-glutamate.

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THE FREE AMINO ACIDS OF HUMAN SPINAL FLUID DETERMINED BY ION EXCHANGE CHROMATOGRAPHY

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THE FREE amino acids in human cerebrospinal fluid (CSF) of eighteen individuals are reported in this paper. In each case the amino acids were determined on 0·75 ml of CSF by an ion-exchange chromatographic micromethod which was developed in this laboratory (HAMILTON, 1958; HAMILTON, BOGUE and ANDERSON, 1960; HAMILTON, 1960, 1962, 1963; DICKINSON, ROSENBLUM and HAMILTON, 1965), and which is based on earlier ion exchange procedures (MOORE and STEIN, 1951; SPACKMAN, STEIN and MOORE, 1958). The present method was shown (DICKINSON *et al.*, 1965) to be capable of determining amino acids at the 10^{-8} mole level to within ± 5 per cent and at the 5×10^{-9} mole level to within ± 10 per cent; detection is to the 10^{-10} mole level.

Available data published prior to 1961 on amino acid concentrations in CSF, compiled by SCHREIER (1962), reported values determined predominately by paper chromatography with the exception of one set obtained by a microbiological method and one set by column chromatography. Most of the data were of a semi-quantitative nature and the procedures used did not detect some of the amino acids and other ninhydrin positive compounds which are now known to be normally present in CSF in very low concentrations.

Since 1961 the most comprehensive report is that of PERRY and JONES (1961). The column chromatographic method they used enabled them to detect a number of unknown ninhydrin positive compounds in human CSF, and they also provided quantitative data on the concentration of the free amino acids in CSF from eighteen patients with neurological diseases and three control subjects. In all but five cases, pooled specimens were needed as their method required 20 ml of CSF for an analysis.

This paper confirms the general findings of PERRY and JONES (1961) and more particularly is in agreement that citrulline, methionine, ethanolamine, and ornithine are normal constituents, that γ-aminobutyric acid was absent from any of the specimens analysed, and that a number of additional unknown peaks are normally found in CSF. In addition, this paper also reports that cystine is a normal constituent of CSF, but in very low concentration. Through the generous cooperation of Dr. MARC VAN SANDE, Laboratory for Neurochemistry, Bunge Institute, Antwerp, Belgium (personal communication, 1964) we were able to compare the present data with those obtained by him on CSF from fifty-two subjects whose CSF was also analysed by ion exchange chromatographic procedures. Qualitatively and quantitatively the findings of VAN SANDE, of PERRY and JONES (1961) and of the present work are in good agreement.

METHODS

Collection of spinal fluid. CSF was made available to this laboratory after being collected for neurological diagnostic procedure by Dr. PIERRE L. LEROY and by the Wilmington Medical Center, Wilmington General Division, Wilmington, Delaware. The patients, eight female and ten male, ranged in age from 4 months to 42 years. Fluid with any evidence of contamination by blood was discarded. When possible, the spinal fluids were stored at −68° immediately after collection. It has been shown that storage at this temperature is necessary to preserve the integrity of the aspartic acid, glutamine, glutamic acid, cystine, methionine and tryptophan values. These amino acids were found to be subject to change in concentration when plasma or a protein-free filtrate of plasma was stored under other conditions (DICKINSON *et al.*, 1965).

Preparation for analysis. Proteins were removed by mixing 0·750 ml of spinal fluid with 0·150 ml of 15% sulphosalicylic acid (J. T. Baker, Analytical Reagent) in a 2·0 ml conical centrifuge tube; the contents were stirred with a fine glass rod and allowed to stand for 10 min. Precipitated protein was packed in an Adams microchemistry centrifuge at 13,500 rev/min for 5 min. Of the clear, colourless supernatant, 0·750 ml (equivalent to 0·625 ml of CSF) was placed directly on the column. The use of sulphosalicylic acid as a protein precipitant for amino acid analysis follows previously reported procedures (HAMILTON, 1962, and DICKINSON *et al.*, 1965), in which the concentration of free amino acids was found to be the same as in a picric acid filtrate.

Ion exchange chromatography. Ion exchange chromatography was carried out on a single column packed with 17·5 μ diameter spherical Dowex 50X8 resin particles with 80 per cent of the particles within ±2 microns of the mean (HAMILTON, 1962). The apparatus and general procedure have been fully described (HAMILTON, 1963). A complete analysis through to arginine, plus column wash (0·2N-NaOH) and column regeneration (pH 2·875 buffer) takes 23 hr, 50 min. The temperature and buffer sequences were those used for plasma analysis; the precision of determination was the same as that reported for the plasma amino acids of the newborn infant (DICKINSON *et al.*, 1965).

Preparation of the concentrated filtrate. A CSF filtrate, 10 times more concentrated than in the regular analysis (hereafter referred to as the X10 filtrate) was prepared from a pooled sample made up of approximately equal volumes of CSF from each of six patients. Protein was precipitated from 7·5 ml of the pooled fluid by the addition of 1·5 ml of 15% sulphosalicylic acid. After centrifugation, 7·5 ml of the supernatant were passed through an 0·9 × 1·8 cm (1·65 g) column of Dowex 2 (chloride form) to remove sulphosalicylic acid in a manner similar to that described by STEIN and MOORE (1954) for the removal of picric acid from picric acid filtrates prepared according to the method of HAMILTON and VAN SLYKE (1943). The filtrate and washings (3 × 5·0 ml of 0·02 N-HCl) were reduced to dryness in a rotary evaporator, and the entire residue was analysed.

RESULTS

The concentrations of the amino acids for each of the patients are given in Table 1. The arithmetic means are compared with the normal pooled adult CSF values reported by PERRY and JONES (1961) and the normal plasma values reported by DICKINSON *et al.*, (1965) Table II, Col. 5, Adult Mean.

The following amino acids were always found in readily detectable amounts: taurine, urea, threonine, serine, glutamine, glutamic acid, citrulline, glycine, alanine, α-amino-*n*-butyric acid, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, ethanolamine, ornithine, lysine, histidine and arginine. The normal occurrence of ethanolamine in CSF was reported in a paper dealing with amine content of normal human CSF (PERRY, HANSEN and JENKINS, 1964). Since asparagine is completely overlapped by serine in this method of analysis, the values reported are of necessity serine — asparagine. Aspartic acid and cystine, although always present, occurred in very small or trace amounts. Tryptophan was present in thirteen of the eighteen subjects.

A small peak in the position of proline was seen in five chromatograms; γ-amino-butyric acid was never observed. Urea and glutamine were always present in relatively high concentrations, more nearly approximating the concentrations of urea and glutamine in plasma. In addition, twelve unknown peaks were nearly always evident. Analysis of the X10 concentrated filtrate clearly demonstrated those amino acids

TABLE 1.—FREE AMINO ACIDS IN HUMAN SPINAL FLUID (μmoles/100 ml)

Patient	Sex	Age (years)	Clinical diagnosis	Urea	Taur*	Asp	Thr	Ser†	Glu(NH₂)	Glu	Pro
1	F	2	Mentally and physically retarded	465	0·70	0·17	1·87	4·13	53·7	0·34	**
2	F	9	Seizures	347	0·62	**	1·39	2·80	59·9	0·62	0
3	M	8	Febrile convulsions	229	0·59	0·10	1·73	3·18	52·1	0·67	0
4	M	3	Feeding problems	409	0·54	0·10	2·62	3·22	54·6	0·56	0
5	M	2	Anaemia	359	0·46	0·10	1·58	3·95	46·4	0·74	0
6	M	16	Mentally retarded	448	0·53	0·14	2·62	2·43	55·8	0·88	0
7	M	0·3	Pneumonitis	††	0·22	**	4·91	3·79	††	2·46	0
8	F	3	Hypoglycemia	668	0·50	0·08	0·77	1·71	38·3	0·61	0
9	F	0·3	Craniosynostosis	††	0·62	**	3·33	12·11	86·5	0·69	0
10	M	9	Traumatic brain injury	444	0·85	0·03	2·43	2·80	††	0·86	0
11	M	13	Scoliosis	402	0·80	0·05	2·39	‡‡	‡‡	0·62	0
12	F	8	Convulsive disorder	341	0·66	0·22	2·13	3·54	55·5	1·10	0·58
13	M	8	Retardation and convulsions	201	0·45	0·11	1·02	1·65	19·4	0·38	0
14	F	22	Uncontrollable psychotic	336	0·94	0·08	2·26	3·55	42·7	0·35	**
15	F	0·3	Convulsive disorder	167	0·86	0·16	2·77	4·77	36·3	0·54	0·46
16	M	42	Motor accident	††	0·53	0·13	3·49	3·36	53·9	0·43	**
17	F	9	Encephalopathy	405	0·78	0·10	1·84	3·90	52·2	0·50	0
18	M	12	None	424	0·54	0·06	3·46	3·44	56·5	0·24	0
			CSF mean		0·63	0·09	2·48	3·78	50·9	0·70	0·06
			S.D.		±0·18	±0·05	±1·01	±2·29	±14·4	±0·49	±0·16
			CSF Mean (Lit.)§§		0·58	0·31	4·79	5·05	22·24	0·75	0·14
			Plasma Mean (Lit.)¶¶		6·63	1·65	16·3	16·6	56·8	5·84	23·5
			Plasma Mean (Lit.)¶¶/CSF Mean		11	18	7	4	1	8	392

TABLE 1. (*Continued*)

Patient	Sex	Age (years)	Clinical diagnosis	Cit‡	Gly	Ala	ABA§	Val	Cys‖	Met	Ileu
1	F	2	Mentally and physically retarded	0·12	0·91	2·02	0·26	1·49	**	0·19	0·53
2	F	9	Seizures	0·11	0·77	1·17	0·30	1·20	**	0·18	0·38
3	M	8	Febrile convulsions	0·22	0·64	2·21	0·34	1·15	**	0·21	0·34
4	M	3	Feeding problems	0·19	0·61	2·22	0·21	1·34	**	0·24	0·45
5	M	2	Anaemia	0·14	0·69	2·19	0·24	0·99	**	0·19	0·35
6	M	16	Mentally retarded	0·22	0·85	2·11	0·18	1·18	**	0·29	0·42
7	M	0·3	Pneumonitis	0·24	0·70	1·89	0·37	1·30	0·14	0·75	0·37
8	F	3	Hypoglycemia	0·21	0·46	1·94	0·21	1·15	**	0·14	0·45
9	F	0·3	Craniosynostosis	0·45	0·51	4·46	0·91	2·70	0·08	0·43	0·61
10	M	9	Traumatic brain injury	0·14	0·66	1·28	0·29	1·31	**	0·18	0·35
11	M	13	Scoliosis	0·18	0·40	3·94	0·27	1·44	**	0·27	0·48
12	F	8	Convulsive disorder	0·19	0·74	1·62	0·62	2·06	0·06	0·22	0·56
13	M	8	Retardation and convulsions	0·08	0·43	0·88	0·24	0·74	**	0·05	0·30
14	F	22	Uncontrollable psychotic	0·13	0·51	2·45	0·38	1·07	**	0·21	0·30
15	F	0·3	Convulsive disorder	0·29	0·82	3·22	0·14	1·31	**	0·16	0·40
16	M	42	Motor accident	0·19	1·07	3·49	0·54	2·82	0·03	0·50	0·86
17	F	9	Encephalopathy	0·19	0·62	2·18	0·37	1·57	**	0·22	0·43
18	M	12	None	0·21	0·51	2·54	0·19	1·41	**	0·27	0·40
			CSF mean	0·20	0·66	2·32	0·34	1·46	0·02	0·26	0·44
			S.D.	±0·08	±0·18	±0·94	±0·19	±0.55	±0·03	±0·16	±0·13
			CSF Mean (Lit.)§§	***	1·50	3·24	0·09	1·42	‖‖	0·27	0·44
			Plasma Mean (Lit.)¶¶	3·02	23·2	34·5	1·65	17·0	7·37	2·14	5·41
			Plasma Mean (Lit.)¶¶/CSF Mean	15	35	15	5	5	368	8	12

TABLE 1. (*Continued*)

Patient	Sex	Age (years)	Clinical diagnosis	Leu	Tyr	Phe	Eth(NH_2)¶	Orn	Lys	His	Try	Arg
1	F	2	Mentally and physically retarded	1·10	0·77	0·67	1·94	0·53	1·61	1·20	**	1·99
2	F	9	Seizures	1·01	0·42	0·45	1·36	0·58	2·21	1·09	0	1·68
3	M	8	Febrile convulsions	0·91	0·83	1·26	0·72	0·43	1·01	1·14	**	1·73
4	M	3	Feeding problems	0·94	1·28	0·90	1·57	0·67	2·37	1·34	**	2·10
5	M	2	Anaemia	0·74	0·69	0·66	0·75	0·74	1·81	0·96	**	1·92
6	M	16	Mentally retarded	0·93	0·67	0·78	1·26	1·02	1·92	1·34	0	2·19
7	M	0·3	Pneumonitis	0·98	2·43	2·74	1·92	0·83	1·95	2·38	0·58	2·30
8	F	3	Hypoglycemia	1·06	0·42	0·61	1·58	0·43	0·91	0·62	0	0·91
9	F	0·3	Craniosynostosis	1·46	1·66	1·38	0·18	0·72	2·30	2·10	**	2·99
10	M	9	Traumatic brain injury	0·94	0·77	0·75	1·30	0·37	1·66	1·31	0·13	2·05
11	M	13	Scoliosis	1·33	0·90	0·77	2·40	0·70	2·61	1·60	**	2·91
12	F	8	Convulsive disorder	1·33	0·83	1·17	1·06	0·56	1·63	0·91	0·22	1·81
13	M	8	Retardation and convulsions	0·62	0·30	0·16	0·93	0·40	0·61	0·83	0	0·80
14	F	22	Uncontrollable psychotic	0·72	0·62	0·66	1·25	0·45	1·47	1·65	0	1·39
15	F	0·3	Convulsive disorder	0·88	0·90	0·56	0·46	0·61	1·73	1·07	**	2·54
16	M	42	Motor accident	2·22	1·15	1·63	0·91	0·48	3·34	1·33	0·19	2·45
17	F	9	Encephalopathy	1·30	0·72	0·58	1·78	0·38	2·00	1·04	0·08	2·24
18	M	12	None	1·22	0·96	0·83	1·68	0·37	2·56	1·52	0·26	2·18
			CSF mean	1·09	0·91	0·92	1·28	0·57	1·87	1·30	0·08	2·01
			S.D.	±0·36	±0·50	±0·58	±0·57	±0·18	±0·66	±0·44	±0·14	±0·58
			CSF Mean (Lit.)§§	0·98	0·66	0·88	1·29	0·38	1·70	1·29	0·14	0·99
			Plasma Mean (Lit.)¶¶	10·1	5·02	5·75	0·16	6·96	17·4	7·99	4·80	8·21
			Plasma Mean (Lit.)¶¶/CSF Mean	9	6	6	0·1	12	9	6	60	4

* Taurine
† Serine + asparagine
‡ Citrulline
§ α-NH_2-*n*-butyric acid
‖ Cystine
¶ Ethanolamine
** Too small to integrate: <0·03 μm/100 ml approximately
†† Off scale
‡‡ Serine overlapped by glutamine
§§ PERRY and JONES (1961)
‖‖ No value reported
¶¶ Table II, Col. 5, Adult Mean (DICKINSON *et al.*, 1965)

whose presence might have been doubted because of their ordinarily low concentrations and it also served to distinguish the twelve unknown peaks from confusion with baseline artifacts. Additional ninhydrin positive compounds, not seen in the standard analysis, also became apparent on the analysis of the X10 filtrate.

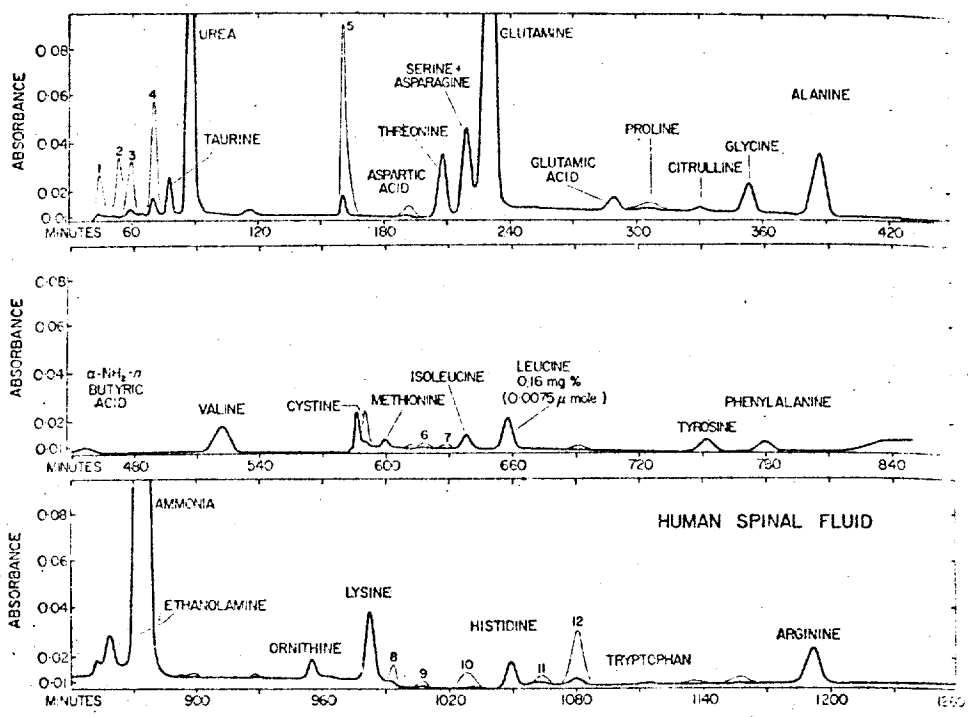

FIG. 1.—A tracing of a chromatogram of a standard analysis of the free amino acids in 0·75 ml of CSF is shown in heavy lines. The light line peaks were obtained from a separate analysis of a filtrate 10 times more concentrated than the standard analysis. Further analytical details are in the text.

In Fig. 1, the small peaks shown at 120, 590, and 860 min were seen in every chromatogram, including blank runs and were, therefore, considered to be artifacts. The peaks in light lines and numbered show the increase obtained in the analysis of the X10 filtrate and were each present in approximately 10 times the amounts seen in a standard analysis. The light lined peaks over aspartic acid, proline and cystine, also obtained from analysis of the X10 filtrate, confirm the findings of these amino acids in the standard analysis. At minutes 610, 640, 900, 930, 1135 and 1160 peaks are shown which were not seen in the standard analysis but which were visible in the analysis of the X10 filtrate.

For an approximation of the concentrations represented by the unknown peaks as they appear in the standard analysis, a visual comparison may be made with the leucine peak for which a concentration figure is given on the chromatogram. In the comments which follow, it should be emphasized that the unknown peaks are not being identified. The intention is to acquaint those workers, who might find identification mandatory, with compounds known to occur in the same area of the chromatogram

as the unknown peak (HAMILTON, 1963; ZACHARIUS and TALLEY, 1962; FRIMPTER and BASS, 1962; FRIMPTER, OHMORI and MIZUHARA, 1963).

Peak 1 falls in the position occupied by cysteic acid, homocysteic acid, the phosphorylated derivatives of serine, threonine, and hydroxyproline, and by glycerophosphoethanolamine. Peak 2 has a maximal absorption at 4400 Å. No compound analysed in this laboratory has occupied that position with the same absorption characteristics. In the X10 filtrate analysis it was apparent from the shape of Peak 2 that another component was also present. Peaks 3 and 4 are in the region where glucosamine phosphate, ethanolamine phosphate, and tyrosine-*O*-sulphate are located. Peak 5 was overlapped by S-(1-2-dicarboxyethyl)-cysteine, an amino acid recently isolated from human urine and guinea pig kidney (KUWAKI and MIZUHARA, 1966). The authors are indebted to Dr. MIZUHARA for a sample of synthetic material. None of the other compounds known to emerge in the vicinity of Peak 5 overlapped, nor did allo-γ-OH-glutamic acid, whose position had not been previously reported; this last amino acid was resolved just after Peak 5. Peak 6 is in the position for cystathionine, and this amino acid has been reported present in the CSF (PERRY and JONES, 1961). Peak 7 is in the position of allo-isoleucine, but as with Peak 6 other numerous possibilities (see Fig. 4, HAMILTON, 1963) exist. Peak 8 appearing close to the trailing edge of lysine lies in the same position as that found for ε-*N*-methyllysine; this amino acid was recently shown to occur in histones (MURRAY, 1964). Peak 9 lies in the position of 1-methylhistidine. Peak 10 is in the position of anserine and Peak 11 is in the position of 3-methylhistidine, but other compounds also emerge close to these positions (HAMILTON, 1963). Peak 12 occupied the position of carnosine, previously inferred to be present in CSF (PERRY and JONES, 1961; HAMILTON and DICKINSON, 1964). In addition this peak was exactly overlapped by added authentic carnosine, it vanished on acid hydrolysis and it was accompanied by an increase in histidine and the appearance of β-alanine. A tentative identification of carnosine is therefore reasonable.

It is perhaps not altogether unexpected that analysis of X10 filtrates of plasma from newborn infants and adults also disclosed unknown peaks (DICKINSON *et al.*, 1965), many of which were in the same position as the CSF unknowns.

DISCUSSION

The formation of CSF in the choroid plexuses, its circulation through the central nervous system and its final return through the arachnoid villi to the venous blood invlove a dynamic exchange between the blood and the central nervous system (see SAMSON WRIGHT, 1965). It was, therefore, of interest to calculate the plasma/CSF ratio of each amino acid as shown in Table 1.

If the amino acids in CSF were the same as in plasma but simply less concentrated, as would be expected of a simple filtrate, the ratios would in each case be the same. If a selective distribution were occurring, as might be expected across membranes functioning in a selective and dynamic way, it would be unlikely that the ratios would be the same for each amino acid. This latter is clearly the case.

It is reasonable to assume that further information about the biochemistry of the central nervous system may be gained by a knowledge of the precise composition of the amino acids in CSF and that such information might lead in turn to some insight into mental and neurological disorders. Such was the expressed interest of PERRY and

JONES (1961) in their investigation of normal individuals and mental defectives. However, these authors found no significant deviations in the amino acid concentrations from pooled normal controls with the exception of high levels of phenylalanine in three cases of phenylketonuria. This observation confirmed a similar one of BOREK, BRECHER, JERVIS and WAELSCH (1950). High levels of argininosuccinic acid an the CSF from patients with argininosuccinicaciduria (ALLAN, CUSWORTH, DENT and WILSON, 1958), of glutamine in hepatic coma (GILON, SZEINBERG, TANMAN and BODONYI, 1959), of aspartic acid in hereditary cerebellar ataxia (LOGOTHETIS, 1958), and of valine, isoleucine, and leucine in maple syrup urine disease (SNYDERMAN, NORTON, ROITMAN and HOLT, 1964) were also reported. In Parkinson's disease low glutamic acid and elevated glycine, serine, threonine, cysteine and methionine levels were observed (BRUCK, GERSTENBRAND, GRUENDIG and TEUFLMAYR, 1964). Other than these few exceptions, significant deviations from the normal of the amino acid concentrations in CSF have not been reported. Few marked differences were found in any of the amino acid concentrations of the eighteen subjects reported in this paper, even though a variety of neurological conditions and a wide age group were represented. However, many mental and neurological disorders remain to be intensively investigated. For example, patient 9, craniosynostosis, showed an elevated serine, but little significance can be attached to this isolated finding. Few systematic studies have to date been published on CSF, largely owing to the difficulties imposed by prohibitive sample size. This is particularly true in paediatric research. It is hoped that the present demonstration with minimal sample requirements indicates the feasibility of such analyses and that significant information of either metabolic or diagnostic import will be more easily acquired.

SUMMARY

(1) The free amino acids in human CSF from eighteen subjects have been determined. The analyses were performed on 0·75 ml of CSF by an ion exchange chromatographic method which is capable of detection to the 10^{-10} mole level.

(2) The amino acids always found in readily detectable amounts were: taurine, threonine, serine, glutamine, glutamic acid, citrulline, glycine, alanine, α-NH_2-*n*-butyric acid, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, ethanolamine, ornithine, lysine, histidine and arginine. Urea was present. Aspartic acid and cystine, though always present, occurred in small or trace amounts. Proline was found in four cases and tryptophan in thirteen cases. In addition, twelve unknown peaks were nearly always evident in every chromatogram.

(3) Filtrates 10 times more concentrated than those used regularly were prepared from pooled CSF and analysed. These analyses clearly confirmed the presence of those amino acids which were normally in very low concentration and they also served to distinguish the twelve unknown compounds from confusion with baseline artifacts.

(4) The distribution of free amino acids in CSF was different from their distribution in blood plasma.

(5) Despite a variety of neurological conditions and a wide age span few marked deviations were found in any of the amino acid concentrations.

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Glutamic Acid Metabolism in the Lactating Dairy Cow [1]

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ABSTRACT A single dose of L-glutamic acid uniformly labeled with carbon-14 was injected into a lactating dairy cow via the portal vein or the jugular vein in four experiments, and the time course of the isotope was followed in respired CO_2, plasma glutamate, plasma glucose and milk constituents. In each experiment the specific activity of plasma glutamate decreased with a half-time of approximately 5 minutes during the first 30 minutes after injection. Expired CO_2 and plasma glucose became labeled rapidly with maximal specific activities occurring at about 12 minutes in CO_2 and 20 minutes after the injection in plasma glucose. Between 34 and 41% of the injected dose appeared in respired CO_2 in the first 3 hours. Milk constituents contained 3 to 8% of the injected dose after 3 hours and 12 to 15% after 48 hours. Lactose accounted for 46 to 54% of all carbon-14 recovered in milk during 48 hours; casein, 27 to 37%; and albumin, 4 to 5%. The specific activity of citrate was very high, whereas that of milk fat was negligible. Aspartate was the most highly labeled amino acid in casein except for glutamate, indicating extensive interconversion of these two amino acids. Proline and arginine had very low specific activities, which means that their synthesis from glutamate was nominal. It is concluded that the amount of carbon from the plasma glutamate pool utilized for gluconeogenesis exceeds that incorporated into milk protein.

Amino acids are specifically required for synthesis of protein, but many of these compounds also play a critically important role as glucogenic precursors when glucose is limiting. These are competing processes for several amino acids which may be utilized either in gluconeogenesis or in protein synthesis.

The present study was undertaken to assess the metabolic fate of a glucogenic amino acid under conditions in which there is a large daily precursor requirement for both protein and carbohydrate. The lactating cow provides a unique model for this type of study since the formation of lactose and milk protein is virtually continuous, and approximately equal amounts of organic precursor [3] are required for each process. Furthermore, the glucose required for lactose synthesis (1, 2) must be derived largely by gluconeogenesis since the amount of alimentary glucose in the ruminant is limited (3, 4).

Glutamic acid was selected for the present study since it is the major component of milk protein,[4] comprising 19.8% of all milk protein (5), including 22.4% of the casein (6), and it is also a glucogenic amino acid (7, 8). Its metabolic fate was assessed by following the time course of appearance of isotope from glutamate-^{14}C in respired CO_2, blood glucose and the major constituents of milk. It was of interest also to determine how conditions which influence gluconeogenesis, such as withholding food or providing an exogenous supply of a glucogenic precursor, affect the metabolic fate of glutamic acid.

The studies reported here show that more of the carbon from glutamate appeared in lactose than in milk protein

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[3] Based on an average composition of milk containing 4.8% lactose (42% carbon in lactose), 3.[illegible] casein and 0.7% albumin (54% carbon in protein); each liter of milk would contain 1.68 g atoms carbon in lactose and 1.78 g atoms carbon in protein.

[4] An undetermined part of the "glutamate" in casein is actually present as glutamine. Barry (9) has estimated that there are approximately equal amounts of each amino acid in casein and has shown that the specific activities of the two amino acids in casein are nearly the same 3 hours after intravenous injection of ^{14}C-glutamate. In view of this rapid interconversion, the two amino acids are considered here as a single component.

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hen the cow was not fed and that this lationship was not appreciably changed feeding the cow, or by providing sub-rate amounts of propionic acid intra-nously. It is concluded that the processes volved in gluconeogenesis sequester a eater part of the carbon flux through the utamate pool than do processes of pro-n synthesis during milk formation in e cow.

MATERIALS AND METHODS

Animal. All experiments were carried t with a mature lactating Jersey cow 56 kg body weight) which had a plastic theter implanted in the portal vein (9). the day preceding each experiment, a cond catheter was inserted into the jugu-r vein. These catheters permitted the in-venous injection of ^{14}C-glutamate and thdrawal of blood samples over precisely ed intervals with minimum disturbance the cow. This approach was critically portant in the lactating animal, espe-lly for studies of gluconeogenesis, since otional disturbance can interfere with cose metabolism and obscure normal re-tionships. For this reason also, the cow d been accustomed to the experimental a and to all procedures used during the periment.

Diet. The cow received a high quality lfa hay ad libitum (about 12 kg/day sted) and a grain concentrate[5] (3.3 twice daily, given at each milking). is ration supplied protein and energy in ess of the NRC recommended allow-ce (10). When "unfed," the animal had ived a full ration on the day preceding experiment, but no food was avail-le from 14 hours before the experiment. ough this treatment does not represent fasted state or even a postabsorptive te, as it would in a monogastric animal, the time of the experiment rumen fer-ntation would have subsided. This pro-dure provided a more standard condition n the fed animal in which, depending the level and rate of intake on the morn-of the experiment, there would be vari-le amounts of CO_2 produced in rumen mentation. A longer period of fasting s rejected to avoid a situation that would preciably interfere with milk formation.

Radioactive glutamic acid (L-glutamic acid-U-^{14}C). Protein was extracted from a *Chlorella pyrenoidosa* culture grown in an atmosphere of $^{14}CO_2$ of high specific activity (25.5 mCi/mmole) and was hydrolyzed in 6 N HCl for 16 hours. Amino acids were separated by chromatography on a column of Dowex-50 (8% cross linked) ion exchange resin, eluting with hydrochloric acid (11). Successive fractions were examined by paper chromatography and autoradiographic techniques, and the pure fractions containing a single amino acid were combined and concentrated under reduced pressure. Purity of the sample was checked by standard procedures of chromatography (see below for solvent system) and autoradiography.

Experimental procedures. Each experiment began at 8 AM to avoid diurnal effects. The cow was connected by face mask to the respiration apparatus for one-half hour before injection of ^{14}C-glutamate. When propionate was administered, the infusion was started 30 minutes before injection of labeled glutamate. The cow was completely milked, after which the labeled glutamate was intravenously administered over a period of 2 minutes. The syringe and catheter were then flushed twice with 15 ml of sterile saline, during an additional minute. Following the first 3-hour period of sample collection, the cow was milked, and then intermittent samples of respired air, blood and milk were collected for 2 days.

Experiment 1: The cow was unfed overnight (14 hours) and L-glutamate-U-^{14}C was injected via the portal vein.

Experiment 2: The cow was unfed overnight (14 hours), and propionate (neutralized to pH 7.0 with NaOH) was infused via the portal vein at the rate of 0.44 mole/hour for 4 hours, commencing 30 minutes before portal injection of L-glutamate-U-^{14}C.

Experiment 3: The cow was fed just before the experiment and was infused with propionate as in experiment 2; the L-glutamate-U-^{14}C was injected via the portal vein.

[5] Feed contained 16% crude protein and consisted of wheat mill run, rolled barley, cane molasses, cottonseed meal, ground grain sorghums, and copra meal in a commercial mix for which the exact composition is not available.

Experiment 4: The cow was unfed (14 hours) and L-glutamate-U-^{14}C was injected via the jugular vein. In experiments 1, 3 and 4 the cow received feed 3 hours after ^{14}C-glutamate injection and was then returned to the normal feeding regimen.

An interval of at least 1 week elapsed between each experiment. Since more than 50% of the ^{14}C had left the cow as CO_2 and milk products in 2 days (52 to 63% of that injected), and the traces of ^{14}C that may have remained in CO_2, glucose and milk products after 7 days were too small to be detected by our methods, it is concluded that residual ^{14}C from one experiment did not affect the results of succeeding experiments.

Analytical methods. Expired CO_2 was collected by drawing air from a face mask through flow meters to an infrared CO_2 analyzer and to a spray tower for absorption of respiratory CO_2 in 4% NaOH solution. Absorbed CO_2 was precipitated as $BaCO_3$ and counted at "infinite-thickness" for calculation of the specific activity. Total radioactivity recovered in respiratory CO_2 was calculated from total air flow, CO_2 concentration and specific activity data.

Blood samples (15 ml) were collected and stored on ice in tubes containing 1 mg each of heparin and sodium fluoride. Samples were obtained at frequent intervals during the first 3 hours after glutamate injection. The blood was centrifuged within a few minutes of collection, and the plasma was stored at −15° until analyzed.

Plasma glucose concentration (table 1) was measured by the glucose oxidase method, and the specific activity of plasma glucose was determined after isolation of the glucosazone (12).

The specific activity of plasma glutamate was assayed by a procedure adapted from that used for algal amino acids (13). The plasma (3 ml) was deproteinized with 5% $ZnSO_4$—0.3 N $Ba(OH)_2$. The filtrate was concentrated to 1 ml under reduced pressure and transferred to a column of Dowex-50 resin (H^+ form; 0.7 cm diameter by 5 cm long). Ten milliliters of distilled water removed free sugars, and the amino acids were eluted with 4 N NH_4OH. The eluate was concentrated in vacuo over concentrated sulfuric acid to remove excess ammonia. The concentrated solution was then spotted onto paper and chromatographed in a solvent containing isobutyric acid, 2000 ml; 1-propanol, 200 ml; 2-propanol, 60 ml; 1-butanol, 60 ml; H_2O, 750 ml; 17 N NH_4OH, 100 ml; and ethylenediaminetetraacetic acid (EDTA), 1.0 g. This system gave good separation of the glutamic acid, free from other amino acids. A pure segment of the glutamate, located by a chromatogram scanner, was cut from the chromatograph, eluted and diluted to 2 ml. One milliliter was used to measure glutamate concentration (14), and the other portion was used for assay of radioactivity at "infinite-thickness." Counting efficiency at "infinite thickness" was established by plating increasing aliquots (5 to 50 uliter) of a ^{14}C-glucose solution that had been standardized against the National Bureau of Standards sodium bicarbonate-^{14}C standard (Standard No. 4924). This standardization was accomplished by mixing carrier glucose with an aliquot of the ^{14}C-glucose, com

TABLE 1

Conditions of experiments

	Exp. no.			
	1	2	3	4
Injected dose, mCi	0.96	0.72	0.72	0.72
Injection route	portal	portal	portal	jugular
Conditions	fasted	fasted and propionate	fed and propionate	fasted
CO_2 production, g atoms carbon/hr	2.91	3.14	3.75	3.45
Mean blood glucose, mg/100 ml	49	53	52	53

busting with Van Slyke reagent, precipitating the CO_2 as $BaCO_3$ and counting at infinite-thickness."

The milk collected at 3 hours and the pooled samples collected between 3 and 18 hours were separated into major components by methods described elsewhere (15, 16). Casein amino acids were separated, purified and crystallized as described earlier (17). All samples were oxidized by the Van Slyke wet-combustion technique; the evolved CO_2 was trapped, precipitated as barium carbonate and counted at "infinite-thickness" to determine specific activity of milk constituents. The internal labeling pattern of milk citrate was determined by the stepwise method of decarboxylation and oxidation (18). The ^{14}C assay was made on a low background Geiger counter, using duplicate planchettes counted for 20 minutes or 10,000 counts to reduce the counting error to 1% or less.

All data have been presented as standardized specific activities derived by dividing the specific activity of the samples (μCi per gram atom carbon) by the tracer dose (mCi), and have the dimensions μCi per gram atom carbon per mCi injected.

RESULTS

Plasma glutamate. The turnover of plasma glutamate was very rapid as shown by the time course of specific activity plotted in figure 1. During the first 30 minutes after injection, the specific activity decreased with a half-time of 4 to 6 minutes, in all experiments. After 30 minutes, when less than 10% of the ^{14}C-glutamate remained in the plasma, the rate of disappearance decreased, presumably reflect-

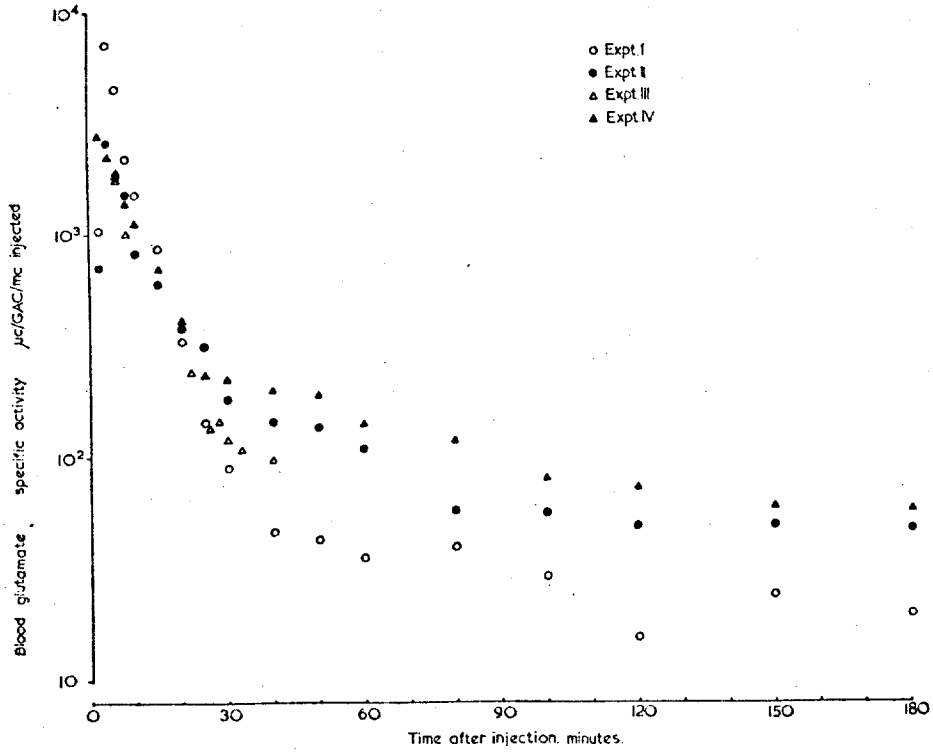

Fig. 1 Specific activity of plasma glutamate (μCi per gram atom carbon per mCi injected) during the first 3 hours after intravenous injection of U-^{14}C-glutamate into the cow. The ^{14}C-glutamate was injected into portal blood in experiments 1, 2 and 3 and into jugular blood in experiment 4. Other experimental variables were: cow unfed in experiment 1; unfed but received propionate intraportally in experiment 2; fed and received propionate intraportally in experiment 3; and unfed in experiment 4.

ing substantial reentry of labeled glutamate into the plasma pool. The plasma glutamate specific activity declined at a slightly slower rate when injection was made via jugular (exp. 4) rather than via the portal vein. The average standardized specific activity for plasma glutamate, calculated for 24 hours after injection was 24 and 22 units in experiments 1 and 2, respectively.

Respired CO_2. Carbon-14 appeared in the respired CO_2 within the first minute after injecting labeled glutamate and reached a maximum specific activity in 10 to 14 minutes (fig. 2). The curve for change of specific activity with time was similar for all treatments, but it had the lowest value when the cow was fed and intravenous propionate administered (exp. 3). During this latter experiment the CO_2 output of the cow was greater than it was at any other time. The cumulative percentage of the injected dose appearing in CO_2 during the first 3 hours ranged from [illegible] to 41% (table 3). During the 24 hours after glutamate injection, the average standardized specific activity of CO_2, estimated from continuous measurements during 3 hours and intermittent measurements later, was 2.30 and 1.85 units for experiments 1 and 2, respectively. The transfer quotient (specific activity ratio for CO_2/plasma glutamate) indicates that 8 to 10% of the respiratory CO_2 comes from oxidation of carbon that equilibrates with the plasma glutamate pool.

Plasma glucose. As with respired CO_2 carbon-14 from glutamate appeared rapidly in plasma glucose (fig. 3). The first sample collected within 3 minutes after injection of the ^{14}C-glutamate, contained isotope and the maximal specific activity occurred between 15 and 25 minutes. This maximum occurred somewhat later, and the

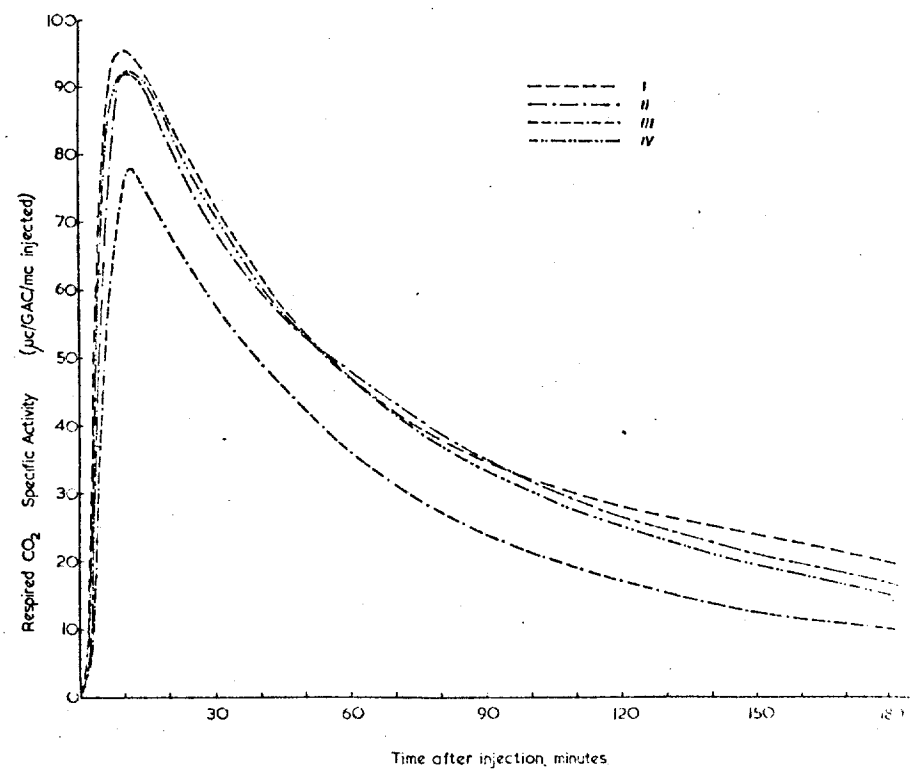

Fig. 2 Specific activity of CO_2 in respired air (μCi per gram atom carbon per mCi injected) after intravenous injection of U-^{14}C-glutamate into the lactating cow. See figure 1 for experimental conditions.

lue was of lower magnitude than in spired CO_2, in all experiments.

The specific activity in glucose was eatest when ^{14}C-glutamate was injected o portal blood, producing a value that s nearly equal to that in respired CO_2 xp. 1, figs. 2 and 3). Lower values reted when ^{14}C-glutamate was injected into gular blood (exp. 4) and when propiate was infused into the fed animal xp. 3). The average standardized specific ivity of plasma glucose, estimated from riodic measurements over 24 hours after ection, was 2.0 and 1.7 units for experiments 1 and 2, respectively. The nearly ual specific activity values for CO_2 and sma glucose indicate that about the me percentage of carbon in CO_2 and cose arose from the glutamate pool.

Milk constituents. The specific activity the major milk constituents, shown in le 2, reflects the relative utilization of bon from glutamate for biosynthesis of various components. The specific activof citrate always exceeded that of all other milk constituents and, in the milk collected at 3 hours, was two to three times as great as that of protein or lactose. These results indicated extensive synthesis of citrate from glutamate carbon, presumably reflecting the close metabolic relationship between citrate and α-ketoglutarate, derived from glutamate, in the tricarboxylic acid cycle. Lactose and casein had intermediate specific activities, but in more than half of the samples lactose had a greater specific activity than casein. Albumin had a lower specific activity than casein which would be anticipated in view of its lower glutamic acid content. In all experiments fat had only very low specific activities even after 48 hours which demonstrated that very little of the carbon from glutamate was utilized for fat synthesis in the lactating cow.

The cumulative recovery of carbon-14 in respired CO_2 and in milk constituents is listed in table 3. Forty to forty-five percent of the carbon-14 injected appeared in respired CO_2 and milk products during the

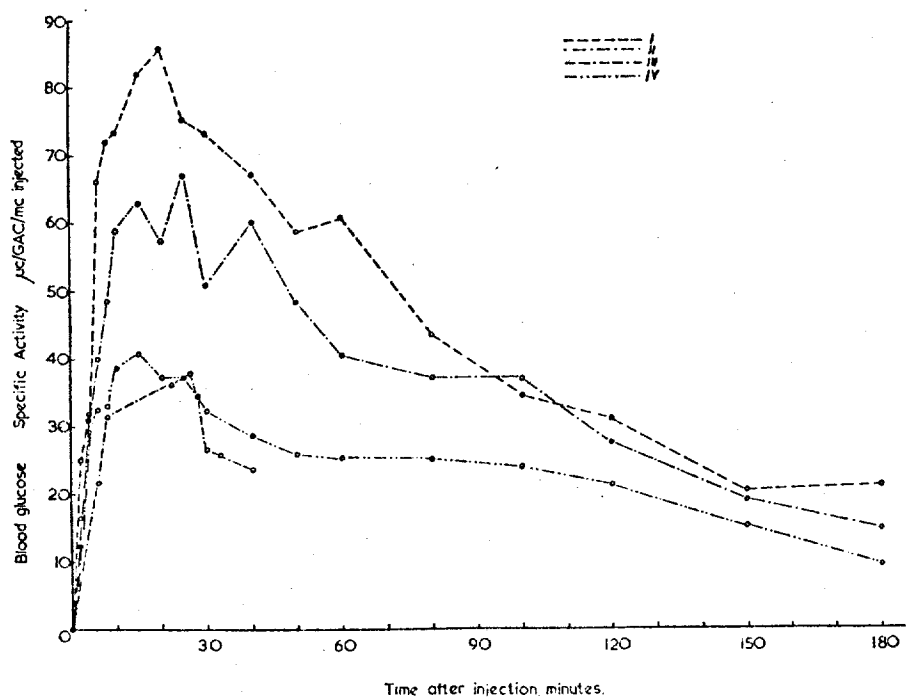

Fig. 3 The time course of plasma glucose specific activity (μCi per gram atom carbon per mCi injected) after intravenous injection of U-^{14}C-glutamate into the lactating cow. See figure 1 for experimental conditions.

first 3 hours, the larger amount appearing after injection of glutamate into jugular blood. From figure 2 it is apparent that considerable amounts of isotope were still appearing in the respired CO_2 3 hours after injection of the labeled glutamate so that the cumulative percentage oxidized would continue to increase significantly at later times. Continuous measurements of CO_2 production were not made, but on the assumption that the rate was constant over 24 hours it may be estimated that an additional 12 to 18% of the injected dose appeared in expired CO_2 during the subsequent 21 hours.

The total carbon-14 appearing in milk constituents during 48 hours was 11 to 15% of that injected. Among the milk constituents, lactose accounted for 46 to 54% and casein, 25 to 37% of the total carbon-14 recovered in milk. In all experiments the total activity in lactose relative to casein increased with time, indicating that at later times after injection the carbon from glutamate was used to a greater extent for glucose than for milk protein synthesis.

Table 4 lists the specific activities for several amino acids recovered from milk casein collected during the first 3 hours and from 3 to 48 hours after ^{14}C-glutamate injection. In all experiments the greatest specific activity occurred in glutamic acid although in several cases the specific activity of aspartic acid was nearly as great. This relationship would be anticipated since both amino acids are closely linked metabolically to intermediates of the tricarboxylic acid cycle. In addition, the high specific activity in aspartate may reflect the intervening role of aspartate in the transfer of carbon out of the mitochondria during gluconeogenesis (19, 20). Serine, alanine and glycine had lower specific activities than aspartic acid but greater than proline and arginine. Although glutamate is known to be an active precursor of proline and arginine in the rat (21), the specific activities of these three amino

TABLE 2

Specific activities of major milk constituents following intravenous injection of U-^{14}C-glutamate in each of four experiments

	Exp. no. 1	2	3	4
	μCi per gram atom carbon per mCi injected			
Milk 1 [1]				
Lactose	4.92	6.67	7.89	3.80
Casein	3.35	4.52	8.71	7.23
Albumin	1.78	3.03	5.74	3.20
Citrate	11.78	18.00	19.47	22.61
Fat	0.12	0.11	0.17	0.18
Milk 2 [2]				
Lactose	2.06	2.26	1.16	1.10
Casein	1.11	1.21	0.83	1.21
Albumin	0.83	0.88	0.77	0.99
Citrate	3.88	4.30	1.87	3.92
Fat	0.24	0.33	0.17	0.22

[1] Milk 1 was collected 3 hours after intravenous injection of ^{14}C-glutamate.
[2] Milk 2 contained pooled samples of milk collected between 3 and 48 hours after labeled glutamate was injected.

TABLE 3

Cumulative recovery of carbon-14 in respired air and milk constituents following intravenous injection of U-^{14}C-glutamate in each of four experiments

	Percentage of injected dose appearing in product							
	Exp. no. 1		2		3		4	
Respired CO_2 (3 hr)	37.6		38.2		34.2		41.4	
Time of sample	3 hr	48 hr	3 hr	48 hr	3 hr	48 hr	3 hr	48 hr
Milk constituents								
Lactose	1.50	6.66	1.52	6.09	3.48	6.73	1.30	7.29
Casein	0.90	3.35	0.91	3.07	3.39	5.44	2.18	5.07
Albumin	0.10	0.47	0.12	0.44	0.45	0.84	0.26	0.71
Citrate	0.13	0.48	0.15	0.46	0.31	0.50	0.28	0.66
Fat	0.07	1.21	0.05	1.33	0.14	1.03	0.12	1.28
	2.70	12.17	2.75	11.39	7.77	14.54	4.14	15.01
Milk and CO_2	40.3		41.0		42.0		45.5	

TABLE 4

Specific activities of casein amino acids recovered from milk 3 hours and 48 hours following intravenous injection of U-^{14}C-glutamate in the cow [1]

Time of sample	Exp. no. 1 3 hr	1 48 hr	2 3 hr	2 48 hr	3 3 hr	3 48 hr	4 3 hr
Amino acids	*μCi ^{14}C per gram atom carbon per mCi injected*						
Glutamate	9.68	3.97	12.6	4.36	37.8	2.65	27.0
Aspartic	6.66	2.56	11.3	—	17.9	1.73	15.1
Serine	2.26	2.36	6.22	1.60	—	1.27	5.46
Alanine	2.68	1.65	5.12	1.99	6.56	0.88	4.08
Glycine	—	1.49	3.43	1.75	5.40	1.27	—
Proline	1.24	0.87	1.54	0.77	3.80	0.61	2.87
Arginine	0.46	0.66	0.08	0.72	0.88	—	—

[1] See Methods for details of experiments.

ids show that only 10 to 20% of the roline and arginine had been derived om glutamate in the cow. Very low levels f isotope were observed in valine, threoine, leucine, lysine and histidine, which e known to be essential amino acids for e cow (22); therefore, their signifint synthesis from glutamate would not expected.

Citrate. Because of the very high spefic activity in citrate, which exceeded that casein glutamate in the 3-hour samples, me citrate samples were partially deaded; and, from the intramolecular disbution of isotope, an attempt was made establish the metabolic relationship been this compound and glutamate. The sults shown in table 5 provide only rtial clues but these indicate that much the citrate was derived directly from etoglutarate by CO_2 fixation, which agrees with evidence from perfused mammary gland experiments (23). This route incorporates CO_2 carbon into the C-6 position of citrate which had the lowest specific activity in the molecule. The remaining carbons of citrate would be derived directly from glutamate, via α-ketoglutarate.

Although the specific activity of citrate was greater than that of casein-glutamate at 3 hours it was less in the later milk samples (see tables 2 and 4). These relationships presumably reflect the greater time interval between synthesis and secretion in milk for casein compared with citrate.

TABLE 5

tribution of carbon-14 in citrate isolated from milk after ^{14}C-glutamate injection

	Exp. no. 1	2	3
	μCi per gram atom carbon per mCi injected		
C_5 [1]	10.45	12.89	13.27
C_3+C_4	16.32	20.12	19.20
	0.24	0.52	1.02
al specific activity	11.68	14.44	14.19
bon-14 recovery n degradation	97.1%	80.2%	72.9%

C_1 and C_5 designate terminal carboxyls; C_6 desigs the middle carboxyl.

DISCUSSION

Glutamic acid is the most abundant component of milk protein comprising 19.8% of the amino acid residues (5). On this basis, one might anticipate extensive incorporation of ^{14}C-glutamate into milk protein. In fact, only 3.5 to 6.3% of the carbon-14 from glutamate was recovered in milk proteins during 48 hours after adding the labeled glutamate to the plasma pool, which indicates that there was extensive utilization of glutamate carbon for many processes other than protein synthesis. On the assumption that the ^{14}C-glutamate had all been removed from the body pool at 48 hours and from the percentage recovery of carbon-14 in milk protein, one may estimate that the flux through the glutamate pool must have been some 16 (100/6.3) to 29 (100/3.5) times greater than the amount of glutamate actually in-

corporated into the protein fractions of milk.

More intriguing, in view of the large glutamate content of milk protein, was the extensive recovery of carbon-14 in the lactose of milk. In all experiments, whether the cow was fed or not, the isotope recovered in lactose over 48 hours was greater than in milk protein; in the nonfed cow receiving ^{14}C-glutamate portally, the differential was nearly twofold in favor of lactose. Thus, it is evident that gluconeogenesis acquires a larger fraction of the glutamate carbon than does protein synthesis during milk formation. One could argue that the greater recovery of isotope in carbohydrate relative to protein simply reflects the transient effects of milking and was, therefore, an experimental artifact. The cow was completely milked just before ^{14}C-glutamate injection; thus, if this condition resulted in a preferential stimulation of gluconeogenesis, then more of the label would be transferred to carbohydrate soon after the injection, when the pool was still highly labeled. This explanation must be rejected, however, as the ratio of carbon-14 recovered in lactose/protein was always greater after 48 hours than it was after 3 hours (table 3).

The most reasonable explanation for the results observed would be that the rate and extent of glutamate catabolism in the cow is much greater than the rate and extent of its utilization for protein synthesis. Several factors support the conclusion that glutamate has a very active and diverse metabolic role. It has been shown that glutamate disappeared from the plasma with a half-life of about 5 minutes during the initial period after injection of the labeled glutamate (fig. 1). This change was accompanied by the very rapid appearance of carbon-14 in respired CO_2 and plasma glucose (figs. 2 and 3), demonstrating that tissue uptake and metabolism of glutamate paralleled its rapid disappearance from the plasma. Consequently, carbon of maximal specific activity appeared in respired CO_2 in about 12 minutes and in plasma glucose in about 20 minutes after the labeled glutamate was added to the plasma glutamate. The high specific activity in citrate and in aspartate, which is derived from oxaloacetate, demonstrated that the carbon from glutamate entered extensively into reactions of the tricarboxylic acid cycle. Since the cycle is intimately involved in gluconeogenesis (20, 24, 25) through the intervening role of oxaloacetate, it is apparent that these relationships favor extensive transfer of carbon from glutamate into carbohydrate synthesis in the intact animal.

A perennial question that has plagued investigators working with intact animals concerns the interpretation of isotope data when labeled carbon from a compound equilibrates extensively with active metabolic pools in the body. Under these conditions, product-precursor relationships become diffuse, and the physiological significance of isotope transfer among metabolites is difficult to assess accurately. This problem has been considered by various investigators (26–29), most recently by Krebs et al. (30) in relation to gluconeogenesis from ^{14}C-lactate and ^{14}C-acetoacetate in kidney slices. Krebs et al. concluded that when there was a "crossing over" of carbon between substrates of respiration and gluconeogenesis (in the tricarboxylic acid cycle), the fate of a label does not reflect the *net* fate of a labeled metabolite. It is this phenomenon which accounts for the extensive transfer of isotope from some even-chain fatty acids to carbohydrate even though animals lack a pathway for the net synthesis of glucose from these substrates. This matter has been reviewed by Weinman et al. (29) and has been discussed for the ruminant in relation to the paradoxical behavior of butyrate-^{14}C (31, 32). Unlike the case with butyrate, there is a pathway for net conversion of carbon from glutamate to glucose, via the tricarboxylic acid cycle. This fact, together with the rapid appearance of substantial levels of carbon-14 in plasma glucose and the recovery of 6 to 7% of the isotope in lactose during the same interval (48 hours) during milk protein acquired 3 to 6% of the glutamate-^{14}C, clearly demonstrates a gluconeogenic role for plasma glutamate in the lactating cow and one which, quantitatively, is more important in gluconeogenesis than in protein synthesis.

The transfer quotient, which is the specific activity ratio of plasma glucose/plasma glutamate, provides one means to

estimating the precursor role of glutamate in glucose synthesis. In experiments 1 and 2, respectively, the standardized specific activities over 24 hours were 2.0 and 1.7 for plasma glucose, and 24 and 22 for plasma glutamate. The ratios for these figures indicate that approximately 8% of the glucose carbon had been derived from plasma glutamate. This value does not necessarily reflect the overall role of glutamate as a glucogenic precursor in the cow since it is valid only for glutamate which equilibrates (in specific activity) with the plasma pool. Within certain cells, the labeled glutamate entering from the plasma pool may be diluted by glutamate derived from protein turnover or glutamate synthesized from unlabeled precursors in the cell. These processes would reduce the specific activity of the glutamate at the site of gluconeogenesis, and the estimate of glucose synthesis based on plasma glutamate would then be too low.

The specific activity of glutamate recovered from casein (see table 4) in the 3-hour and 48-hour milk samples was considerably lower than the average value measured in plasma glutamate. This difference reflects a dilution by the mammary gland which has been shown to incorporate carbon from fatty acids and carbohydrate into glutamate (33). Thus, an estimate of the quantitative precursor role of glutamate in the mammary gland based on plasma glutamate specific activity would be erroneously low, and it seems likely that a similar situation would occur in all tissues, including the liver where most gluconeogenesis occurs. It would be necessary to have the specific activity of glutamate at the site of gluconeogenesis to get a more accurate estimate of its precursor role in the cow. This information was not obtained; thus, the estimate based on plasma glutamate, while almost certainly too low, is the best value available.

The transfer quotient for casein based on the specific activity of mammary gland (casein) glutamate (table 4) ranged from 0.23 to 0.36 (specific activity ratio for casein/glutamate) for the various milk samples. The lower figure corresponds closely to the glutamate composition of casein (22.4%), and the higher values reflect the presence of carbon-14 in other amino acids that the cow synthesizes.

Any quantitative values for product-precursor relationships are necessarily approximations in an organism as complex as the intact mammal. In any case, such evaluations would be applicable only to those conditions under which the measurements were carried out, since many variables could affect these relationships and lead to different results. For example, data from experiments 3 and 4 indicate that availability of additional gluconeogenic material and injection of labeled glutamate into peripheral circulation to avoid an initial passage of the amino acid through the liver are both factors which divert somewhat more glutamate toward protein synthesis. Recoveries of carbon-14 in milk casein and albumin were higher in both experiments, but there was no simultaneous decrease in lactose. Although the quantitative aspects of glutamate utilization in the lactating cow are uncertain, it seems clear that when there are both protein synthesis and gluconeogenesis, the latter process will acquire a greater part of the glutamate carbon, even in those conditions which presumably provide ample levels of alternate glucogenic substrate.

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1961

·labolism of Glutamic Acid, Valine and ·inine by the Lactating Goat [1]

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ABSTRACT The metabolic fate of uniformly ^{14}C-labeled L-glutamic acid, L-valine and L-arginine, injected intravenously as a pulse dose into lactating goats, was studied in a series of six experiments. Glutamic acid was the most rapidly and extensively oxidized of the amino acids. In the first 3 hours after injection, respired CO_2 contained about 40% of the carbon-14 from glutamate, and the peak specific activity in CO_2 was reached within 10 to 12 minutes. The slower oxidation of valine and arginine was apparent from carbon-14 recoveries in CO_2 during 3 hours which amounted to 15% and less than 10%, respectively. The maximum specific activity occurred in CO_2 at 20 and 45 minutes following the injection of valine and arginine, respectively. Glutamate was the most glucogenic of the three amino acids. Carbon-14 from glutamate appeared earliest and in largest amounts in plasma glucose, while smaller amounts of ^{14}C from valine and arginine appeared at a slower rate. More of the carbon-14 from glutamate appeared in lactose than in milk protein, whereas the largest part of the carbon-14 from valine and arginine was recovered in milk protein. Citrate had high specific activity, especially in the glutamic acid experiments, whereas milk fat had very low specific activity after the injection of all three amino acids. It is concluded that, in the goat, processes involving gluconeogenesis acquire a greater part of the glutamate flux than processes involved in milk protein synthesis. The reverse relationship is true for valine and arginine. The relatively slow metabolism of valine and arginine has the effect of conserving these amino acids for protein synthesis; it also results in a prolonged availability of the carbon from these amino acids for gluconeogenesis.

·an earlier paper (1), concerning the ·olic fate of uniformly labeled ^{14}C-L-·mic acid injected intravenously into ·tating dairy cow, it was reported ·the amount of glutamate carbon uti-·l for synthesis of carbohydrate was ·er than that utilized for synthesis of ·n during milk formation. While gluta-·has long been recognized as a gluco-·amino acid (2, 3), the observed re-·indicating a very sparing use of pre-·d glutamate as a source of the gluta-·of milk protein, was surprising, par-·ly since glutamate comprises 22% ·casein molecule (4).

·present study extends the investiga-·d glutamate metabolism to another ·of lactating ruminant, the goat, ·in addition, compares the metabolism ·other glucogenic amino acids, valine ·arginine, with that of glutamate. The ·are evaluated in terms of the rate ·xtent of amino acid oxidation, the ·ity with which carbon from each ·acid appears in plasma glucose, and the extent of utilization of each amino acid for synthesis of the major constituents of milk, as indices of their roles in biosynthetic processes.

METHODS

Experimental animals. The three lactating goats used in these experiments were pure-bred animals obtained from a milking herd; one of the goats was of the Saanan breed (No. 2343) and the other two were Toggenbergs. Other characteristics of the goats and the conditions of each experiment are given in table 1. Permanent indwelling portal catheters had been inserted into the ruminal veins of two of

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TABLE 1

Conditions of experiments

	Amino acid injected					
	Glutamate		Valine		Arginine	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Goat no.	2343	2343	2344	2343	2343	2344
Body weight, kg	38.6	38.6	50.4	41.8	37.2	49.0
Injected dose, mCi	0.223	0.125	0.134	0.134	0.207	0.111
Route of injection	Portal	Portal	Portal	Jugular	Portal	Jugular
Condition of animal	Unfed	Unfed and Propionate	Unfed	Unfed	Unfed	Unfed
CO_2 production, moles/hour	0.96	0.78	0.99	0.92	0.93	0.81
Milk yield, kg/day	1.6	1.4	0.9	1.8	1.4	1.1

the goats under surgical anesthesia several days before the experiments, according to the method described by Parker et al. (5). The goats were housed indoors, together, on wood shavings, and were trained to be well accustomed to all procedures used during the metabolic experiments.

Diet. Each goat ingested approximately 1.5 kg per day of long alfalfa hay and in addition received twice daily, at milking, 0.5 kg of a grain concentrate. The protein and energy provided by this amount of food was in excess of the estimated requirements for lactating goats based upon data cited by Morrison (6). All animals were on full feed prior to the experimental period, but no feed was available to the animal for 14 hours before the start of each experiment. This period without food does not produce a fasted condition in the goat since a considerably longer period of time is required for the rumen to empty. It does provide a relatively standard condition with regard to intraruminal fermentation and CO_2 production but avoids the prolonged period necessary for a fasting condition which would interfere with milk production.

Uniformly-labeled ^{14}C-amino acids. These were prepared by hydrolyzing the protein from *Chlorella pyrenoidosa* labeled during photosynthesis in an atmosphere containing $^{14}CO_2$ of high specific activity (25.5 mCi/mmole). The procedure used to isolate and establish the radiochemical purity of the amino acids was described earlier (1). Though amino acids synthesized during $^{14}CO_2$ exposure were diluted by protein and preformed amino acids in the microorganisms, the specific activity of the amino acids isolated was sufficiently high that only tracer amounts of the amino acids were injected, thus avoiding the problems that could occur from injection of larger amounts which might disturb normal blood concentrations.

Experimental procedures. On the day preceding an experiment a plastic catheter was inserted downward in each external jugular vein under local anesthesia. The catheter was stitched in place, filled with a heparinized saline solution and closed with a small plug. At 8 AM on the morning of each experiment, the goat was transferred to a small pen in the metabolism laboratory and connected by face mask to the respiration apparatus for 0.5 hour prior to the metabolism experiment. The experiment began with the injection of the labeled amino acid through the plastic cannulae, either into portal blood or jugular blood. The ^{14}C-amino acid was always injected near 9 AM to avoid any influence of diurnal variation on metabolic behavior. The amino acid solution was injected over a period of 2 minutes, an approximate circulation time for the blood, in order to distribute the material throughout the total blood volume. The syringe and catheter was flushed twice with 15 ml of sterile saline during an additional 1 minute. The injection was made via the jugular catheter; a second catheter inserted into the other jugular vein was used for drawing blood samples. The use of plastic catheters allows the injection of tracer and the

l of blood samples without notice- disturbance to the experimental ani- condition of critical importance in bolic studies. This is especially true udies involving gluconeogenesis since emotional disturbance of the animal affect adrenal activity with subse- changes in liver glycogen and blood se levels.

spired CO_2 was collected continuously plasma samples intermittently during first 3 hours after injection of the led amino acids. At 3 hours after in- on, the first milk sample was collected, the animal was then fed. Subse- tly, intermittent samples of respired blood, and milk were collected for a d of 2 days, under the feeding condi- already described.

experiment glutamate 2, the goat re- d a solution of sodium propionate, dized to pH 7.0, at the rate of 0.1 per hour beginning 0.5 hour before ion of the ^{14}C-glutamate and continu- r 3 hours thereafter. The purpose of experiment was to test the glucogenic or of glutamate when substrate ts of an alternate glucogenic pre- r were available to the animal.

h goat was milked out completely diately before the injection of the ino acid. The milk collected at 3 after the injection and an aliquot e composite sample collected between d 48 hours after the injection were d, by procedures described elsewhere , to isolate the major components the milk. A sample of each compon- was oxidized by the Van Slyke wet com- ion technique, the CO_2 precipitated as m carbonate and counted for radio ity as planchets of "infinite thickness" e an end-window, low-background, r counter. All samples were counted sufficient period to reduce the prob- rror of counting to less than 2%.

he procedure for assessing the specific ity of respired CO_2 and plasma glucose een described earlier (1).

ts of measurement. All specific ac- s have been expressed in units of curies per gram atom of carbon di- by the microcuries injected per kilo- of body weight. Specific activity expressed in these standard units provides a basis for comparing results in different animals when body weight and the amount of isotope injected into experimental animals varies.

RESULTS

Respired CO_2 The change with time in specific activity of respired CO_2 is shown by the curves in figure 1. It is clear that a greater part of the glutamate pool underwent oxidation early whereas the oxidation of valine was considerably slower and that of arginine was slowest of all. Table 2 summarizes pertinent results on the rate and extent of appearance of ^{14}C in respired CO_2 after injecting the three ^{14}C-labeled amino acids. Although there was a significant lag during the injection of each amino acid, the first ^{14}C was detected in respired CO_2 even before the injection was completed, appearing within the first minute with glutamate, after 1.3 minutes with valine, and after 2 minutes with arginine. These time intervals have been corrected for the 25-second period that elapses between intravenous injection of sodium carbonate-^{14}C and the first appearance of isotope in respired CO_2. Thus, these intervals represent the time required for the labeled amino acids to leave the blood, enter body cells, undergo deamination and subsequent metabolism to the level of CO_2, and for the latter to diffuse from the cells back into the blood. The results provide direct evidence for the great speed of this sequence of processes in the intact animal.

The specific activity in CO_2 increased rapidly, reaching maxima of 15 and 18 standard units in trials 1 and 2, respectively, at 10 to 12 minutes after the start of the ^{14}C-glutamate injection. When valine was administered the specific activity maxima for CO_2, which were less than 5 standard units, were only 30% as great as in glutamate experiments, and occurred 15 minutes later, or about 25 minutes after valine injection. After ^{14}C-arginine injection, CO_2 specific activity maxima of 1 to 2 standardized units were reached about 60 minutes after the injection. Relative to the glutamate results, the average specific activity in CO_2 during 3 hours following the injection was about 40% as great after

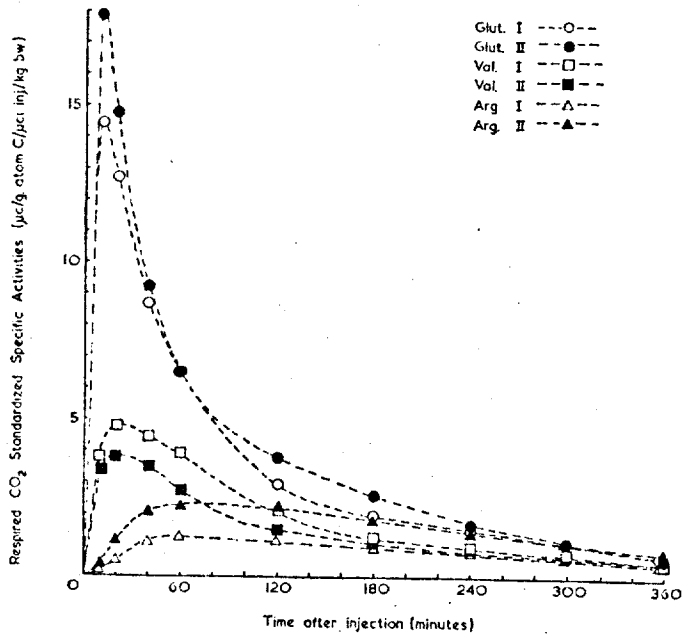

Fig. 1 The specific activity of expired CO_2 in the first 6 hours after injection of U-[illegible] glutamic acid, U-^{14}C-valine, or U-^{14}C-arginine, each in two experiments (details in text).

valine and about 20% as great after arginine. The percentage of the injected carbon-14 recovered in CO_2 during 3 hours of continuous collection was 7 to 10% for arginine, 14 to 16% for valine, and 39 to 44% for glutamate. The high values for glutamate oxidation are in close agreement with results observed in the lactating cow (1) and support the conclusion that glutamate undergoes rapid and extensive [illegible]bolism in the lactating ruminant.

Plasma glucose. The time cou[illegible] specific activity in plasma glucose aft[illegible]jecting each of the ^{14}C-labeled amino [illegible] is shown in figure 2. There were signif[illegible] amounts of carbon-14 in plasma [illegible] within 2 minutes after the injec[illegible] ^{14}C-glutamate and a maximum spec[illegible]

TABLE 2

Rate and extent of appearance of ^{14}C in respired CO_2 and plasma glucose

Amino acid	Respired CO_2				Carbon-14 recovery during 0–3 hr.	Plasma glucose		
	Elapsed time [1] for		Specific activity [2]			Elapsed time [1] until maximum glucose spec. activity	Specific [illegible]	
	First $^{14}CO_2$	Maximum spec. activ.	Maximum	Average (0–3 hr.)			Maximum	[illegible]
	min	*min*			%	*min*		
Glutamate 1	0.8	8.5	14.6	6.0	44.2	18	9.8	[illegible]
Glutamate 2	0.8	11.2	17.8	6.35	38.6	18	11.2	[illegible]
Valine 1	1.4	24	4.9	2.7	15.9	40	2.7	[illegible]
Valine 2	1.2	20	3.8	2.1	14.0	45	1.8	[illegible]
Arginine 1	1.8	60	1.2	0.9	6.6	60–120	2.2	[illegible]
Arginine 2	2.0	60	2.3	1.9	9.6	60–90	3.6	[illegible]

[1] Time after commencement of injection. The elapsed time for first appearance of $^{14}CO_2$ has been [illegible] for the 25-second period after intravenous injection of $^{14}CO_2$ until the first ^{14}C appears in respired [illegible] these data represent the cumulative interval for the metabolism of some of the ^{14}C-amino acids to CO_2 [illegible] transfer of the latter from the cell to blood.

[2] Specific activity in standardized units: μCi ^{14}C/gram atom carbon per μCi injected/kg body weight.

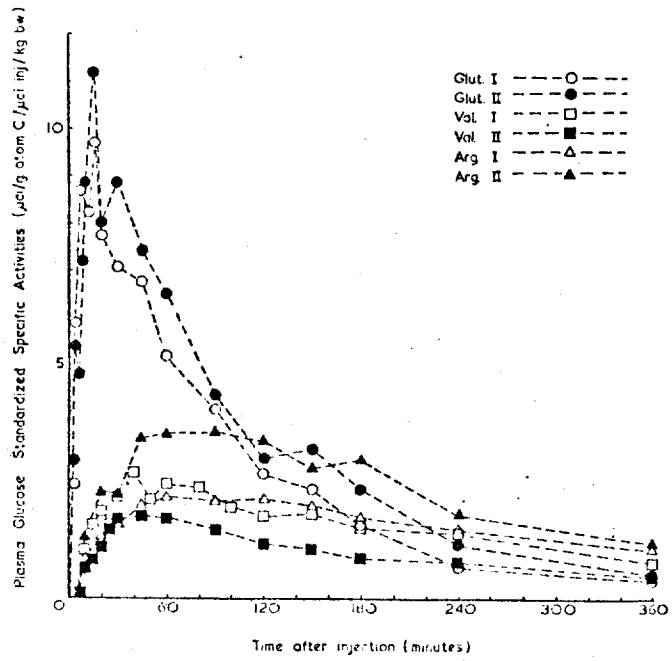

Fig. 2 The specific activity of plasma glucose in the first 6 hours after injection of U-^{14}C-glutamic acid, U-^{14}C-valine, or U-^{14}C-arginine, each in two experiments (details in text).

of 10 to 12 standard units, was reached after approximately 18 minutes. Carbon-14 appeared in plasma glucose much more slowly after injection of ^{14}C-valine or ^{14}C-arginine. In comparison with that for ^{14}C-glutamate, the maximum specific activity of glucose (fig. 2 and table 2) was only 30% and 20% as great for ^{14}C-valine and ^{14}C-arginine, respectively. The corresponding specific activity maxima occurred approximately 45 minutes after injection of ^{14}C-valine and from 60 to 120 minutes after the injection of ^{14}C-arginine. The average specific activity in plasma glucose during 3 hours after injection of each amino acid was greater for arginine than for valine but greatest of all after injection of glutamate. These relationships demonstrate that, relative to the other two amino acids, a greater part of the glutamate flux was utilized in gluconeogenesis in the early hours following injection of the labeled amino acids. However, the specific activity fell very rapidly with time after ^{14}C-glutamate, while it remained almost on a plateau, decreasing very slowly, after injection of labeled arginine and valine. In fact, 3 hours after injection the ^{14}C from arginine was still being utilized at near maximum values for gluconeogenesis, and by 6 hours, for both amino acids, the specific activity in plasma glucose had exceeded that in the glutamate trials.

During the first 3 hours, the average specific activity in respired CO_2 exceeded that in plasma glucose after injecting glutamate and valine, but not after injecting arginine (table 2). One may conclude from these results that during the catabolism of the amino acids a relatively larger proportion of the valine- and glutamate-carbon was oxidized to CO_2 and a smaller proportion incorporated into glucose than was the case for arginine-carbon.

Milk constituents. The specific activities of the major milk constituents (table 3) were strikingly different for the three amino acids, reflecting the strong glucogenic propensity of glutamate and the preferential utilization of carbon from valine and arginine for protein synthesis. The specific activity of citrate generally exceeded that of lactose for all three amino

TABLE 3

Standardized specific activities [1] of milk constituents after injecting uniformly ^{14}C-la[illegible] L-glutamate, L-valine, and L-arginine intravenously into lactating goats

Constituent	Glutamate		Valine		Arginine	
	1	2	1	2	1	2
			Milk 1 [2]			
Lactose	1.26	0.24	1.01	0.49	0.82	0.32
Casein	0.20	0.22	16.76	3.85	2.14	2.14
Albumin	0.27	0.29	7.17	3.54	1.62	1.58
Citrate	1.86	1.09	3.93	1.54	0.99	0.21
Fat	0.04	0.09	0.11	0.02	0.01	0.03
			Milk 2 [3]			
Lactose	0.18	0.11	0.30	0.23	0.09	0.16
Casein	0.11	0.05	2.25	1.53	1.79	2.30
Albumin	0.12	0.13	1.25	0.68	0.76	0.83
Citrate	0.08	0.13	0.46	0.20	0.14	0.21
Fat	0.07	0.01	0.11	0.01	0.01	0.01

[1] Specific activities have been calculated on a basis of the relative injected dose of ^{14}C and are expressed in standardized units: μCi/^{14}C gram atom carbon per μCi injected/kg body weight.
[2] Milk 1 was collected 3 hours after injection of each ^{14}C-amino acid.
[3] Milk 2 is the cumulative sample collected between 3 and 48 hours after injection.

acids, and in the glutamate experiments these two substances had the greatest specific activities among the milk constituents. In contrast, the specific activity of the milk proteins, casein and albumin, was greater than that of any other milk constituent in the trials with valine and with arginine.

The cumulative recovery of injected carbon-14 in the various constituents is listed in table 4. These calculations reflect the fraction of the flux through each amino acid pool which was utilized for synthesis of milk protein, carbohydrate, or fat during 3 hours and 48 hours after injection of each ^{14}C-amino acid. The 48-hour recovery of carbon-14 from glutamate in total milk products, 2.5 to 6% of that injected, was present mainly in lactose and, to a smaller extent, in milk protein. In the case of valine the milk product recovery was 18 to 25% and, for arginine, 17 to 22% of the total injected. Milk protein alone accounted for 87 and 92% of the total carbon-14 recovered in milk products after injection of valine and arginine, respectively.

The specific activity of milk fat (table 3) was low in all experiments indicating that very little of the carbon flux from any of the amino acids had been utilized for fat synthesis in the lactating goat. A similar result was obtained when glutamate was injected into the lactating cow (1).

The route of injection of the L-amino acids did not seem to have [illegible] major influence on their metabolism. Although the observations are limited, jugular vs. portal injection had little effect on the total carbon-14 recovered in milk during 3 hours after injection of valine or arginine. Similarly, most of the carbon from these amino acids which was recovered in milk products was present in protein, irrespective of the route of administration. If one compares the results in terms of total carbon-14 recovered in milk products, it appears that a somewhat greater amount was recovered after jugular injection of valine, whereas the recovery favored the portal injection of arginine. Our results are too limited to decide whether these differences are real or simply represent experimental variation.

^{14}C-glutamate was injected into portal blood in both experiments with goats. In earlier studies in the cow (1), the effect of jugular vs. portal injection indicated somewhat greater utilization of ^{14}C-glutamate for carbohydrate synthesis after portal injection but the total ^{14}C in lactose exceeded that in milk protein after either route of injection. As in the case with valine and arginine, then, the two routes of injection would not appear to have a major influence on whether the amino acid will be used largely for synthesis of carbohydrate or protein during milk formation.

TABLE 4

Cumulative percentage of injected carbon-14 appearing in milk constituents after injection of uniformly ^{14}C-labeled L-amino acids

Constituent	Amino acid injected											
	Glutamic acid				Valine				Arginine			
	1		2		1		2		1		2	
	3[1]	48[1]	3	48	3	48	3	48	3	48	3	48
Casein	0.06	1.38	0.14	0.62	6.22	14.43	2.67	19.65	0.66	19.29	0.79	14.14
Albumin	0.02	0.32	0.04	0.30	0.54	1.49	0.50	2.05	0.10	1.74	0.11	1.09
Lactose	0.38	2.34	0.15	1.13	0.35	1.35	0.32	2.72	0.23	1.06	0.10	0.95
Citrate	0.03	0.06	0.03	0.08	0.06	0.12	0.04	0.12	0.01	0.06	0.00	0.05
Fat	0.03	1.90	0.15	0.42	0.09	0.98	0.02	0.35	0.01	0.35	0.02	0.49
Total ^{14}C in milk products	0.52	6.00	0.51	2.55	7.25	18.37	3.56	24.89	1.01	22.50	1.02	16.72

[1] Time after injection, in hours.

DISCUSSION

The glucogenic nature of several amino acids was first established, and later confirmed, in experimental animals by methods which in some cases, could be questioned physiologically (2, 10–13). The results observed after injection of large amounts of an amino acid into animals that have been treated with phlorhizin, fasted for a prolonged period, or made diabetic do not necessarily reflect the metabolic fate of that compound in the normal animal. Furthermore, the problem of imbalance and toxicity (9) may occur when a large amount of a single amino acid is administered which may influence the results obtained.

The present investigation utilized tracer methods for studying metabolic relationships in the normal, intact animal and, thus, is free from pharmacological implications which might be attached to earlier studies. The technique provides a means for evaluating the metabolic fate of amino acids in the animal with minimal physiological insult and for assessing their glucogenic potential.

The lactating ruminant has several characteristics making it uniquely suited for studying gluconeogenesis from amino acids. There is a large and persisting precursor requirement for milk protein and carbohydrate, each of which comprises about 25% of the carbon in milk of the goat and cow (14). In addition, the ruminant has a paucity of alimentary glucose (15–17) because of rumen fermentation processes. Consequently, there is extensive gluconeogenesis in these animals, and amino acids provide one source of carbon for meeting this metabolic requirement. Since amino acids are also needed in large amounts for milk protein formation, there will be a competition among metabolic processes for these metabolites. The amount of carbon from a given amino acid utilized for synthesis of carbohydrate, in relation to protein, provides an index of the glucogenic potential of that amino acid in the lactating ruminant.

The technique of pulse labeling the plasma amino acid pool allows one to assess the temporal distribution of carbon from each amino acid and its quantitative

utilization, in relation to the flux through the pool, in various metabolic processes. In the present studies it was demonstrated that glutamate was rapidly and extensively metabolized, with processes involved in gluconeogenesis acquiring a larger part of the glutamate carbon than protein synthesis. This result was surprising in one respect, namely, that glutamate comprises nearly one-fourth of the amino acid residues in casein (4), and consequently, one would anticipate extensive incorporation of this amino acid into milk protein. On the other hand, the glucogenic propensity is consistent with the metabolic behavior of glutamate reported by Lusk (2) and others (3); with results obtained in the lactating cow (1); and with the known route for metabolism of glutamate via α-ketoglutarate and the tricarboxylic acid cycle, which is closely involved in gluconeogenesis (18, 19).

Valine and arginine, which are also considered to be glucogenic amino acids (3) behaved quite differently from glutamate. The rate and extent of their utilization for synthesis of plasma glucose and lactose was considerably lower than that from glutamate, whereas the recovery of carbon-14 in milk protein was much greater. It is clear that among amino acids with a glucogenic potential and in a system where the requirement for glucose precursors is high, there will be great differences in the extent of utilization of each amino acid to meet this requirement.

The speed with which carbon from the amino acids appeared in plasma glucose varied markedly. There was a sharp crest of specific activity which appeared in glucose (fig. 2) about 18 minutes after glutamate injection. However, after valine, and especially after arginine, carbon-14 appeared in glucose very slowly, producing an extended plateau rather than a peak in the specific activity, as with glutamate. One can conclude that carbon from glutamate would be readily available for glucose synthesis whereas that from valine and arginine would become available over a prolonged interval. The physiological implications of the individual differences in the delay of transfer of carbon from amino acids to glucose has been pointed out previously (20). The present results prov additional support for the conclusion th protein metabolites provide the animal w a sustained supply of glucogenic precur which become converted into glucose c protracted periods.

The glucogenic potential of the am acids is not simply related to the extent catabolism. It is true that the most gl cogenic of the three amino acids, glu mate, was also most extensively catab ized. On the other hand the oxidation valine was nearly twice that of argin (table 2), yet the average specific activ of plasma glucose was greater after a nine-^{14}C than it was after valine-^{14}C. Th although a relatively greater part of t valine flux was undergoing oxidation, c bon from arginine was appearing in gre amounts in the plasma glucose.

The utilization of the amino acids f milk synthesis (table 3) demonstrates other metabolic characteristic of the a minal. Although glutamate is the ma constituent of milk protein (4), relatiu little of the ^{14}C from glutamate and, th a small part of the total flux through t glutamate pool was actually incorpora into milk protein (table 4). Much m of the glutamate underwent oxidation entered pathways of gluconeogenesis. C sequently, one may conclude that in t lactating goat, as in the lactating cow, t metabolic processes involved in gluco genesis acquire a greater part of the glu mate carbon than does protein synthe Glutamate is nonessential, nutritiona and it is clear that it participates widel metabolic processes. Most of the glutam acid utilized for protein formation was tually synthesized by the goat.

With the other two amino acids, t specific activity of milk protein was eral times higher than any other milk stituent. Valine, in contrast to glutam is nutritionally essential in all higher mals so far investigated, including the (21), man (22), dog (23), and the (24). It is less certain whether argin nutritionally essential. It is formed by mals during urea synthesis, but, at leas the rat, the rate of synthesis appears adequate for optimal growth (21). T

can synthesize arginine, as shown by transfer of significant amounts of carbon-14 from acetate, propionate (24), glucose (25) and glutamic acid (1), but the of ^{14}C was considerably lower than in nonessential amino acids like glutamate, aspartate, serine, and alanine. Thus, the rumen microbes make the animal or less independent of a dietary supply of essential amino acids, the animal itself appears to have a limited ability to synthesize arginine. This amino acid is a potentially critical nutrient for whose requirements may, under some circumstances, exceed synthetic capabilities the ruminant animal.

The cumulative recovery of carbon-14 (Table 3) in milk constituents demonstrates that valine and arginine were preferentially conserved for protein synthesis, compared to glutamate. In fact, the recovery in milk protein of valine and arginine was about equal to, and may have exceeded, the amount oxidized to CO_2. It appears that metabolic processes are geared to conserve those amino acids which cannot be synthesized by the animal to insure their availability for protein synthesis. This sort of priority arrangement would be advantageous for survival since once catabolism of carbon chain is initiated, the "essential" amino acids are lost to the animal for protein synthesis. Slowing their catabolism, presumably by having less active catabolic enzymes, would reduce this problem.

The rapid utilization of glutamate for gluconeogenesis presumably reflects the widespread distribution and very active role of transaminating enzymes which interact with glutamate, converting it to the tricarboxylic acid cycle intermediate, α-ketoglutarate. On the other hand, the branch chain amino acids, such as valine, are metabolized extensively in peripheral tissue (26). α-ketoisovalerate produced peripherally from valine would have to return to the liver before it could enter the gluconeogenic process and appear in substantial amounts in plasma glucose. This more tortuous route for valine metabolism and a slower rate of activity for the enzymes involved in its catabolism presumably account for the very slow rate at which the carbon from valine appears in glucose, in relation to glutamate carbon.

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2047

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EVERLY*, J.L., Department of Anatomy, George Washington University, Washington, D.C.
(Sponsored by F.F. Allen) Light microscopic examination of monosodium glutamate induced lesions in the brain of fetal and neonatal rats.

Monosodium glutamate (MSG) was injected subcutaneously into several strains of fetal and neonatal rats ranging in age from the 16th day of gestation to the 21st day postpartum. Dosages varied from 0.25 to 4.0mg/gm body weight. The animals were sacrificed at intervals of 30 minutes, 1, 2, 3 hours after injection and the brains were removed, sectioned, and stained for examination with the light microscope. The development and nature of the lesions in the brain were studied. Lesions occurred in the cerebral cortex, hippocampus, thalamus, and hypothalamus. The earliest changes consisted of lysis of cytoplasmic organelles in neurons followed by edema of brain parenchyma and finally neuronal necrosis.

2101

Estimation of Monosodium Glutamate in Food Products

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A procedure for estimating monosodium glutamate is described in which all food substances are extracted with water if an insignificant amount of starch is present, or with acetone-water solution when starch is present. An aliquot of the concentrated extract is chromatographed on a column of Dowex 50W—X8 (H^+ form). The glutamic acid is retained on the column and eluted with 1*N* HCl. The glutamic acid content of the eluate is readily determined by the Sorensen formaldehyde titration procedure for amino acids, and the monosodium glutamate can be calculated.

The determination of glutamic acid has been investigated extensively by Forman (1), Cosme *et al.* (2), and others (3–9). However, these investigations and methods were always applied to protein hydrolysates and not prepared foods. A procedure was needed that would separate amino acids from a wide range of complex mixtures in which monosodium glutamate is used. It was necessary to devise a system of extracting glutamic acid from food containing relatively large amounts of starch, sugar, spices, fats, oils, etc.

Experimentation with many extraction procedures and solvents showed water-acetone mixture (1 + 1) to be an efficient solvent for extracting amino acids where starch and other colloidal materials not soluble in this mixture were present.

Fats were easily removed by activated carbon. Sugars, although extracted with amino acids in the water-acetone mixture, do not interfere with subsequent separation of the amino acids, since they are not absorbed on the ion exchange column. Salts also do not interfere with the adsorption and elution of the amino acids.

This report of the Associate Referee, E. Fernandez-Flores, was presented at the 82nd Annual Meeting of the Association of Official Analytical Chemists, Oct. 14–17, 1968, Washington, D.C.

This paper describes a specific method for the determination of monosodium glutamate as glutamic acid in prepared foods such as soups, condensed broths, dry mixes, bouillon cubes, and canned green beans.

METHOD

Apparatus and Reagents

(a) *Chromatographic column.*—50 × 2 cm id.

(b) *Adsorbent.*—Dowex 50W—X8 (H^+ form), 100–200 mesh.

(c) *Sodium hydroxide.*—50% and 0.1*N* NaOH.

(d) *Hydrochloric acid.*—0.8*N*, 1*N*, and 10% HCl.

(e) *Formaldehyde.*—37%.

(f) *Activated carbon.*—Darco G-60.

Sample Preparation

For products in dry form, reduce ca 40 g to powder in a mortar and weigh 10 g sample into 250 ml beaker.

For undiluted, concentrated soups, homogenize entire undiluted content of can in blender and weigh 20 g sample into 250 ml beaker.

For consomme (clear, condensed) soup, weigh 20 g into 250 ml beaker.

Dilute samples to ca 70 ml with water at room temperature and mix until all water-soluble substances are in solution. Add 6 g carbon and mix thoroughly. (For products containing starch, also add 60 ml acetone to precipitate starch and to aid in solution of sample.) Let stand ½ hr. Filter through 60 ml coarse fritted glass funnel containing an asbestos pad, using vacuum. Wash flask and residue with six 25 ml portions of water, or if acetone was added, with six 25 ml portions acetone-water solution (1 + 1). Collect filtrate and washings in 400 ml beaker. Add 2 drops 10% HCl and evaporate to ca 40 ml. (The HCl prevents conversion of glutamic acid to pyrrolidone carboxylic acid.) Quantitatively transfer sample to 50 ml volumetric flask, dilute to volume with water, and mix. Use 25 ml aliquot for each determination.

Determination

Transfer 25 ml aliquot to a prepared column of Dowex 50W—X8, 100–200 mesh, 20 cm high × 2 cm id, and with flow rate adjusted to ca 0.5 ml/min. When all the sample has entered the resin, wash column wall with ca 10 ml water and let wash pass into resin bed. Add 120 ml 0.8*N* HCl and maintain flow rate of 0.5 ml/min. The 0.8*N* HCl will elute any serine, threonine, and aspartic acid. After all the 0.8*N* HCl has passed into the resin, follow with 170 ml 1*N* HCl and adjust flow rate to 25–30 drops/min, collecting eluate in 400 ml beaker. The 1*N* HCl will elute glutamic acid from column. (Any glycine present would elute after 200 ml 1*N* HCl has passed through.) Nearly neutralize eluate with 50% NaOH; then carefully adjust to pH 7 with 0.1*N* NaOH, using a pH meter.

Sorensen Formol Titration Procedure

Neutralize 25 ml 37% formaldehyde to pH 7 with 0.1*N* NaOH and add to the prepared neutral sample. Mix 10 min with magnetic stirrer and titrate to pH 8.9 with 0.1*N* NaOH, using a pH meter.

Determine blank by titrating to pH 8.9 a mixture of 25 ml neutralized formaldehyde and 170 ml 1*N* HCl neutralized to pH 7.

Calculate monosodium glutamate as follows:

$$[(\text{ml NaOH used in titration} - \text{blank}) \times \text{normality} \times 0.147 \times 100]/\text{wt of sample} = \%\ \text{glutamic acid}$$

$$\%\ \text{glutamic acid} \times 1.15 = \%\ \text{monosodium glutamate}$$

Before using resin column again, remove any remaining amino acids by passing 150 ml 4*N* HCl through column. Wash with water until silver nitrate solution gives a negative test for chlorides.

Discussion and Recommendation

Five dried foods and five samples of foods in liquid form including concentrates (Table 1) were analyzed for monosodium glutamate. Analyses of replicate portions of the condensed chicken broth (undiluted) and canned green beans indicated that reproducibility of the results was satisfactory. About the same amount of monosodium glutamate as that found in the analyses was added to each of the ten samples, which were then analyzed to determine recoveries. Recoveries from dried food products in Table 1 ranged from 93.0 to 102.0%. Recoveries from liquid products were 91.6–105.0%.

The analyses of a wide range of dried and liquid food products in which monosodium glutamate is used and analyses of the same products with known amounts of added monosodium glutamate show that the method gives reproducible results and that the recoveries are satisfactory for estimating the monosodium glutamate in food containing starch, fats, and colloidal materials.

It is recommended that the method be subjected to collaborative study.

Table 1. Recovery of monosodium glutamate in dried and liquid foods

Product	Monosodium Glutamate, g/100 g			
	Found	Added	Total Found	Recovery, %
Dried Foods				
"Instant" chicken-flavored broth	12.24	10.00	22.15	99.5
Beef bouillon (granular)	9.75	10.00	19.85	100.5
Chicken bouillon cubes	11.34	9.58	20.55	97.8
Beef bouillon cubes	10.84	9.58	19.76	92.2
Vegetable soup mix	7.18	5.0	12.28	100.8
Liquid Foods [a]				
Condensed pea soup	0.31	1.00	1.31	100.0
Condensed chicken broth	1.23			
	1.30			
	1.34			
	1.23	1.00	2.38	106.7
Condensed chicken broth with rice	0.32	1.00	1.37	103.7
Condensed beef broth	0.79	1.00	1.78	99.4
Canned green beans (seasoned)	0.13	0.958	1.03	94.6
	0.20	0.958	1.07	92.4

[a] Condensed soups were analyzed without dilution.

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2102

5005

Quantitative and Qualitative GLC Analysis of Free Amino Acids in Fruits and Fruit Juices

By ENRIQUE FERNANDEZ-FLORES, DAVID A. KLINE, ARTHUR R. JOHNSON, and BARRY L. LEBER (Division of Food Chemistry and Technology, Food and Drug Administration, Washington, D.C. 20204)

A method utilizing gas-liquid chromatography and the Sorensen formol titration procedure has been devised for the qualitative and quantitative analysis of amino acids in fruits. Forty-four samples representing 22 varieties of fruits were analyzed for 15 free amino acids. The amino acids were determined as N-trifluoroacetyl *n*-butyl esters. In a majority of the fruits, aspartic acid was the major component, while hydroxyproline was the minor component when present. All fruits were found to have different quantitative profiles as well as a distinct qualitative distribution of the free amino acids.

The separation and determination of amino acids in food products has long been of great interest because of their important role in nutrition. Elsden and Synge (1) investigated the separation of amino acids by using starch as a medium for partition chromatography. An important pioneering step in the field of amino acid analysis was made by Stein and Moore (2). Using various solvents, they fractionated a synthetic amino acid mixture, separating 17 amino acids on a starch column.

Extending these investigations, Moore and Stein (3) made a systematic study of the separation of amino acids by elution analysis on columns of synthetic ion exchange resins. Dowex-50, a sulfonated polystyrene resin, was used to develop a chromatographic technique for separating amino acids in protein hydrolysates. This resin has also been found applicable to a wide variety of problems of biochemical interest. Consequently, a result of this work was development of the amino acid analyzer.

Paper chromatography of amino acids in citrus fruits was reported by Underwood and Rockland (4), Knight (5), Coussin and Samish (6), and Kliewer (7). Since positive identification and accurate quantitation proved difficult, analysts turned to gas-liquid chromatography (GLC). Greater sensitivity and rapid separation, identification, and quantitation of amino acids demonstrated the higher efficiency of GLC. Early GLC amino acid analysts such as Bayer (8) and Ikekawa (9) employed N-trifluoroacetyl methyl ester derivatives. However, Darbre and Islam (10) found the methyl ester unsuitable because of its instability and inability to be accurately quantitated.

Gehrke *et al.* developed and refined a satisfactory N-trifluoroacetyl (N-TFA) *n*-butyl ester method for amino acids (11–14). After several other volatile amino acid derivatives (N-TFA methyl esters, trimethylsilyl esters, etc.) had been tested, the N-TFA *n*-butyl ester derivative was selected for the present study because of its reliability and the reproducibility of results obtained in preliminary tests. The method used for this study employs the N-TFA ester derivative of Gehrke.

METHOD

Apparatus and Reagents

(a) *Gas chromatograph.*—Perkin-Elmer Model 900 with flame ionization detector and dual 6′ × ¼″ od glass columns containing 0.65% ethylene glycol adipate (EGA) on 60–80 mesh acid-washed Chromosorb W. *Operating conditions:* program column from 80 to 205°C at 4°/min; inlet 200°C; manifold 205°C; helium flow 45 ml/min; hydrogen flow 22 psi; compressed air flow 55 psi; and chart speed 30″/hr.

(b) *Vacuum apparatus.*—Rinco rotary vacuum for evaporation of NH_3; vacuum pump with cold-finger condenser trap for evaporation of acidic solvents.

(c) *Amino acids.*—Purified (Calbiochem AA-3).

(d) *Ion exchange resin.*—Dowex 50W-X8 (H^+ form), 100–200 mesh.

(e) *Standard solutions.*—0.020 and 0.10*N* NaOH and 1*N* NH_4OH solution.

(f) *HCl gas.*—Anhydrous.

(g) *Anhydrous methanol.*—Place 4 g Mg turnings and 500 ml reagent grade methanol in 1 L round-bottom flask. Leave solution at room temperature until Mg has dissolved and reaction ceases; cautiously heat to reflux temperature and reflux 2 hr. (Distill solution in all-glass apparatus.) Discard first 10–15 ml distillate after temperature reaches

64–65°C. Collect anhydrous methanol in flasks dried at 110°C. Protect distilling and refluxing systems from moisture with $CaCl_2$ drying tubes.

(h) *Acidic methanol.*—Weigh 100 ml anhydrous methanol in 250 ml Erlenmeyer flask with stopper. Bubble in ca 4.5 g dry HCl gas to yield ca 1.25N solution. Keep reaction flask in ice to prevent overheating during reaction. (As much as possible, avoid exposure of acidic methanol to humidity.)

(i) *Anhydrous butanol.*—Add 40 g $CaCl_2$ to 500 ml butanol and distill, protecting system from moisture with $CaCl_2$ drying tubes. Discard first 10–15 ml distillate after solution reaches 117°C and collect remainder in flasks dried at 110°C.

(j) *Acidic butanol.*—Follow procedure for acidic methanol.

(k) *Anhydrous methylene chloride.*—Follow procedure for anhydrous butanol, but collect fraction distilling at 37–40°C.

(l) *Trifluoroacetic anhydride (TFA).*

Preparation of Sample

(a) *Fruit juices.*—Weigh 100 g fruit juice into 500 ml volumetric flask. Dilute to volume with 80% alcohol and mix; add additional 80% alcohol if needed to adjust volume. Let stand ≥1 hr and filter through rapid paper.

(b) *Whole fruit.*—Weigh 100 g whole or cut fruit into beaker; transfer to high-speed blender, add 300 ml 80% alcohol, and blend 10 min. Transfer quantitatively to 500 ml volumetric flask and dilute to volume with 80% alcohol. Let stand ≥1 hr and filter.

Determination

Transfer 100 ml aliquot to 250 ml beaker. Evaporate on steam bath to ca 30 ml to eliminate alcohol. Add 50 ml water and transfer quantitatively to 50 × 2 cm id chromatographic column containing ca 20 ml Dowex 50W-X8. Drain solution through column at <0.5 ml/min.

When sample has completely entered resin, wash column with 600 ml water at rapid flow rate. After all water has passed through resin, elute amino acids with 120 ml 1N NH_4OH at 0.5 ml/min; collect eluate in 300 ml round-bottom flask. Vacuum concentrate to ca 25 ml, using rotary vacuum with 60°C water bath. Transfer quantitatively to 50 ml round-bottom flask and evaporate to dryness, using rotary vacuum at 60°C. Place flask in vacuum desiccator overnight at 40°C.

Add stirring bar and 10 ml acidic methanol, and stopper flask. Stir ½ hr with magnetic stirrer and vacuum-evaporate to dryness. Add 10 ml acidic butanol, attach adapter with Drierite tube, and stir 2½ hr in 100°C oil bath. Vacuum-evaporate to dryness. Add 4 ml CH_2Cl_2-TFA (3 + 1). Store in 1 dram vial with Teflon-lined cap and let stand ≥2 hr. Reaction mixture is now ready for GLC analysis. (Sample size depends on conditions and sensitivity needed to keep major peaks on scale.)

Table 1. Relative molar response factors (RMR) and area correction factors (ACF) for GLC of amino acids

Amino Acid	RMR	ACF
Alanine	0.508	1.97
Valine	0.735	1.36
Glycine	0.331	3.02
Isoleucine	0.862	1.16
Leucine	0.935	1.07
Threonine	0.719	1.39
Proline	0.847	1.18
Serine	0.723	1.38
4-Aminobutyric acid	0.625	1.60
Hydroxyproline	0.917	1.09
Phenylalanine	1.25	0.80
Aspartic acid	1.00	1.00
Glutamic acid	1.10	0.91
Tyrosine	0.870	1.15
Lysine	0.709	1.41

Calculations

To quantitate amino acids, first prepare synthetic mixture of all natural amino acids to be analyzed in fruits by combining milliequivalent amounts of each acid (*see* Table 1). Dissolve composite in 200 ml 0.1N HCl and transfer 5 ml aliquot (ca 20 mg) to 50 ml round-bottom flask. Evaporate to dryness on rotary evaporator and derivatize under conditions described, beginning "Add stirring bar and 10 ml acidic methanol, and stopper flask." Chromatograph ca 1 μl aliquot. From this chromatogram (Fig. 1), determine relative molar response factor (RMR) and area correction factor (ACF) for each acid. These factors (Table 1) are necessary because the amino acids do not have equal area responses.

Determine RMR for any amino acid as peak area (PA) of that acid divided by peak area of aspartic acid. (Since aspartic acid is present in all fruits in appreciable, if not predominant, amounts, it was chosen for calculation of RMR.) RMR (amino acid) = PA (amino acid)/PA (aspartic acid).

To have all acids respond 100% with respect to aspartic acid, the area correction factor is necessary: ACF (amino acid) = PA (aspartic acid)/PA (amino acid) = 1/RMR (amino acid). The ACF for aspartic acid was arbitrarily assigned a value of unity.

To determine the amount of each amino acid in a fruit, the relative percentage area distribution of the amino acids must first be known: Relative % area (amino acid) = 100 × PA (amino acid) × ACF (amino acid)/total corrected PA (all amino acids).

The final calculation involves the total milliequiv-

$$= \sum_i \frac{PA_{(i)}}{RMR_{(i)}}$$

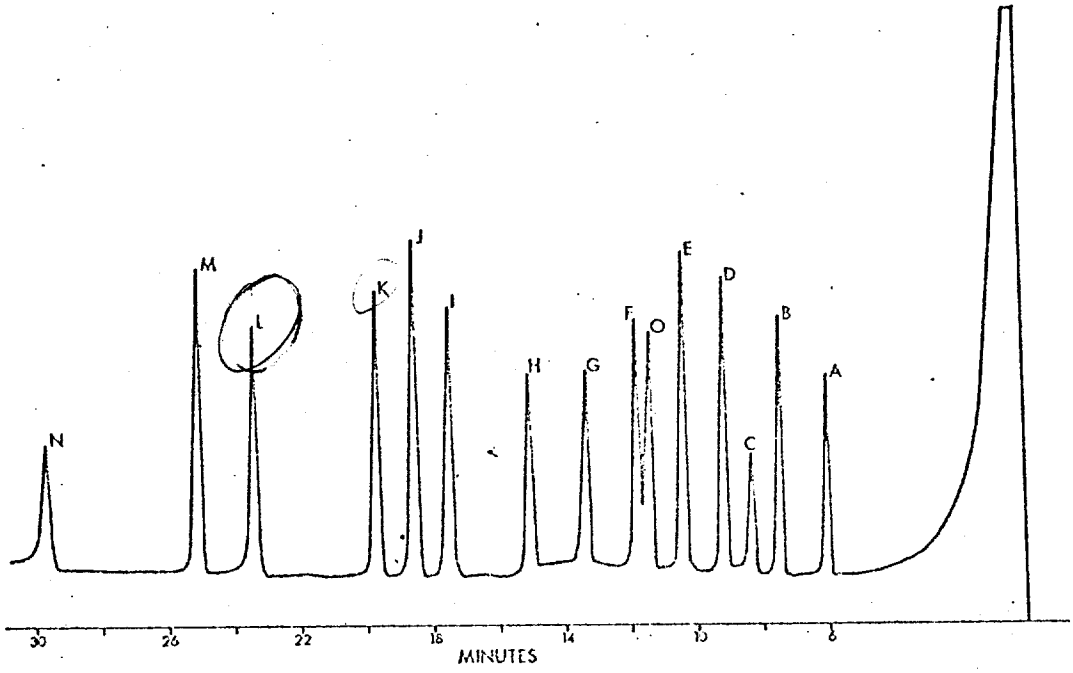

FIG. 1—Chromatogram of 15 amino acids used in composite: A, alanine; B, valine; C, glycine; D, isoleucine; E, leucine; O, threonine; F, proline; G, serine; H, aminobutyric; I, hydroxyproline; J, phenylalanine; K, aspartic; L, glutamic; M, tyrosine; N, lysine.

alents of amino acid as determined by the Sorensen formol titration procedure:

For 20 g aliquot (100 ml) of sample, follow procedure for amino acid derivatization up to addition of acidic methanol. Dissolve aliquot in 30–40 ml water and bring to pH 8.4, using 0.1 and 0.020*N* NaOH. Neutralize 37% formaldehyde solution to pH 8.4, using NaOH, and add 10 ml to sample. Back-titrate to pH 8.4, using 0.020*N* NaOH, and record volume of titrant used. Meq total amino acid/100 g sample = 0.020*N* × ml titrant × 5.

Calculate amount of each amino acid in 100 g sample as mg amino acid/100 g sample = relative % area (amino acid) × gram equivalent weight (amino acid) × meq total amino acids per 100 g sample/100. The amount of each acid present in the fruits is shown in Table 2. The gram equivalent weight is equal to the molecular weight in this case.

Discussion

A previous study of the fruit amino acids was made, using 1.5% OV-17 phenyl silicone on Gas Chrom Q. The operating conditions were the same except that programming was from 65 to 250°C. All fruits included in the EGA study were analyzed on the OV-17 column and only one other amino acid, arginine, was identified. Because arginine was present in only a few fruits, and then only in trace quantities, it was not considered important in the present study using EGA.

Complete separation of aspartic acid and phenylalanine was not obtained on OV-17, whereas EGA was effective; leucine and isoleucine had the same retention times on OV-17 but were distinctly separated on EGA. Hence, the EGA column proved to be the best available despite its inability to detect arginine because of the acidity of fruit asparagine and glutamine. Typical chromatograms representing cantaloupe and kiwi (Chinese gooseberry) are shown in Figs. 2 and 3, respectively.

For accurate identification of amino acids, the composite had to be re-evaluated about once a week due to the slight shifting of the peaks for some amino acids. The sensitivity would be less than 0.5 mg/100 g sample.

The quantitative and qualitative distribution of amino acids in fruits could be useful as an index for determining the authentic identity of fruits. For example, apples, blueberries, and cranberries contain only minimal amounts of amino acids.

Table 2. Analysis of amino acids; results reported as mg each amino acid/100 g fruit

Sample	Alanine	Valine	Glycine	Isoleucine	Leucine	Threonine	Proline	Serine	Aminobutyric	Hydroxyproline	Phenylalanine	Aspartic	Glutamic	Tyrosine	Lysine	Total mg Amino Acid/100 g Fruit
Apples	1.0	Tr[a]	0.2	[b]			Tr	0.3	0.3			0.6	0.9			3.3
Avocado	6.0	1.8	2.2	1.2	1.8		6.0	4.2	5.0		3.2	21.0	13.6	5.8	3.8	75.6
Banana	1.7	6.2	3.5	0.7	11.3	3.4	3.6	5.7	6.8		0.8	21.9	36.7	2.3	4.8	109.4
Blackberry	80.9	6.0	3.2	2.0	3.4	6.2	5.2	23.3	25.1		Tr	56.2	17.4	Tr	Tr	228.9
Cantaloupe	107.4	7.3	13.2	1.3	4.2		9.1	12.7	36.8	3.9	5.8	33.6	54.3	Tr	2.7	292.3
Cantaloupe (carbon)	122.6	8.8	10.8	1.3	4.0		7.1	14.4	24.5	1.1	1.2	37.5	55.9	Tr	Tr	289.2
Cranberry	Tr	Tr	Tr		Tr		Tr	1.3	3.9			6.2	2.1	1.6	Tr	15.1
Figs	42.3	12.3	4.0	9.6	5.4		29.7	19.3	14.8		4.3	256.5	38.1	Tr	Tr	436.3
Grapefruit (pink meat)	5.9	1.1	2.7	Tr	Tr	1.9	12.4	8.7	20.5		1.4	71.5	17.1		3.5	146.7
Grapefruit (white meat)	5.9	0.7	2.8	Tr	Tr		20.4	9.4	21.6		10.2	87.1	11.5	Tr	3.2	172.8
Grapes (black)	13.4	2.8	Tr	1.4	1.7	27.0	4.4	3.9	19.1	1.8	2.2	6.5	43.6	5.8	1.8	135.4
Grapes (red)	5.9	2.3	Tr	1.0	2.4		39.7	3.7	14.7	1.5	1.4	8.9	36.6	2.9	2.6	123.6
Grapes (white)	32.2	5.0	3.0	3.0	6.4		39.1	7.3	22.4	1.6	6.1	2.1	35.0	6.0	3.6	172.8
Kiwi	2.1	3.3	4.6	2.1	2.6		4.7	2.6	24.6		1.9	2.1	14.4	1.8	2.4	71.2
Lemon	7.9	1.0	1.8	0.4	0.4		38.9	13.3	5.2		1.3	33.8	13.4	Tr	0.9	118.3
Lemon (Sunkist #4)	7.4	0.8	1.9	Tr	Tr	1.2	29.3	11.9	4.9		1.0	28.5	13.2		Tr	100.1
Lime	9.0	1.1	1.6	1.1	0.9		4.9	10.1	8.5			59.4	11.0		1.7	109.3
Nectarine #1	7.3	Tr	Tr	Tr	Tr		Tr	8.8	Tr		1.8	200.5	9.6		Tr	227.0
Nectarine #2	5.5	Tr	Tr				2.4	10.0			Tr	202.4	7.3		Tr	227.6
Orange #1	6.4	0.8	1.8	Tr	Tr	1.5	47.6	11.1	16.6		3.8	55.5	13.1		4.3	162.5
Orange #4	6.2	1.5	2.6	Tr	Tr	1.5	56.9	8.7	12.7		5.1	34.3	8.7	Tr	3.4	141.6
Orange #5	8.3	1.5	2.7	Tr	Tr	2.1	65.9	12.6	27.6		5.4	45.6	11.8		4.0	187.5
Orange #6	7.0	1.0	3.6	Tr	1.1	1.4	55.3	8.5	31.2		9.5	36.7	10.8	Tr	3.8	169.9
Orange #2 (Calif. Valencia)	5.3	1.0	1.9	Tr	Tr	Tr	48.3	8.6	7.7		4.5	29.2	9.1	Tr	3.6	119.2
Orange #3 (Sealed Sweet)	3.9	0.6	1.5	Tr	Tr	Tr	43.2	5.0	13.0		1.6	15.2	7.3	Tr	1.2	92.5
Peaches (Freestone)	0.8	Tr	Tr	Tr	Tr	1.0	Tr	2.1	0.8		Tr	105.6	1.6			111.9
Pears	0.5	1.2	0.4	1.7	Tr		1.8	1.6	Tr			23.3	1.3	Tr	Tr	31.8
Pineapple	8.8	2.1	5.5	1.4	2.5	2.9	2.8	7.5	3.2	3.9	3.1	36.6	18.4	10.6	3.8	113.1
Pineapple (Mexican type)	4.2	1.2	3.0	0.6	2.0		3.2	4.7	2.2	3.7	1.1	29.3	6.9	2.6	4.2	68.9
Plums #1 (purple)	1.6	Tr	Tr	0.6	Tr		2.6	1.1	3.3			98.3	3.4			110.9
Plums #2 (purple)	1.8	Tr	Tr	0.7	Tr		2.7	1.2	5.8			93.8	4.0			110.0
Plums (yellow)	24.8	5.1	Tr	2.7	2.6	2.5	49.7	9.9	33.5		3.1	184.9	7.9			326.7
Prunes (California)[c]	21.4	6.8	7.2	6.3	3.2		35.0	5.1	28.7		9.5	185.5	14.4	8.8	Tr	331.9
Prunes (French)[c]	25.7	7.8	8.7	11.3	7.0		90.6	8.1	49.7		7.0	199.2	7.2	7.0	Tr	430.3
Prunes (Imperial)[c]	28.4	26.3	9.2	17.7	10.1	52.2	13.6	29.2	14.3		61.6	518.4	18.6	37.2	43.8	880.6
Prunes (Robe de Sergent)[c]	19.4	5.9	8.3	10.1	4.8		45.5	6.9	70.4		7.6	282.4	5.7	Tr	9.2	476.4
Strawberry #1	10.8	0.7	1.9	0.5	1.9	2.0	1.3	5.0	8.6			24.6	4.1		1.5	62.9
Strawberry #2	3.7	0.4	0.5	Tr	0.6	Tr	0.6	1.2	2.7			20.5	5.8	0.5	0.7	37.2
Strawberry #3	7.4	0.7	1.4	Tr	1.1	Tr	1.5	3.3	6.4			41.2	10.1	Tr	Tr	73.1
Strawberry #4	4.2	0.5	0.8	0.5	0.8	Tr	1.1	2.3	7.0			21.3	8.4	1.5	0.5	48.9
Strawberry #6	3.6	0.3	0.8	Tr	Tr	1.1	Tr	2.7	7.7		Tr	45.5	13.7	Tr		75.4
Strawberry #7	11.2	1.4	1.6	0.8	1.0	4.4	1.1	5.2	6.8		1.2	60.1	44.4	0.9		140.1
Watermelon	4.9	7.7	3.6	10.0	3.8		5.8	7.7	3.6	Tr	19.7	6.2	29.1	38.9	6.8	147.8
Blueberry	0.5	0.2	0.3	Tr	Tr	Tr										1.5

[a] Tr = trace. [b] Absence of value means none found. [c] Dry prunes.

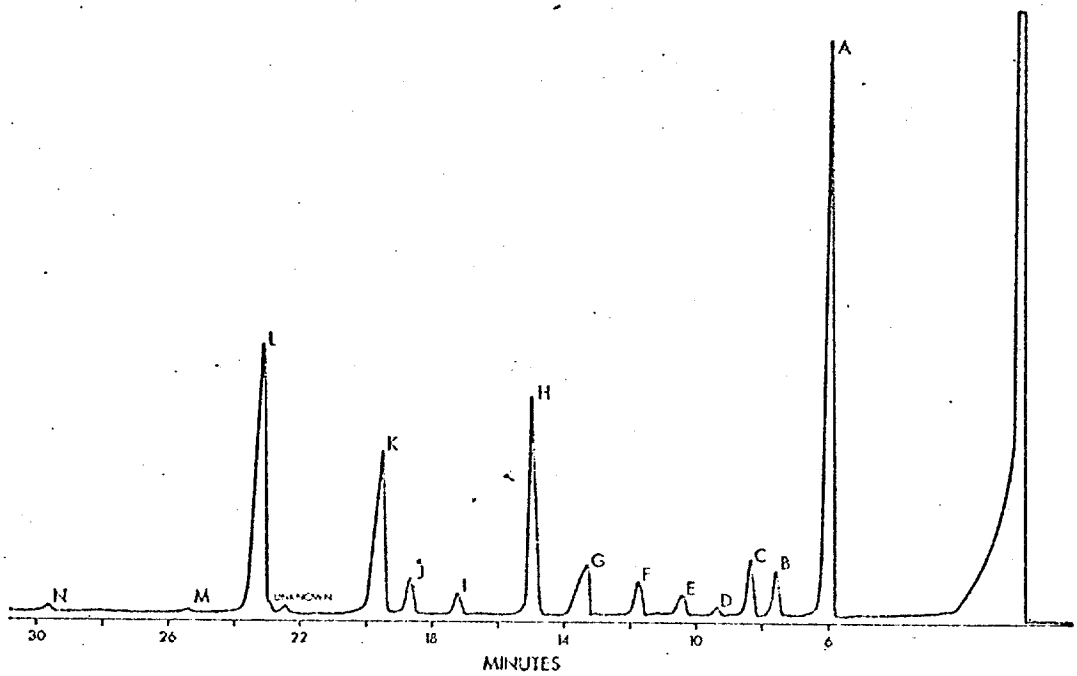

FIG. 2—Chromatogram of cantaloupe: A, alanine; B, valine; C, glycine; D, isoleucine; E, leucine; F, proline; G, serine; H, aminobutyric; I, hydroxyproline; J, phenylalanine; K, aspartic; L, glutamic; M, tyrosine; N, lysine.

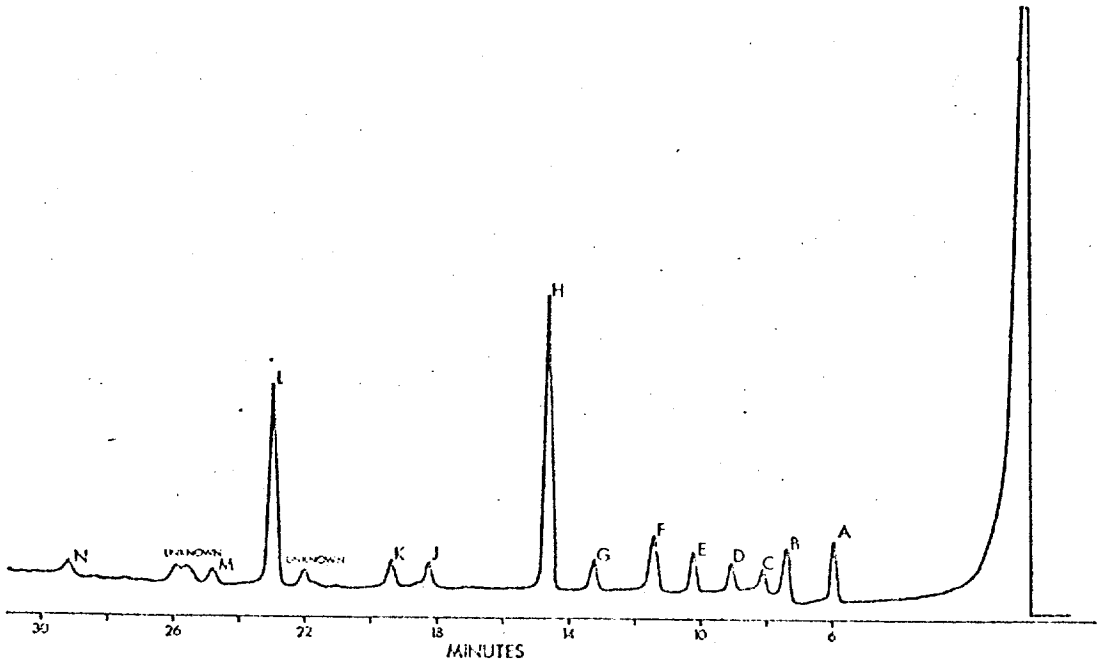

FIG. 3—Chromatogram of kiwi: A, alanine; B, valine; C, glycine; D, isoleucine; E, leucine; F, proline; G, serine; H, aminobutyric; J, phenylalanine; K, aspartic; L, glutamic; M, tyrosine; N, lysine.

On the other hand, fresh figs, nectarines, and cantaloupe have significantly greater amounts of amino acids. All fruits had aspartic acid, but very few contained hydroxyproline.

The free amino acid compositions of 6 orange juice samples (4 factory and 2 fresh fruit) follow the same pattern with only small variations. The same can be said of the 6 strawberry samples. Also, the total amount of amino acid in different samples of one fruit has been found to be very similar. For example, the 2 purple plum samples differed by less than 1% in total weight of amino acids.

The ease of operation and the simple procedures of this method suggest its applicability to other foods (meat, cereals, vegetables, cooked foods, etc.).

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Glutamate Effects in the Developing Chicken

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Intravenously administered glutamate caused a transient arrest of the electrogram of the corpus striatum in chickens from 16 days of incubation to the time of hatching. In animals examined 5 days after hatching glutamate injection had no noticeable effect on the EEG. The glutamate administration also caused in chickens up to the time of hatching a drop in the tissue conductivity of the corpus striatum. This either developed rather rapidly and was then often accompanied by a slow potential change resembling the potential change accompanying spreading depression, or the impedance drop developed more slowly and was then usually accompanied by uncharacteristic and very slow changes in potential. These phenomena were absent in 5-day-old chickens. Spreading depression was elicited by local application of 25% KCl solution in chickens incubated for 18 days or longer. The effects of intravenous glutamate administration resemble some of the concomitants of spreading depression and asphyxiation, supporting the postulate that the latter are caused by a glutamate release from the intracellular compartment of the brain. It also suggests that the blood-brain barrier is quite permeable for glutamate before the time of hatching.

Introduction

Glutamate applied electrophoretically to nerve cells causes depolarization and firing by an increase in the ion permeability of the plasma membrane (2, 9, 17). It has been suggested that this amino acid, which is present in a relatively high concentration in central nervous tissue (10 mM/kg; 1, 27), is released during spreading depression (SD) and during asphyxiation (32, 34). This would result in an increase in membrane permeability which causes a movement of extracellular electrolytes (mainly sodium chloride), accompanied by water to maintain osmotic equilibrium, into the intracellular compartment (39). The resulting depolarization of cellular elements (4, 7) can account for the slow potential change (SPC) during SD and asphyxiation, and for the flattening of the EEG during spreading depression. The transport of extracellular material explains the swelling and chloride accumulation in certain cellular elements (30, 31, 33, 41), and the contraction of the extracellular space observed in electron micrographs of

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Figure 1 shows the effect of such an injection on the blood pressure of a newly hatched chicken. Consistently only a relatively small rise in blood pressure was recorded in these animals. Although chickens before hatching are smaller, their circulation is enlarged by the extraembryonic (viteline and allantoic) circulations, which can be expected to enhance their tolerance to the large intravenous injection. Ether or urethane narcosis was used for chickens after, and when necessary before, hatching.

The electrogram of the corpus striatum recorded with a Grass polygraph was led off monopolarly with a silver–silver chloride electrode placed on the surface of the corpus striatum. A similar electrode insulated to the tip, placed 1.0–1.5 mm deep in the tissue was used to record slow potential changes. The tissue impedance was measured with the equal-ratio bridge described previously (39) using 1000-cycle sinusoidal current attenuated to about 10 mv. Silver–silver chloride electrodes, about 1 mm in diameter placed on the surface of the corpus striatum were used for the impedance measurement. They were covered with paraffin oil to prevent drying. The experiments were carried out in a room heated to 30–32 C.

Results

Effect of Glutamate on the Electrogram of the Corpus Striatum. Glutamate depolarizes neurons when applied electrophoretically. This may cause excitation of the neuronal structures involved, but when pronounced, such a depolarization arrests all activity. If the barrier is permeable for glutamate, excitatory effects as well as a depression of the EEG can, therefore, be expected. The youngest chickens investigated had been incubated for 16 days, shortly after the development of the EEG (15 days; 8). Light ether narcosis was used in these experiments.

Figure 2A shows the effect of glutamate administration to an 18-day-incubated chicken. Record (a) shows the EEG before glutamate administration. After 1 min of injection the amplitude had decreased markedly (b) and continued to decrease until at the end of the administration (2 min) the electrogram had flattened out. Shortly afterward high-frequency waves developed (c). This convulsive activity was accompanied by movements

FIG. 1. The blood pressure of a chicken on the day of hatching is recorded with a fine cannula introduced into the right innominate artery. The system dampens the cardiac pressure variations. The bar indicates the injection of a 100 mM *l*-sodium glutamate solution at 1.2 ml/min for 2 min. The arrow indicates a convulsive movement. On the ordinate the blood pressure is plotted in mM Hg.

central nervous tissue fixed during spreading depression and asphyxiation (36, 37). Finally the transport of extracellular material into the intracellular compartment accounts for the increase in tissue impedance observed during asphyxiation and spreading depression (10, 20, 24, 39, 40). Most of the current used for the impedance measurement is transported by extracellular ions (6). A loss of extracellular material can therefore be expected to cause an increase in tissue impedance. The postulate that these changes are produced by glutamate is supported by the demonstration of a glutamate release from the retina during spreading depression (35).

The above concept suggests that an increase of the glutamate concentration in the extracellular space of the brain by the administration of this amino acid should cause some of the phenomena postulated to be due to a glutamate release from tissue elements during spreading depression and asphyxiation. The blood-brain barrier (BBB) of adult animals prevents an increase of the glutamate concentration by intravenous administration of the amino acid (13, 19, 27). There is, however, considerable evidence for a greater permeability for amino acids of the barrier in young animals, although it seems well established that even during fetal life the BBB is impermeable for protein-bound dyes such as trypan blue (3, 11, 12, 22). Glutamate administered to newly born rats increased the glutamate concentration in the brain and caused convulsions and dysrhythmia of the EEG in 10-day-old animals (13). An accumulation of lysine occurred in the brain of young mice after intravenous injection of this amino acid (18). The administration of γ-aminobutyric acid elevated the level of this amino acid in the brains of newly hatched chickens (28). Changes in the EEG and behavioral changes were observed after the administration of glutamate and γ-aminobutyric acid in chickens during the first weeks after hatching (15, 16, 25, 26).

In the present investigation the effects of glutamate administration on the EEG, the steady potential and the impedance of the corpus striatum were examined in chickens from 14 days of incubation to 5 days after hatching.

Methods

White Leghorn eggs incubated at 38C hatched after 20–21 days. When investigated before hatching the egg was opened at one side and the head pulled out of the opening. The right jugular vein was dissected and cannulated with a fine Teflon tube. The head was then fixed in a holder and the hemisphere was exposed. A 100 mM solution of 1-sodium glutamate, the pH adjusted to 7.2, was injected intravenously at a rate of 1.2 ml/min for 1.5 –2 min. The blood circulation did not seem to be seriously affected by the administration of the relatively large amount of glutamate solution.

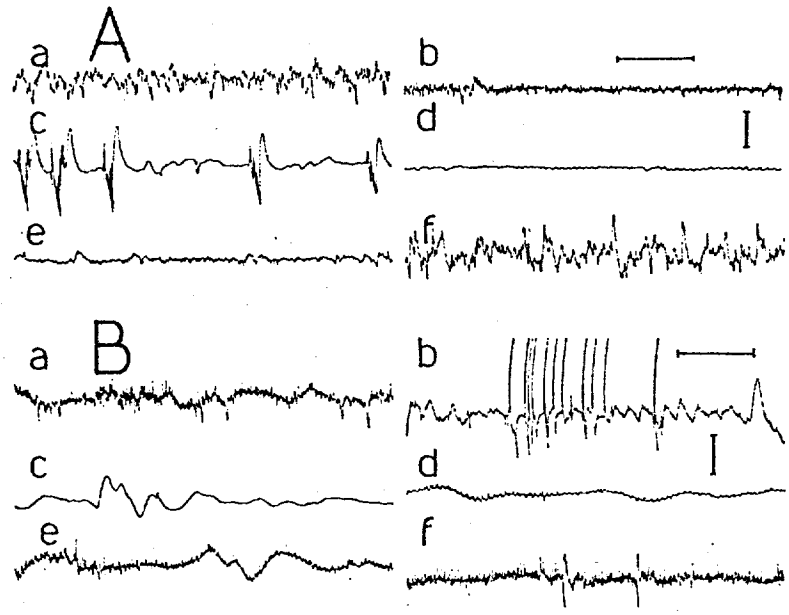

FIG. 2. A, shows the electrogram of the corpus striatum in an 18-day-incubated chicken; (a) before *l*-sodium glutamate (100 mM) administration. (b) after 1-min injection of the amino acid at 1.2 ml/min, (c) directly after the 2-min injection, (d) 1 min after the injection, (e) and (f) 5 and 10 min after the injection, respectively. B, shows the EEG of a 16-day-incubated chicken; (a) before injection, (b) after 1.5-min glutamate administration, (c), (d), (e), and (f) 1, 5, 7, and 11 min after the end of the 2-min injection. The vertical calibration line indicates 50 μv, the horizontal line 5 sec in both records A and B.

of the animal. The record then remained flat for the ensuing 2 min (d). A low-amplitude activity was present 5 min after the end of the injection (e) and after 10 min an electrogram comparable with the preinjection record was again recorded (f). Figure 2B shows an experiment in a 16-day-incubated chicken which is quite similar to that of Fig. 2A. Convulsive activity developed after 1.5 min of glutamate injection (b). The electrogram recovered its original appearance in about 11 min. The injection of the same amount of a physiological salt solution or an isotonic glucose solution did not affect the electrogram in 16- and 18-day-incubated chickens.

This effect of glutamate administration was observed in all experiments on chickens incubated for 16, 18, 20, and 21 days (Table 1). In only one of seven chickens examined 2 days after hatching was the EEG flattened out; in four it decreased in amplitude but at no time was the EEG completely abolished; in two chickens the electrogram was not affected. In none of six chickens investigated 5 days after hatching did glutamate

TABLE 1

THE EFFECT OF GLUTAMATE ADMINISTRATION ON THE EEG

Days of incubation				Days after hatching	
16	18	20	21	2	5
6/6	4/4	5/5	6/6	1(4)/7	0/6

The numerator indicates the number of experiments in which the electrogram flattened out; the denominator, the number of chickens investigated. In the group of chickens examined 2 days after hatching the figure in brackets indicates the number of experiments in which the amplitude of the EEG declined without being completely flattened out. The glutamate effect is expressed as a fraction.

administration have an effect on the electrogram. This does not seem in agreement with the observation of Kramer, Sherman, and Seifter (16) that glutamate causes in chicken 2–8 days after hatching a change of the EEG from the alert pattern to a slow-wave electrogram as seen in sleep. In the present experiments chickens 5 days of age were investigated under light ether narcosis which may have masked such a change.

During the injection chickens incubated for up to 20 days became invariably unreactive to strong mechanical stimuli. This response returned slowly when the electrogram recovered. The stimulus response was attenuated but not completely abolished in chickens examined on the day of hatching. The respiration in these animals was not notably affected by the glutamate administration.

Effect of Glutamate on the Tissue Conductivity. Since glutamate administration flattens the EEG in chickens up to about 2 days after hatching, this amino acid seems to pass the BBB. Therefore, if the transport of extracellular material into the intracellular compartment during SD and asphyxiation is due to a release of glutamate from cellular elements into the extracellular compartment, then the intravenous administration of this compound can be expected to cause an increase of the impedance of the corpus striatum. Urethane was administered in experiments in which the tissue impedance was measured.

Figure 3 shows the effect of glutamate administration on the tissue conductivity, the reciprocal of the impedance, in three 16- to 19-day-incubated chickens. The conductivity, which was expressed as a percentage of the preinjection value (100%), started to decrease shortly after the beginning of the injection and dropped in a few minutes to roughly 50% of the preinjection value. In part of the experiments the curve was more or less S shaped (Fig. 3, a and b) and this value was reached in 5–7 min; in others the decrease was more gradual and often had not reached this level after 10–12 min (Fig. 3c).

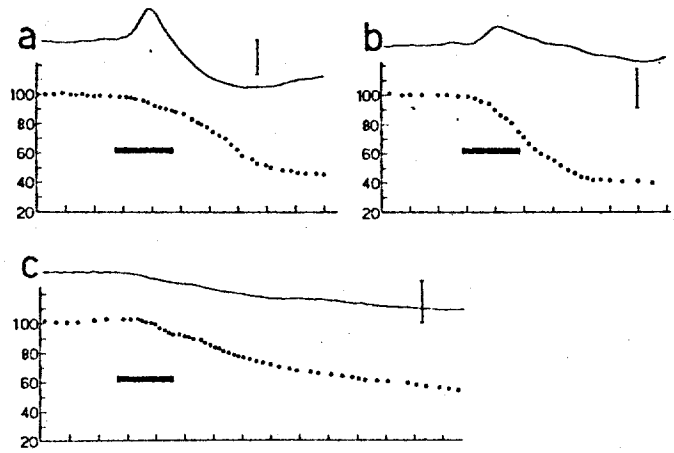

FIG. 3. A, b, and c show the slow potential changes and loss of conductivity of the corpus striatum caused by intravenous glutamate administration in chickens incubated for 16, 17, and 19 days, respectively. The vertical calibration lines indicate 5 mv for the SPC. The conductivity (reciprocal of the impedance) is expressed as a percentage of the preinjection value. The bars indicate the injection of a 100 mm *l*-sodium glutamate solution at 1.2 ml/min for 2 min. Time in minutes is plotted on the abscissa.

Glutamate administration, as shown in Table 2, caused similar losses in tissue conductivity in chickens from 14 days of incubation to the time of hatching. In two instances (14 and 15 days of incubation) this ffect was not found. This may demonstrate the increasing technical difficulty of performing the injection and impedance measurement in very young

TABLE 2

LOSS IN CONDUCTIVITY AFTER GLUTAMATE ADMINISTRATION

	Days of incubation								Days after hatching	
No.	14	15	16	17	18	19	20	21	2	5
1	+	+	++	++	+	++	++	++	+	–
2	–	++	++	++	+	+	±	±	–	–
3	+	+	++	+	++	+	±	+	–	–
4	++	++	+	+	++	±	++	++	+	–
5	+	++	++	+	+	++	++	++	±	–
6	+	–	+	+	++	±	++	+	++	–

A gradually developing conductivity loss without initial slow potential change deflection is indicated by +, and when the drop in conductivity was small by ±. A faster, S-shaped drop in conductivity accompanied by an initial slow potential change is indicated by ++.

chickens, or it may indicate that in these animals the glutamate sensitivity had not yet developed. Of six chickens examined 2 days after hatching two did not respond with a conductivity loss to the glutamate injection and none of the six 5-day-old animals showed a change in conductivity. The injection of Ringer's solution in chickens at a responsive age had no significant effect on the tissue conductivity.

Slow Potential Changes Elicited by the Glutamate Injection. Simultaneous with the impedance measurements the SPC was recorded from a closely adjacent area of the corpus striatum. Figure 3 shows three of these simultaneous records. In Fig. 3a, there is a deflection starting shortly after the beginning of the injection which resembles the SPC recorded during SD (see below). Such deflections may be followed by very slow and gradual potential changes. Figure 3b shows a similar record, only the amplitude of the initial deflection is smaller. In Fig. 3c, there is no trace of an initial deflection; only a very gradual change in potential occurs. There was, in general, a correlation between the presence of the initial deflection and the S-shaped relatively rapid conductivity change (Fig. 3 a and b). The slow, gradual conductivity loss was usually accompanied by uncharacteristic and very slow potential changes (Fig. 3 c). In Table 2 the experiments in which a relatively fast conductivity loss accompanied by an initial SPC deflection were observed are marked ++; the ones showing the gradual conductivity loss without initial deflection as +. In about half the experiments the initial deflection and fast loss in conductivity were seen. No age group seems to have a special tendency to respond in either ways to the glutamate injection.

Spreading Depression. This has been elicited in the corpus striatum of birds by the local application of a 25% KCl solution (5, 23). Figure 4 shows such an experiment in a 3-day-old chicken in which both the SPC was recorded and the tissue impedance was measured. The SPC is relatively large and shows an initial positivity (which was lacking in most experiments) followed by a larger negativity of the monopolar striatal electrode. The SPC is accompanied by an approximately 20% drop in conductivity which is of relatively short (about 4 min) duration. In other experiments on chickens of the same (or greater) age, the conductivity loss had the same time course but was not so large (usually not more than 10%). SD was observed in younger chickens as shown in Table 3. The youngest age at which the SPC of SD could be demonstrated was after 18 days of incubation. The time course of the SPC was often slower in these young animals.

Other stimuli were applied such as electrical (d-c) stimulation, isotonic KCl solution, and 150 mM glutamate solution. The solutions were applied

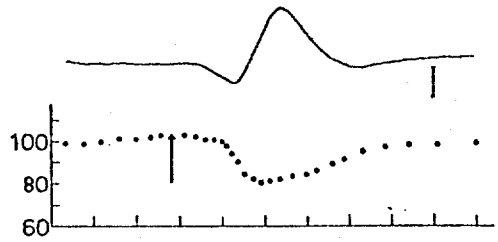

FIG. 4. Shows the slow potential change during spreading depression elicited by local application of a 25% KCl solution (arrow) and the conductivity drop of the corpus striatum (expressed as a percentage of the pre-SD value). Time in minutes is plotted on the abscissa.

to the surface of the corpus striatum and in some instances injected into the hemisphere. Although these stimuli are very effective in eliciting SD in the mammalian cortex, they only seldom caused SD in chickens investigated up to an age of 5 days after hatching. Corner *et al.* (8) were unable to cause SD by electrical stimulation in chickens younger than 2 weeks.

Discussion

Intravenous glutamate administration to chickens up to 2 days after hatching caused some of the changes characteristic for SD and asphyxiation, such as the arrest of the EEG, slow potential changes and an increase in tissue impedance. Furthermore, it eliminated all reactions to strong mechanical stimuli. This may indicate that under these conditions glutamate readily passes the BBB. The alternate possibility that the glutamate injection affects the blood circulation and in this way causes the EEG arrest and impedance changes can be excluded on the following grounds. The consistent recovery of a normal EEG about 10 min after the injection even in the youngest animals investigated (16 days of incubation) excludes the possibility that the circulation is permanently damaged. When after the

TABLE 3

SPREADING DEPRESSION IN CHICKENS

Days of incubation							Days after hatching	
15	16	17	18	19	20	21	2	5
0/6	0/12	0/9	8/26	9/12	10/12	9/15	17/21	13/15

The incidence of SD elicited by stimulation with a 25% KCl solution is expressed as a fraction, the numerator of which indicate the observation of SD, the denominator the total number of chickens tested.

recovery of the EEG these chickens were decapitated they bled profusely and the EEG flattened out in 2–3 min. A transient failure of the circulation during and after the glutamate administration might explain the temporary arrest of the EEG. However, the blood pressure showed a slight rise during the injection in chickens at the day of hatching (Fig. 1), which consistently exhibited all the symptoms of glutamate administration (Tables 1 and 2). The lack of an effect of the injection of a physiological salt solution or an isotonic glucose solution on EEG and tissue impedance excludes the possibility that blood dilution would have caused the symptoms typical of the injection of the glutamate solution. It can, therefore, be concluded that the latter are actually caused by the amino acid.

The similarity of the effects of glutamate administration to young chickens and some of the changes occurring during SD and asphyxiation supports the postulate that the latter are caused by a release of glutamate from cellular elements.

The effects on the EEG, impedance, and steady potential were absent in chickens 5 days after hatching, suggesting that between 2 and 5 days after hatching the permeability of the BBB for glutamate decreases markedly. Krämer, Sherman, and Seifter (16) observed behavioral changes (wing drop) and a change from an alert EEG to a sleep pattern after glutamate administration to 2- to 8-day-old chickens. These much less severe effects indicate that a residual permeability remains for some time after the BBB has lost the much greater permeability for glutamate of the prehatching age.

The arrest of the EEG was transient in chickens up to 2 days after hatching. After about 10 min the record had regained much of its preinjection appearance. Amino acids injected intravenously tend to disappear rapidly from the blood (42). Although a large amount of glutamate was injected (about 0.25 mM) the glutamate concentration in the blood may have decreased sufficiently during this time to stop and even reverse the movement of the amino acid from blood into brain. The metabolism of the nervous tissue may then have further decreased the concentration of the amino acid in the extracellular material. Another factor which may explain the transient nature of the EEG arrest is the desensitization shown by the effects of glutamate on membrane permeability (14, 21, 29, 38), allowing the membrane to repolarize in the presence of the amino acid.

The reversibility of the electrogram contrasts with the conductivity drop which 10 min after the glutamate injection did not show any sign of abating. Several reasons for this difference can be suggested. The electrogram is a function of nervous elements, whereas possibly other structures, such as neuroglia, which may not exhibit desensitization, could also take up extracellular material and thus contribute to the impedance rise. Fur-

thermore, only repolarization may be involved in the recovery of the electrogram, whereas for a reversal of the impedance increase an active transport is necessary to return the material which moved into the intracellular compartment back into the extracellular space.

Stimulation of the retina charged with ^{14}C glutamate with the unlabeled amino acid causes a release of the label (35). Such a mechanism may result in a regenerative release of glutamate, the increasing glutamate concentration in the extracellular spaces enhancing the glutamate permeability of the plasma membrane until a maximum is reached. A regenerative release of glutamate may be the basic mechanism in SD, explaining the all-or-none character of this phenomenon. Since SD is spontaneously reversible one has to assume the presence of an additional mechanism which returns the glutamate permeability of the plasma membrane to the pre-SD level. This could be the desensitization of the plasma membrane for glutamate.

Two reaction forms of the corpus striatum can be expected during intravenous administration of glutamate. The glutamate entering the brain may cause a regenerative release of this amino acid from the cellular elements as in SD. Alternatively the glutamate concentration in the brain may rise so slowly that desensitization prevents the regenerative release of glutamate. Evidence for both reaction forms were found (Fig. 3). In some instances the injection resulted in a SPC resembling that of SD (Fig. 3a and b). This was accompanied by a loss of tissue conductivity which differed from that accompanying SD in its magnitude (about 50% against about 10% for the loss accompanying SD) and by its long duration. This can be explained by the continued movement of glutamate across the BBB during (and probably for some time after) the injection in addition to the regenerative release from the tissue elements at the start of the administration. In other instances (Fig. 3c) the slow potential change was uncharacteristic and the conductivity loss developed more slowly. In these instances no regenerative glutamate release may have occurred but the glutamate concentration in the brain may have increased slowly by a movement of the amino acid across the BBB resulting in a more gradual uptake of extracellular material by the intracellular compartment.

Spreading depression was postulated to be dependent on glutamate sensitivity of cellular elements in the nervous tissue (32, 34). This phenomenon was observed in chickens as young as 18 days of incubation. Although in younger chickens the glutamate sensitivity was demonstrated, they did not show SD. Glutamate sensitivity of the cellular elements, although a necessary condition, may not by itself be sufficient to insure the possibility of eliciting SD. For the propagation of this phenomenon other factors may be necessary which are lacking in chickens of less than 18 days' incubation.

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Studies on the Metabolism of L(+)-Glutamic acid. III. Effects of the Administration of L(+)-Glutamic Acid on Carbohydrate Metabolism.

by

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Dedicated to Prof. H. Reinwein, Kiel, on his 60th birthday.

In our previous reports (1,2) the behavior of α-ketoglutaric acid, pyruvic acid, L-alanine and aspartic acid in rabbit serum was described after the administration of L-glutamic acid. We observed a rise in α-ketoglutaric acid, a decline of pyruvic acid and a considerable increase in L-alanine and were able to demonstrate that the rise in L-alanine exceeded the pyruvate supply of the blood 5-10-fold. From this we concluded a resupply of the pyruvic acid necessary for the formation of alanine from the carbohydrate metabolism, particularly since the degree of alanine increase was independent from the quantity of glutamic acid injected (2 and 4 mMol/kg), from the simultaneous administration of pyruvate, and from the nature of the application. Seitz, Engelhardt-Gölkel and Schaffry (3) have confirmed the increase in α-ketoglutaric acid in humans, but they observed no decline of pyruvic acid. We will therefore once again recall the findings of Kamin and

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Handler (4), who have demonstrated a pronounced excretion of aspartic acid in the urine as an end product of transamination, while we found no indication of any appreciable excess production of aspartic acid.

An account will be given in this report of the effect of glutamic acid on carbohydrate metabolism in vivo, based on determinations of the blood sugar level and liver glycogen. The experiments in chapter 1 deal with the effect of glutamic acid on the blood sugar elevated by glucose loads in the rabbit (1a), and on the blood sugar level lowered by insulin (1b), and on the behavior of the blood sugar of animals with alloxan diabetes (1c). In capter 2 an account will be given of the behavior of liver glycogen of rats after the administration of glutamic acid to normal animals (2a), to animals whose adrenal function was blocked (2b), to adrenalectomized animals (2c) and to animals with pyridoxin deficiency (2d).

1. Influence of L(+)-glutamic acid on the blood sugar.

There are numerous, in some cases contradictory, studies (5,8) on the behavior of the normal blood sugar after glutamic acid in humans and animals. Our own blood sugar findings after the administration of glutamic acid were also inconsistent (9,10), so that it seemed to us desirable to analyze the mechanism of the effect of glutamic acid on carbohydrate metabolism on the basis of blood sugar determinations under various conditions.

1a) Effect of the administration of L(+)-glutamic acid on the blood sugar level raised by glucose loading.

Solutions of 4 mMol glucose/kg were administered through a stomach tube to 6 rabbits weighing approximately 3 kg and kept fasting for 12-14 hours. The blood sugar was determined according to Hagedorn-Jensen and the "true glucose" according to the method of Nelson (11) modified by Frank and Kirberger. Two days later 2 mMol/kg L(+)-glutamic acid was administered to the same rabbits simultaneously with the glucose and the blood sugar was once again determined. Three days after this experiment 3 of these animals received 8 mMol glucose/kg and after 2 more days again 8 mMol glucose/kg together with 2 mMol glutamic acid/kg through a stomach tube. The glucose quantities correspond to 50 and 100 g in adults weighing 70 kg, while the glutamic acid quantity corresponds to 20 g.

For these last experiments we used the same animals with twice the glucose quantity, since it has been demonstrated by Himwich (5) that the metabolic action of intravenously or orally administered glutamic acid disappears after one day. The simultaneous oral administration of glutamic acid leads to a decline in the blood sugar loading curve (Fig. 1). We have attempted to confirm this finding on healthy adult experimental subjects under analogous conditions, but found no decline in the blood sugar loading curve in the 4 persons studied.

1b) Effect of L(+)-Glutamic acid on blood sugar depressed by insulin.

If 3.1 U insulin is administered subcutaneously to a rabbit kept fasting for 12 hours, hypoglycemic shock develops 2-3 1/2 hours after the injection which can be eliminated with 5 ml 20% glucose solution (Fig. 2a).

If 2 hours before the expected onset of shock 2 mMol/kg glutamic acid is administered to the same animal per os, the blood sugar does not decline further, but there is a slow rise, as a result of which the shock is prevented (Fig. 2b). The shock can also be prevented by the simultaneous administration of insulin and glutamic acid (Fig. 2b).

Fig. 2c shows experiments, once again on the same animal, in which the glutamic acid was administered before the insulin injections. Experiment 91, in which the glutamic acid was administered 30 min before insulin, again shows no shock, while in experiment 90, glutamic acid administered 60 min before insulin could no longer prevent shock. These experiments were carried out at 3-day intervals on 3 rabbits in a total of 6 experimental series.

1c) Effect of L-glutamic acid on the behavior of the blood sugar of rabbits with alloxan diabetes.

For these experiments rabbits weighing 2 and 3 kg were used that had been rendered diabetic by a single intravenous injection of 150 mg/kg alloxan in 5% solution. The alloxan

was freshly prepared according to Vanino (11a). The animals were brought into the experiment one week after the alloxan injection to allow possible liver and kidney damage to subside.

Two mMol/kg sodium glutamate in 10% solution was administered intravenously to 6 rabbits kept fasting for 8-10 hours. Table 1 shows the blood sugar values determined in these experiments. The maximum blood sugar values were found 120 min after the injection, increases being the greater, the more severe the diabetes, in other words the higher the initial blood sugar level of the animals was.

In order to assure that this rise in the blood sugar level was really the effect of glutamic acid and not the effect of a hypertonic salt solution, in two other experiments we investigated the effect on the blood sugar of 4 mMol/kg glutamic acid administered to rabbits with alloxan diabetes as a suspension through a stomach tube. The blood sugar increase observed was 86 and 108 mg%. The administration in one of the glutamic acid solutions of isotonic sodium chloride solution did not lead to any significant rise in blood sugar.

In order to determine whether in this rise of the blood sugar glucose was liberated from the liver through the action of adrenalin, or whether glutamic acid itself was responsible for the formation of glucose in the intermediate metabolism, we sought to block the adrenalin effect by means of adrenolytic substances. Dihydrated ergot alkaloids in a dose of 0.1 mg/kg administered to rats can suppress the effect of 0.1 mg/kg

adrenalin on blood sugar up to 96% according to the studies of Rothlin (12), Schneider (13) and Kroneberg (11). The most active substances from this group are dihydroergocristine, dihydroergocornine and dihydroergocryptine, which are contained in equal parts in Hydergine and have no individual effect on the blood sugar, as we ourselves observed in confirmation of the findings of Kroneberg (11).

In a preliminary experiment we injected 2 mMol/kg sodium glutaminate intravenously to rabbits with alloxan diabetes and repeated this experiment 3 days later 20 min after the administration of 0.5 mg Hydergine subcutaneously and followed the blood sugar in both experiments. Fig. 3 shows the blood sugar under the influence of Hydergin and glutamate, as shown by broken curves B and C. The 3 curves were obtained at 3 day intervals, the solid line curve A without Hydergine coming between the other two in time.

2. Influence of L(+)-glutamic acid on liver glycogen.

2a) Normal rats.

Ten mMol/kg sodium glutamate in a 10% solution was injected i.p. 3 times at 2-hour intervals to 10 male albino rats weighing between 150 and 200 g that had been fed ad libitum with oats up to the beginning of the experiment. The animals were killed by decapitation 2 hours after the last injection and the liver glycogen was determined in double determinations according to Pflüger (see Lohmann, 15). Ten other rats, under otherwise identical conditions, received the corresponding quantity of sodium chloride solution i.p. instead of the glutamic

acid solution. These animals were also killed 2 hours after the 3rd injection and their liver glycogen was determined.

In Fig. 4a the glycogen content in g% fresh liver as mean value of the double determinations of the individual animals, as mean values of the animals treated with glutamic acid and as mean values of the control animals is shown in the form of columns. Statistical evaluation took place as a series of observations with a constant characteristic (16) according to the following formula:

for mean value $a = \frac{1}{k} \cdot \sum x$ and for the spread $\sigma^2 = \frac{1}{k} \cdot \sum (x - a)^2$.

The judgement value to determine the significant difference was established according to the formula

$$\varphi = \frac{a_2 - a_1}{\sqrt{\frac{\sigma_2^1}{n_2} \frac{\sigma_2^2}{n_1}}}$$

The probability limits were laid down according to the 3σ-value of a Gauss distribution with $Q \approx 3 + \frac{10}{n} = 3 + \frac{10}{n_1 + n_2} = 3.5$.

Since in our case a judgement value of Q = 9.5 is obtained, the decrease in liver glycogen after i.p. administration of 3 times 10 mMol/kg glutamic acid is statistically confirmed. Table 2 gives the statistical calculation.

In another experiment 10 rats received 2 instead of 3 injections of 10 mMol/kg sodium glutamate i.p. and once again the liver glycogen was determined in comparison with 10 animals treated with sodium chloride solution. Fig. 4b shows the glycogen content in g% fresh liver. It is not known why the liver glycogen of

one of the animals treated with glutamic acid remained high. Possibly the injection went into the intestine. The statistical evaluation of this experiment only gives a significant difference if this one animal is excluded from the calculation. If the rats receive 10 mMol/kg sodium glutamate only once, there is no statistically confirmed drop in glycogen, so that these results are not given.

In all animals treated with glutamate injections we found an abundance of protein-containing fluid in the abdominal cavity, which in another experiment was quantitatively pipetted off from 5 rats which received twice 10 mMol/kg glutamate. Next, after deproteination, it was subjected to two-dimensional paper chromatography and the amino nitrogen was determined according to Van Slyke (18). The sugar content of the exudate corresponded to the blood sugar of the animals. Paper chromatography showed such a predominance of glutamic acid over the other amino acids that we expressed the quantity of amino-N found as glutamic acid.

It is apparent from these findings that about 90% of the quantity of glutamate injected is actually reabsorbed by the animal and that the mobilization of liver glycogen cannot be a process of dilution by the quantity of injected fluid, since the quantity of injected fluid of 4.0 ml on the average would only necessitate the mobilization of 5 mg glucose to achieve a sugar content of 125 mg %, which would correspond roughly to

a 0.1 g% decrease in liver glycogen.

2b) Influence of L(+)-glutamic acid on the liver glycogen of rats with a blocked adrenal function.

In order to clarify the mechanism of action of the mobilization of liver glycogen by glutamic acid we also studied this effect under the influence of the dihydrated ergot alkaloids contained in Hydergine, dihydroergocornine, dihydroergocristine and dihydroergocryptine. These proved valuable because of their suppression of certain effects on the sympathetic nervous system during our study of the "adrenergic effect of glutamic acid" on the blood sugar of rabbits with alloxan diabetes (see section 1c).

Ten rats fed ad libitum with oats until the beginning of the experiment received 0.5 mg/kg Hydergine s.c. 20 min before the i.p. injection of 10 mMol/kg sodium glutamate. Both injections were repeated 2 hours later, the animals were killed after a further 2 hours and the liver glycogen was determined. Thus the experimental conditions were the same as those shown in Fig. 4b, save the administration of Hydergine. Fig. 5 shows the glycogen content in g% fresh liver in comparison with 10 control animals that also received Hydergine. The mean value a for the animals treated with glutamate was 0.13 g% with a spread σ of $\pm$ 0.168 g%. The control animals had a mean value of a = 2.77 g%, with a spread of $\sigma = \pm 1.99$ g%. The judgement value for the significant difference was 6.67 with a limit of probability of 3.55.

Thus despite the considerable variation in the glycogen content in the control animals, the statistical significance was also demonstrated in this experiment.

Since the rats treated with glutamate showed distinct hypoglycemic symptoms towards the end of the experiment, we studied the blood sugar in 4 animals during such an experiment and found that in comparison with normal values at the beginning it distinctly dropped towards the end of the experiment (Fig. 6). The controls with glutamate alone and those with Hydergin alone showed no variation in blood sugar.

2c) Influence of L(+)-glutamic acid on liver glycogen and blood sugar of adrenalectomized animals.

In another series of experiments we studied the influence of i.p. injections of glutamate on the liver glycogen and blood sugar of adrenalectomized male rats. The animals were adrenalectomized bilaterally in one session and in the next 8 days they received 0.4 ml/100 g adrenal extract (Pancortex) twice daily i.m. as well as a diet consisting of oats and 2% sodium chloride solution. On the day of the experiment the animals received twice 10 mMol/kg sodium glutamate i.p. at 2-hour intervals. At various time intervals after the second injection the animals showed pronounced hyperglycemic symptoms, ending in severe shock with violent tetanic spasms. At the peak of shock the animals were decapitated, and the sugar from the blood of the head and the liver glycogen were determined. Table 4 shows the results of this experiment. Large injections of glutamic acid

lead to a fall in liver glycogen and blood sugar in adrenalectomized animals, as they do in animals with the adrenal function blocked by Hydergine. The fact that the hypoglycemic symptoms occurred earlier and more intensively in this case may be due to the absence of a reactive adrenocortical preparation with a sufficient supply of glycocorticoids and to the completely successful elimination of the adrenergic effect.

2d) Influence of L(+)-glutamic acid on the liver glycogen of animals with pyridoxin deficiency.

Male albino rats (Sprague-Dawley) were kept on a pyridoxin-free diet, consisting of 63% vitamin-free corn starch, 18% vitamin-free casein, 4% cellulose powder, 4% salt mixture according to McCollum and 11% cod liver oil. The following was added per 100 g diet: 100 mg choline hydrochloride, 1 mg thiamine, 2 mg lactoflavin, 10 mg nicotinic acid amide, 0.2 mg vitamin K (Synkavit). Each animal received 180 γ deoxypyridoxine daily through a stomach tube. After 5-8 days the animals gained no further weight and after 3 weeks they showed all the symptoms of severe pyridoxine deficiency.

Since it was found that these rats, due to the state of hunger caused by the severe alterations in the mucosa of the muzzle and throat, had extremely low glycogen values, the animals were fed during the last week before the experiment with a liquid diet enriched with calories (150 g vitamin-free casein dissolved with pepsin, 500 g glucose, 20 g salt mixture,

50 g cellulose powder, 40 mg deoxypyridoxine, added vitamins as above, water ad 1000 ml) 3 times daily through a stomach tube. One half the animals treated in this manner received twice 10 mMol/kg sodium glutamate at 2-hour intervals i.p. and the liver glycogen was determined after a further 2 hours. The other half of the animals received sodium chloride solution instead of glutamate.

The glycogen values of the treated and control animals can be seen in table 5. In the animals treated with glutamate the mean value was a = 0.59 g%, the spread $\sigma = \pm 0.196$%. In the control animals the mean value was a = 0.54 g%, the spread $\sigma = 0.46$ g%. The judgement value was 1.06, the limit of probability 4.0. Thus there is no significant difference between the two groups.

Discussion

Since no concordant results could be arrived at after numerous investigations (5-10) on the behavior of the normal blood sugar in humans and animals, we believed we could obtain more unequivocal results on the influence of glutamic acid on glycemia by testing it on the experimentally varied blood sugar level. We therefore investigated the effect of L(+)-glutamic acid on the blood sugar elevated by glucose loading or alloxan treatment or lowered by the administration of insulin.

Our experiments have shown that alimentary hyperglycemia can always be partially suppressed in the rabbit by glutamic acid. We were not able to confirm the finding of Küper (6)

on humans, who carried out an experiment with the same results on a 23 year old healthy experimental subject. This discrepancy may be due to the fact that our experimental subjects were members of the institute staff who continued their predominantly sedentary occupations during the experiments.

The partial suppression of alimentary hyperglycemia by glutamic acid would seem to suggest that the influence of a single dose or isolated massive doses of glutamic acid on the carbohydrate metabolism cannot invariably be interpreted as an adrenergic effect (19,20), but that glutamic acid may also exert its action in metabolism after transamination to α-keto-glutaric acid by activation of the citric acid cycle, or by consumption of pyruvic acid in transamination (2). Apparently the reaction products, α-ketoglutaric acid and alanine, are not utilized for the immediate resynthesis of glucose, but in the citric acid cycle.

The effect of glutamic acid on emergence from hypoglycemic shock has often been described and studied (8, 19). Since in addition to glutamic acid other substances (p-aminobenzoic acid, glycocoll, arginine and protein hydrolysates) also have this effect, although their action is different in brain slices, we cannot assume a central site of action for glutamic acid. On the other hand Weil-Malherbe (19,20) was able to show that by injection of small quantities of glutamic acid there was a pronounced rise of adrenaline which had decreased during hypo-

glycemic shock (21). The consequence of this increased adrenaline is then the emergence from shock and the resultant rise in blood sugar. This adrenergic effect, which is also cited by Weil-Malherbe to explain the increase occasionally observed in the blood sugar after the administration of glutamic acid to normal glycemic persons, can also be demonstrated according to our experiments when the glutamic acid is administered 30 min before insulin. Thus it would appear to persist roughly until the glutamic acid level and the shifts in intermediate metabolic products are normalized again. Since this occurs within 2-3 hours, the administration of glutamic acid 2-3 hours before the anticipated onset of shock can no longer prevent it. The observation of Himwich (5) that the glutamic acid effect on metabolism has already disappeared by the next day is carried further by the finding that only 2-3 hours are required for the normalization of the metabolism.

Findings by Kraus and Rech (22) are contrary to these observations. These authors have demonstrated an insulin-potentiating effect of glutamic acid when it is administered on the evening before an administration of insulin dose which normally does not elicit shock. We have repeated these experiments and were not able to confirm these findings.

The increase in blood sugar after the administration of glutamic acid to animals with alloxan diabetes is not due to the glyconeogenesis which is increased in diabetes, but

according to our experiments should also be regarded as an adrenergic effect, since it can be suppressed with dihydrated ergot alkaloids.

Thus two different mechanisms of the glutamic acid effect on blood sugar can be distinguished: on the one hand a blood sugar-depressing effect in alimentary hyperglycemia, and on the other a blood sugar-increasing adrenergic effect in alloxan diabetes and insulin shock. Both the glycolytic and the adrenergic effects are eliminated upon the administration of glutamic acid to normal glycemic animals. In alimentary hyperglycemia the glycolytic effect predominates, proceeding in the same direction as the catabolic metabolism, while in alloxan diabetes, with a disturbed glucose breakdown this glycolytic effect is apparently not exercised, instead of which the adrenergic effect leads to a liberation of glucose from the liver rich in glycogen and therewith to a considerable rise in blood sugar. This is the higher, the more pronounced the diabetes is, that is to say the lower the capacity of the organism to break down glucose.

Having shown that the effect of glutamic acid on carbohydrate metabolism need not be due solely to an adrenergic effect (19,20), but that the depression in the blood sugar loading curve which has been demonstrated must be explained by some other mechanism, it could be statistically demonstrated that sufficiently large injections of L(+)-glutamic acid lead to an almost complete disappearance of the liver glycogen

of fed rats. This mobilization of glycogen is independent of the intact adrenal medullary system, as had been demonstrated by its elimination by pharmacological and by surgical means. Schober (23), using histochemical methods, showed a complete loss of glycogen from the liver and adrenals in the rabbit after intravenous injection of 6 times 0.54 g glutamate. Since he also found a disappearance of chromaffinity of the adrenals, he interpreted the loss of glycogen as an adrenergic effect. In our opinion, based on the comprehensive studies of Weil-Malherbe (19), there can be no doubt of the increased production of adrenaline and noradrenaline by the adrenals after the administration of glutamic acid, although we find ourselves in opposition to Schober, since in view of the mobilization of the liver glycogen we cannot attribute any great significance to this adrenergic effect.

In this elimination of the adrenergic effect the finding that the administration of glutamic acid leads to a drop of the blood sugar to hypoglycemic values seems to us of interest. According to the studies of Weil-Malherbe (21) the drop in the blood sugar after insulin is a temporal consequence of the fall in adrenalin, but at this point we would like to omit any discussion of the possible causes of this phenomenon. Since we have suppressed the adrenaline action either pharmacologically or surgically, by these methods one obtains the same metabolic situation with low (or absent) adrenaline action as after the

administration of insulin, and thus the blood sugar is given the possibility of falling, which occurs either by an increased decomposition in the periphery, as in the case of insulin shock, or in case of glutamic acid admininistration, by removal of pyruvic acid by way of transamination to α-ketoglutaric acid and alanine. The fall in adrenaline thus seems to be a postulate for the onset of hypoglycemic shock, the other postulate being the decomposition of glucose which comes about in some manner.

We sought to obtain support for our postulated mechanism of action of the influence of L(+)-glutamic acid in carbohydrate metabolism by its transamination to α-ketoglutaric acid and alanine by studies on pyridoxine-deficient animals with reduced transaminase activity. According to Kühnau (24) vitamin B_6 is essential for genuine transamination; yet since an avitaminotic organism strives to liberate all vitamin reserves for processes essential to life, including transamination in the liver, the vitamin deficiency of an animal can only be regarded as an influence on enzymatic reactions under special experimental conditions, as has been demonstrated by the works of Awapara, Elvehjem, Meister, McHenry, Snell and associates (25). Therefore for these experiments the rats received deoxypyridoxine as antivitamin in addition to a diet free of vitamin B_6. Despite forced feeding with a diet rich in carbohydrate we were not able to increase the liver

glycogen of the animals to an average concentration of 2 g% in order to create conditions favorable for our experiments, but our results showed nevertheless that there was no difference in the glycogen content of the liver of the treated animals and the controls.

If we calculate the significant difference between the pyridoxine-deficient animals treated with glutamate and the first of our experimental series, we get a judgment value of Q = 6.0 with a limit of probability of 3.66. Thus statistical confirmation is obtained that an intact transamination system is necessary for the mobilization of liver glycogen by glutamic acid.

In order to obtain a quantitative indication of the percentage of administered glutamic acid eliminated by the organism, we examined the excretion of glutamic acid in the urine of an 80 kg experimental subject kept fasting for 10 hours after oral intake of 25 g glutamic acid in the form of a suspension. The urine was examined by paper chromatography in half hour portions and the amino nitrogen was determined after splitting the urea with urease and adsorption of the ammonia produced onto permutite according to Van Slyke. Excretion of glutamic acid began 1 hour after intake and within 3 hours amounted to 0.65 g, that is 2.6% of the quantity taken.

Our findings in some degree differ from the well-known earlier studies of Wilson and Lewis (26) and of Butts, Blunden and Dunn (27), who administered glutamate to rats kept fasting

for 24-48 hours through a stomach tube and found significant increases in the liver glycogen in comparison with the fasting control animals. We have repeated these experiments and were able to confirm them essentially. Ten rats kept fasting for 48 hours received 2 ml 10% sodium glutamate solution for 10 hours every two hours. Next the liver glycogen was determined in comparison with control animals. It was 0.45 g% on the average with a spread of error of 0.15 to 1.13 g% and in the control animals 0.02 to 0.03 g%.

We believe that these findings are only in apparent disagreement with the glycogen-mobilizing effect of glutamic acid observed by us, since the initial metabolic situation of the organism is undoubtedly of importance for the direction of the reaction, so that in the case of fed animals with an abundant supply of glycogen there is a breakdown of glucose, while a severely fasting organism builds up its glycogen supply via the citric acid cycle.

Summary.

Please refer to English in original text.

Figures.

1. Glucose loading curve. Rabbit. 4 mMol/kg glucose per os. Broken line: without added glutamic acid. Solid line: together with 2 mMol/kg suspended L(+)-glutamic acid per os. a) Determination of "true" glucose according to Frank, Kirberger (1); b) according to Hagedorn-Jensen.

2. Blood sugar curves after 3 IU regular insulin (↓) s.c., rabbits (2.8 kg). a) and c): ⚡ hypoglycemic shock, 5 ml 20% glucose solution i.v. b) and c) 2 mMol L(+)-glutamic acid/kg per os.

3. Blood sugar curve in alloxan diabetes. Curve A: 2 mMol/kg sodium glutamate i.v. (↓). Curves B and C: 0.5 mg/kg Hydergin s.c. After 20 min 2 mMol/kg sodium glutamate i.v.

4a. Behavior of liver glycogen in fed rats after 3 injections of 10 mMol/kg sodium glutamate i.p. A: Control animals. B: animals treated with glutamate; C: Mean values of the control animals. D: Mean values of animals treated with glutamate.

4b. Like 4a, but two injections of 10 mMol sodium glutamate/kg i.p.

5. Behavior of liver glycogen of fed rats after two injections of 10 mMol/kg sodium glutamate i.p. and 20 min previously 0.5 mg/kg Hydergine s.c. A: Controls, Hydergin animals. B: Glutamate-Hydergine animals. C: Mean value of control animals. D: Mean value of glutamate-Hydergine animals.

6. Behavior of blood sugar of fed rats (↑). Injection of 10mMol/kg sodium glutamate i.p., 20 min previously 0.5 mg/kg Hydergine s.c.

Tables.

1. Changes in blood sugar in alloxan-diabetic rabbits after i.v. injection of 2 mMol/kg sodium glutamate (10% solution). Determination according to Hagedorn-Jensen: data in mg%. Key: 1. Animal no. 2. Before the injection. 3. Min after the injection. 4. Largest increase in blood sugar.

2. Influence of 3 times 10 mMol/kg sodium glutamate on the liver glycogen of normal rats. Key: 1. Animals treated with glutamic acid. 2. Control animals. 3. Animal no. 4. Liver glycogen.

3. Change in the contents of the abdominal cavity of rats 2 hours after injection of twice 10 mMol/kg sodium glutamate (=Glu) as 10% solution. Key: 1. Rat no. 2. Body weight. 3. Injected Glu-quantity. 4. Exudate found in the abdominal cavity. 5. NH_2-N calculated in mg Glu.

4. Influence of twice 10 mMol/kg sodium glutamate on liver glycogen and blood sugar of adrenalectomized rats. 1. Animal no. 2. Body weight in g. 3. Glutamic acid i.p. in mg. 4. Onset of shock after 2 injections, in min. 5. Blood sugar sub finem, in mg %. 6. Liver glycogen in g%.

5. Influence of twice 10 mMol/kg sodium glutamate on liver glycogen of animals with pyridoxine deficiency. Key: 1. Animal no. 2. Animals treated with Glu. 3. Control animals. 4. Liver glycogen.

Bibliography.

See footnote references.

Untersuchungen über den Stoffwechsel der L(+)-Glutaminsäure

III. Mitteilung

Wirkungen von L(+)-Glutaminsäuregaben auf den Kohlenhydratstoffwechsel

Von

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(Direktor: Prof. Dr. J. Kühnau)

(Der Schriftleitung zugegangen am 28. Mai 1955)

Herrn Prof. Dr. H. Reinwein, Kiel, in Verehrung zum 60. Geburtstag gewidmet

In den vorausgegangenen Mitteilungen[1,2] berichteten wir über das Verhalten von α-Ketoglutarsäure, Brenztraubensäure, L-Alanin und Asparaginsäure im Serum des Kaninchens nach Verabreichung von L-Glutaminsäure. Wir hatten einen Anstieg von α-Ketoglutarsäure, einen Abfall von Brenztraubensäure sowie eine starke Vermehrung des L-Alanins beobachtet und konnten nachweisen, daß der Anstieg des L-Alanins den Pyruvatvorrat des Blutes um das 5–10fache übersteigt. Wir schlossen daraus auf eine Nachlieferung der zur Alaninbildung benötigten Brenztraubensäure aus dem Kohlenhydratstoffwechsel, zumal die Höhe des Alaninanstieges unabhängig war von der injizierten Glutaminsäuremenge (2 und 4 mMol/kg), von gleichzeitiger Pyruvatgabe sowie von der Applikationsart. Seitz, Engelhardt-Gölkel und Schaffry[3] haben beim Menschen den Anstieg von α-Ketoglutarsäure bestätigen können; sie fanden aber keinen Abfall von Brenztraubensäure. Deswegen soll noch einmal an die Befunde von Kamin und Handler[4] erinnert werden, die als ein Endprodukt der Transaminierung eine starke Ausscheidung von Asparaginsäure im Harn nachgewiesen haben, während wir keinen Anhalt für eine erhebliche Mehrbildung von Asparaginsäure fanden.

Wir berichten in dieser Mitteilung über die Wirkung von Glutaminsäuregaben auf den Kohlenhydratstoffwechsel in vivo auf Grund von Bestimmungen des Blutzuckerspiegels und Leberglykogens, und zwar betreffen die Versuche im I. Kapitel die Glutaminsäurewirkung beim

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[1] V. Klingmüller u. K. H. Vogelgesang, Ber. ges. Physiol. exp. Pharmakol. **162**, 369 [1954]; diese Z. **300**, 97 [1955].

[2] V. Klingmüller, J. Gayer u. F. Bramstedt, diese Z. **300**, 107 [1955].

[3] W. Seitz, A. Engelhardt-Gölkel u. J. Schaffry, Klin. Wschr. **33**, 228 [1955].

[4] H. Kamin u. P. Handler, Amer. J. Physiol. **164**, 654 [1951].

Kaninchen (1a) auf den durch Glucosebelastung erhöhten Blutzucker, (1b) auf den durch Insulin erniedrigten Blutzuckerspiegel und (1c) auf das Verhalten des Blutzuckers alloxandiabetischer Tiere. Im 2. Kapitel teilen wir das Verhalten des Leberglykogens von Ratten mit, und zwar nach Glutaminsäuregaben (2a) an Normaltiere, (2b) an Tiere mit blockierter Adrenalinausschüttung, (2c) an adrenalektomierte Tiere und (2d) an Pyridoxin-Mangeltiere.

1. Einfluß von L(+)-Glutaminsäure auf den Blutzucker

Über das Verhalten des normalen Blutzuckers nach Glutaminsäure bei Mensch und Tier liegen zahlreiche, sich zum Teil widersprechende Untersuchungen vor[5-8]. Auch unsere Blutzuckerbefunde nach Glutaminsäuregaben waren uneinheitlich[9,10], so daß es uns wünschenswert erschien, den Mechanismus der Einwirkung der Glutaminsäure auf den Kohlenhydratstoffwechsel an Hand von Blutzuckerbestimmungen unter verschiedenen Bedingungen genauer zu analysieren.

1a) Wirkung von L(+)-Glutaminsäuregaben auf den durch Glucosebelastung erhöhten Blutzuckerspiegel

An 6 Kaninchen von etwa 3 kg Gewicht, die seit 12—14 Stdn. nüchtern waren, wurden Lösungen von 4 mMol Glucose/kg per Schlundsonde verabfolgt und der Blutzucker sowohl nach Hagedorn-Jensen als auch als „wahre Glucose" nach der von Frank und Kirberger modifizierten Methode von Nelson[11] bestimmt. 2 Tage später wurde an die gleichen Kaninchen gleichzeitig mit der Glucose 2 mMol/kg L(+)-Glutaminsäure verabreicht und wiederum der Blutzucker verfolgt. 3 Tage nach diesem Versuch erhielten 3 dieser Tiere 8 mMol Glucose/kg und nach weiteren 2 Tagen wiederum 8 mMol Glucose/kg zusammen mit 2 mMol Glutaminsäure/kg gleichzeitig durch eine Schlundsonde. Die Glucosemengen entsprechen 50 und 100 g bei 70 kg schweren Erwachsenen, die Glutaminsäuremenge entspricht 20 g.

Wir haben für die zuletzt erwähnten Versuche mit der doppelten Glucosemenge die gleichen Tiere benutzt, da auch Himwich[5] nachgewiesen hat, daß die Stoffwechselwirkung intravenös oder oral verab-

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[5] W. A. Himwich, Amer. J. Physiol. [1954], persönl. Mitteilung.
[6] R. Küper, Dissertat. Hamburg 1954.
[7] R. Torres, Rev. Neuro-Psiquatr. **17**, 32 [1954].
[8] W. Mayer-Gross u. J. W. Walker, Nature [London] **160**, 334 [1947]; Biochem. J. **44**, 92 [1949].
[9] V. Klingmüller, Biochemie, Physiologie und Klinik der Glutaminsäure Editio Cantor K. G. Aulendorf Württ. 1955, im Druck.
[10] V. Klingmüller u. J. Gayer, Verh. Dtsch. Ges. Inn. Med. **60**, 960 [1954].
[11] H. Frank u. E. Kirberger, Biochem. Z. **320**, 359 [1950]; N. Nelson, J. biol. Chemistry **153**, 375 [1944].

folgter Glutaminsäure nach einem Tage abgeklungen ist. Die gleichzeitige orale Verabfolgung von Glutaminsäure führt zu einer Depression der Blutzuckerbelastungskurve (Abb. 1). Wir versuchten, diesen Befund an erwachsenen gesunden Versuchspersonen unter analogen Bedingungen nachzuprüfen, fanden aber bei den 4 untersuchten Personen keine Depression der Blutzuckerbelastungskurve.

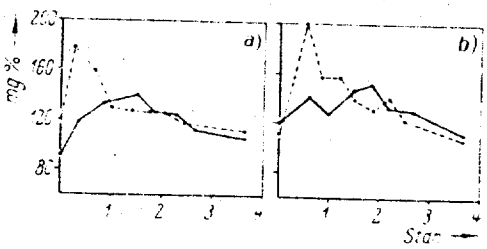

Abb. 1. Glucosebelastungskurve: Kaninchen 4 mMol/kg Glucose per os: (----) ohne Glutaminsäurezusatz, (——) gleichzeitig mit 2 mMol/kg suspendierter L(+)-Glutaminsäure per os; a) Bestimmung der „wahren" Glucose nach Frank, Kirberger[11]; b) nach Hagedorn-Jensen.

1b) Wirkung von L(+)-Glutaminsäure auf den durch Insulin erniedrigten Blutzucker

Verabfolgt man einem 12 Stdn. nüchternen Kaninchen 3 I. E. Altinsulin subcutan, so tritt 2—3½ Stdn. nach der Injektion ein hypoglykämischer Schock ein, der mit 5 ml 20-proz. Glucoselösung unterbrochen werden kann (Abb. 2a).

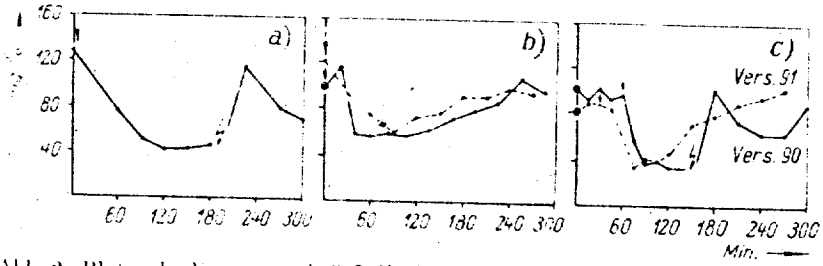

Abb. 2. Blutzuckerkurven nach 3 I. E. Altinsulin (,) subc., Kaninchen (2,8 kg). a) und c): ⚡ Hypoglykämischer Schock, 5 ml 20-proz. Glucoselösung, i. v. b) und c): ⊙ 2 mMol L(+)-Glutaminsäure/kg per os.

Werden demselben Tier 2 Stdn. vor dem erwarteten Schockeintritt 2 mMol/kg Glutaminsäure per os verabfolgt, so sinkt der Blutzucker nicht weiter ab, sondern es kommt zu einem langsamen Anstieg, wodurch der Schock verhindert wird. (Abb. 2b). Ebenso kann der Schock durch gleichzeitige Gabe von Insulin und Glutaminsäure verhindert werden (Abb. 2b).

Abb. 2 c zeigt, wieder bei demselben Tier, Versuche, bei denen die Glutaminsäure vor den Insulininjektionen gegeben wurde. Der Versuch 91, bei dem wir die Glutaminsäure 30 Min. vor dem Insulin gaben, zeigt wiederum keinen Schockeintritt, während bei Versuch 90 die Glutaminsäure, 60 Min. vor dem Insulin gegeben, den Schock nicht mehr verhindern konnte. — Diese Versuche wurden in 3tägigem Abstand an 3 Kaninchen in insgesamt 6 Versuchsserien durchgeführt.

I c) Wirkung von L-Glutaminsäure auf das Verhalten des Blutzuckers alloxandiabetischer Kaninchen

Für diese Versuche wurden Kaninchen von 2 und 3 kg Gewicht verwendet, die durch einmalige intravenöse Injektion von 150 mg/kg Alloxan in 5-proz. Lösung diabetisch gemacht worden waren; das Alloxan wurde frisch nach Vanino[11a] dargestellt. Sie wurden eine Woche nach der Alloxaninjektion in den Versuch genommen, damit eventuelle Leber- und Nierenschädigungen abklingen konnten.

An 6 Kaninchen, die seit 8—10 Stdn. nüchtern waren, wurden 2 mMol/kg Natriumglutaminat als 10-proz. Lösung intravenös verabfolgt. In Tab. 1 sind die bei diesen Versuchen ermittelten Blutzuckerwerte zusammengestellt. Die Maximalwerte des Blutzuckers lagen bei 120 Min. nach der Injektion; dabei sind die Anstiege um so größer, je stärker der Diabetes, je höher also die Blutzuckerausgangslage der Tiere ist.

Um sicherzustellen, daß es sich bei diesem Blutzuckeranstieg tatsächlich um eine Wirkung der Glutaminsäure und nicht um den Effekt einer hypertonischen Salzlösung handelt, prüften wir in zwei weiteren Versuchen an alloxandiabetischen Kaninchen den Einfluß von 4 mMol/kg als Aufschwemmung durch die Schlundsonde verabfolgte Glutaminsäure auf den Blutzucker. Die hierbei beobachteten Blutzuckervermehrungen betrugen 86 und 108 mg%. Die Verabfolgung einer der Glutaminsäurelösung isotonischen Kochsalzlösung führte zu keiner signifikanten Blutzuckersteigerung.

Tab. 1. Blutzuckerveränderungen bei alloxandiabetischen Kaninchen nach intravenöser Injektion von 2 mMol/kg Natriumglutaminat (10-proz. Lösung). Bestimmung nach Hagedorn-Jensen; Angaben in mg%.

Tier Nr.	Vor der Injektion	Min. nach der Injektion							Größte BZ-Vermehrung
		20	40	60	90	120	150	180	
1	287	318	320	326	342	359	351	320	72
2	270	300	304	322	352	352	320	304	82
3	206	248	258	293	—	313	306	297	107
4	475	515	565	610	718	760	742	683	285
5	533	572	632	674	754	821	803	726	288
6	182	202	229	238	270	266	262	256	88

[11a] L. Vanino, Handb. d. präparativen Chemie; Verlag Ferd. Enke, Stuttgart, 2. Aufl. 1923, Bd. 2, S. 235.

Um zu klären, ob bei dieser Blutzuckererhöhung Glucose durch Adrenalinwirkung aus der Leber freigesetzt wurde oder ob hier die Glutaminsäure selbst für die Glucosebildung im Zwischenstoffwechsel verantwortlich war, suchten wir die Adrenalinwirkung durch adrenolytische Substanzen auszuschalten. Die dihydrierten Mutterkornalkaloide vermögen in der Dosis von 0,1 mg/kg an der Ratte nach Untersuchungen von Rothlin[12], Schneider[13] und Kroneberg[14] die Blutzuckerwirkung von 0,1 mg/kg Adrenalin zu 96% zu unterdrücken. Die am stärksten wirksamen Substanzen dieser Reihe sind das Dihydroergocristin, Dihydroergocornin und Dihydroergokryptin, die im Hydergin

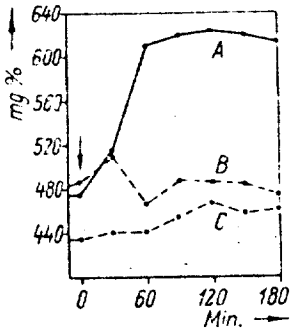

Abb. 3. Blutzuckerkurve bei Alloxandiabetes: Kurve A: 2 mMol/kg Na-Glutaminat i. v. (↓); Kurve B und C: 0,5 mg/kg Hydergin subc.; nach 20 Min. 2 mMol/kg Na-Glutaminat i. v.

zu gleichen Teilen enthalten sind und selbst keine Eigenwirkung auf den Blutzucker haben, wie wir in Bestätigung der Beobachtungen von Kroneberg[14] fanden.

Wir injizierten den alloxandiabetischen Kaninchen im Vorversuch 2 mMol/kg Natriumglutaminat intravenös, wiederholten diesen Versuch 3 Tage später 20 Min. nach der Verabreichung von 0,5 mg/kg Hydergin subcutan und verfolgten in beiden Versuchsanordnungen den Blutzucker. In Abb. 3 stellen die beiden gestrichelten Kurven B und C den Blutzucker unter dem Einfluß von Hydergin und Glutaminat dar. Dazu wurden die 3 Kurven in jeweils dreitägigem Abstand gewonnen, wobei die ausgezogene ohne Hydergin erzielte Kurve A zeitlich in der Mitte lag.

2. Einfluß von L(+)-Glutaminsäure auf das Leberglykogen

2a) Normale Ratten

10 männlichen Albinoratten mit Gewichten zwischen 150 und 200 g, die bis zum Versuchsbeginn ad libitum mit Hafer gefüttert wurden, wurde in jeweils 2-stdg. Abstand 3mal 10 mMol/kg Natriumglutaminat als 10-proz. Lösung intraperitoneal injiziert. 2 Stdn. nach der letzten Injektion wurden die Tiere durch Dekapitation getötet und das Leber-

[12] G. Rothlin, Bull. Schweiz. Acad. Med. Wiss. **2**, 249 [1946/47].
[13] P. B. Schneider, Schweiz. Arch. Neurol. **65**, 283 [1950].
[14] G. Kroneberg, persönl. Mitteilung.

glykogen nach Pflüger (vgl. Lohmann[15]) in Doppelbestimmungen bestimmt. 10 weiteren Ratten wurde unter sonst gleichen Versuchsbedingungen statt der Glutaminsäurelösung die entsprechende Menge Kochsalzlösung intraperitoneal verabfolgt. Die Tiere wurden ebenfalls 2 Stdn. nach der 3. Injektion getötet und ihr Leberglykogen bestimmt.

In Abb. 4a ist der Glykogengehalt in g% Frischleber als Mittelwerte der Doppelbestimmungen bei den einzelnen Tieren, als Mittelwerte der mit Glutaminsäure behandelten Tiere sowie der Kontrolltiere als Säulen dargestellt. Die statistische Auswertung erfolgte als Beobachtungsreihe mit stetigem Merkmal[16] nach den Formeln:

$$\text{für den Mittelwert } a = \frac{1}{k} \cdot \Sigma x \text{ und für die Streuung } \sigma^2 = \frac{1}{k} \cdot \Sigma (x - a)^2 .$$

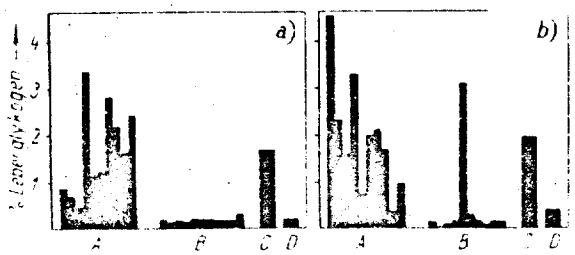

Abb. 4a. Verhalten des Leberglykogens gefütterter Ratten nach 3maliger Injektion von je 10 mMol/kg Na-Glutaminat intraperitoneal. A = Kontrolltiere; B = mit Glutaminat behandelte Tiere; C = Mittelwert der Kontrolltiere; D = Mittelwert der mit Glutaminat behandelten Tiere.

Abb. 4b. Wie Abb. 4a, jedoch 2malige Injektion von je 10 mMol Na-Glutaminat/kg intraperitoneal.

Die Urteilszahl zur Feststellung der signifikanten Differenz wurde nach der Formel

$$Q = \frac{a_2 - a_1}{\sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}}$$

ermittelt. Die Zufallsgrenzen wurden entspr. dem 3 σ-Wert einer Gaußschen Verteilung festgelegt mit $Q \approx 3 \cdot \frac{10}{n} = 3 \cdot \frac{10}{n_1 + n_2} = 3.5$.

Da sich für unseren Fall eine Urteilszahl von $Q = 9.5$ ergibt, ist die Abnahme des Leberglykogens nach intraperitonealer Glutaminsäuregabe von 3mal 10 mMol/kg statistisch gesichert. Die statistische Berechnung zeigt Tab. 2.

In einem weiteren Versuch wurde 10 Ratten statt 3mal nur 2mal je 10 mMol/kg Natriumglutaminat intraperitoneal injiziert und wieder das Leberglykogen im Vergleich zu 10 mit Kochsalzlösung behandelten Tieren bestimmt. Den Glykogengehalt in g% Frischleber zeigt Abb. 4b. Warum das Leberglykogen bei dem einen mit Glutaminsäure behandelten Tier hoch blieb, ist uns nicht ersichtlich; vielleicht erfolgte die Injektion in den Darm. Die statistische Auswertung dieses Versuches

[15] K. Lohmann in C. Oppenheimer u. L. Pincussen, Die Fermente und ihre Wirkungen, 3, 414; G. Thieme Verlag, Leipzig 1936.

[16] H. Gebelein u. H. J. Heite, „Statistische Urteilsbildung", Springer-Verlag Berlin 1951.

ergibt eine signifikante Differenz nur dann, wenn dieses eine Tier in der Berechnung nicht berücksichtigt wird. Wird den Ratten nur einmal 10 mMol/kg Natriumglutaminat verabfolgt, so kommt es zu keinem statistisch zu sichernden Glykogenabfall, so daß wir auf die Wiedergabe der Ergebnisse verzichten.

Bei allen mit Glutaminatinjektionen behandelten Tieren fanden wir in der Bauchhöhle reichlich eiweißhaltige Flüssigkeit, die wir in einem weiteren Versuch bei 5 Ratten, denen wir 2mal 10 mMol/kg Glutaminat injizierten, quantitativ abpipettierten, nach Enteiweißung mit Wolframat[17] zweidimensional papierchromatographisch untersuchten sowie den Aminostickstoff nach van Slyke[18] bestimmten. Der Zuckergehalt des Exsudats entsprach dem Blutzucker der Tiere. Die papierchromatographische Untersuchung ergab ein so erhebliches Überwiegen der Glutaminsäure über die übrigen Aminosäuren, daß wir in Tab. 3 die gefundene Menge Amino-N als Glutaminsäure ausgedrückt haben.

Tab. 2. Einfluß von 3mal je 10 mMol/kg Natriumglutaminat auf das Leberglykogen normaler Ratten.

1 Mit Glutaminsäure behandelte Tiere			2 Kontrolltiere		
3 Tier Nr.	4 Leberglykogen (x) in g%	$(x-a)^2$	Tier Nr.	Leberglykogen (x) in g%	$(x-a)^2$
1	0,22	0,00124	11	0,86	0,706
2	0,13	0,00305	12	0,70	1,0
3	0,16	0,00063	13	1,16	0,292
4	0,12	0,00423	14	0,50	1,44
5	0,18	0,00003	15	3,27	2,46
6	0,21	0,00063	16	1,22	0,23
7	0,20	0,00022	17	2,84	1,30
8	0,16	0,00063	18	2,22	0,27
9	0,17	0,00022	19	1,67	0,0009
10	0,30	0,01322	20	2,42	0,52
$\Sigma x = 1{,}85$; $\Sigma(x-a)^2 = 0{,}02410$; $a = 0{,}185$ g%; $\sigma = \pm 0{,}49$ g%			$\Sigma x = 16{,}86$; $\Sigma(x-a)^2 = 8{,}2189$; $a = 1{,}70$ g%; $\sigma = \pm 0{,}91$ g%		

Tab. 3. Änderung des Bauchhöhleninhalts von Ratten 2 Stdn. nach der Injektion von 2 mal je 10 mMol/kg Natriumglutaminat (= Glu) als 10-proz. Lösung.

1 Ratten Nr.	2 Körper-Gewicht	3 Injizierte Glu-Menge in		4 Gefundenes Exsudat in der Bauchhöhle		
		mg	ml	ml	Glucose in mg%	NH_2-N ber. in mg Glu
1	135	396	3,96	8,1	124	42,0
2	120	352	3,52	6,7	143	12,2
3	118	348	3,48	7,2	108	30,4
4	150	440	4,40	10,8	129	43,0
5	122	360	3,60	6,8	108	20,7

[17] S. Hier u. O. Bergeim, J. biol. Chemistry 161, 717 [1945].
[18] D. D. van Slyke, Ber. dtsch. chem. Ges. 43, 3170 [1910].

Aus diesen Befunden geht hervor, daß etwa 90% der injizierten Glutaminatmenge vom Tier tatsächlich resorbiert worden sind und daß es sich bei der Mobilisierung von Leberglykogen nicht um einen Verdünnungseffekt durch die injizierte Flüssigkeitsmenge handeln kann, da die zugesetzte Flüssigkeitsmenge von durchschnittlich 4,0 ml zur Erlangung eines Zuckergehaltes von 125 mg% nur die Mobilisierung von 5 mg Glucose nötig machen würde, was einer Abnahme des Leberglykogens um etwa 0,1 g% entsprechen würde.

2 b) Einfluß von L(+)-Glutaminsäure auf das Leberglykogen von Ratten mit blockierter Adrenalinausschüttung

Um den Wirkungsmechanismus der Mobilisierung von Leberglykogen durch Glutaminsäure zu beleuchten, untersuchten wir diesen Effekt

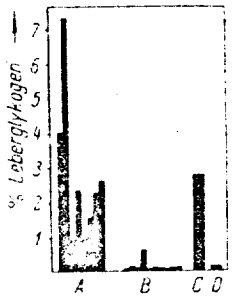

Abb. 5. Verhalten des Leberglykogens gefütterter Ratten nach 2maliger Injektion von je 10 mMol/kg Na-Glutaminat intraperitoneal und 20 Min. vorher von 0,5 mg/kg Hydergin subcutan. — A = Kontrollen; Hydergin-Tiere; B = Glutaminat-Hydergin-Tiere; C = Mittelwert der Kontrolltiere; D = Mittelwert der Glutaminat-Hydergin-Tiere.

ebenfalls unter dem Einfluß von im Hydergin kombinierten dihydrierten Mutterkornalkaloiden, Dihydroergocornin, Dihydroergocristin und Dihydroergokryptin; sie sind uns wegen der Unterdrückung bestimmter Sympathicuswirkungen bei der Untersuchung der „adrenergischen Wirkung der Glutaminsäure" auf den Blutzucker alloxandiabetischer Kaninchen von Wert gewesen (s. Abschn. 1 c).

10 bis Versuchsbeginn mit Hafer ad libitum gefütterten Ratten wurden 20 Min. vor der intraperitonealen Injektion von 10 mMol/kg Natriumglutaminat 0,5 mg/kg Hydergin subcutan verabfolgt, beide Injektionen 2 Stdn. später wiederholt, die Tiere nach weiteren 2 Stdn. getötet und das Leberglykogen bestimmt. Die Versuchsbedingungen waren also die gleichen wie bei dem in Abb. 4b dargestellten Versuch bis auf die Verabfolgung von Hydergin. Abb. 5 zeigt den Glykogengehalt in g% Frischleber im Vergleich zu 10 Kontrolltieren, die ebenfalls Hydergin bekamen. Der Mittelwert a für die mit Glutaminat behandelten Tiere beträgt 0,13 g%, mit einer Streuung σ von $\pm$ 0,168 g%. Die Kontrolltiere haben einen Mittelwert $a = 2{,}77$ g%, die Streuung beträgt $\sigma = \pm 1{,}99$ g%. Die Urteilszahl für die signifikante Differenz ist 6,67 bei einer Zufallsgrenze von 3,55. Die statistische Signifikanz ist also auch bei diesem Versuch trotz der großen Schwankung des Glykogengehaltes bei den Kontrolltieren gewahrt.

Da die mit Glutaminat behandelten Ratten gegen Ende des Versuches deutliche hypoglykämische Symptome zeigten, verfolgten wir bei 4 Tieren während eines solchen Versuches den Blutzucker, der von

normalen Werten am Anfang zu deutlich hypoglykämischen gegen Ende des Versuches abfiel (Abb. 6). Die hierzu durchgeführten Kontrollen mit Glutaminat allein sowie mit Hydergin allein zeigten keine Blutzuckerschwankungen.

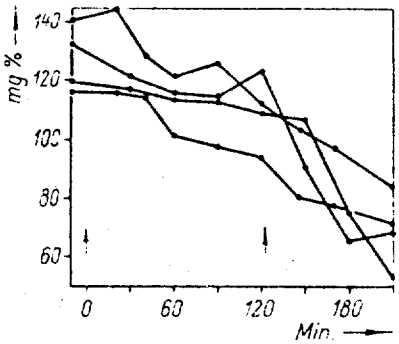

Abb. 6. Verhalten des Blutzuckers gefütterter Ratten. (↓): Injektion von 10 mMol/kg Na-Glutaminat intraperitoneal; 20 Min. vorher 0,5 mg/kg Hydergin subcutan.

2 c) Einfluß von L(+)-Glutaminsäure auf Leberglykogen und Blutzucker adrenalektomierter Tiere

In einer weiteren Versuchsserie untersuchten wir den Einfluß von intraperitonealen Glutaminatinjektionen auf Leberglykogen und Blutzucker adrenalektomierter männlicher Ratten. Die Tiere wurden in einer Sitzung doppelseitig adrenalektomiert und erhielten in den folgenden 8 Tagen 2 mal täglich 0,4 ml/100 g Nebennierenextrakt (Pancortex) intramuskulär sowie eine aus Hafer und 2-proz. Kochsalzlösung bestehende Diät. Am Versuchstage wurde den Tieren 2 mal 10 mMol/kg Natriumglutaminat in 2-stdg. Abstand intraperitoneal verabreicht. In verschiedenem zeitlichem Abstand nach der zweiten Injektion zeigten die Tiere starke hypoglykämische Symptome, die im ausgeprägten Schock mit heftigen Streckkrämpfen endeten. Auf der Höhe des Schocks wurden die Tiere dekapitiert, der Blutzucker aus dem Kopfblut sowie das Leberglykogen bestimmt. Tab. 4 zeigt die Er-

Tab. 4. Einfluß von 2 mal 10 mMol/kg Na-Glutaminat auf Leberglykogen und Blutzucker adrenalektomierter Ratten.

Tier Nr.	Körpergewicht in g	Glutaminsre. i. p. in mg	Schockeintritt nach 2 Inj. in Min.	Blutzucker sub finem in mg%	Leberglykogen in g%
1	132	390	110	21	0,14
2	120	350	55	24	0,17
3	110	322	30	25	0,10
4	117	344	20	35	0,14
5	117	344	5	28	0,13
6	95	280	85	41	0,13
7	120	350	65	26	0,16
8	100	294	40	32	0,15

gebnisse dieses Versuches. Auch bei adrenalektomierten Tieren führen hohe Glutaminsäureinjektionen wie bei den Tieren mit durch Hydergin blockierten Adrenalinwirkungen zur Senkung von Leberglykogen und Blutzucker. Daß die hypoglykämischen Symptome hier eher und intensiver auftreten, mag seine Erklärung in dem Fehlen eines reaktionsfähigen Nebennierenrindenapparates mit ausreichender Bereitstellung von Glykocorticoiden sowie in der durch die Operation doch vollständiger gelungenen Ausschaltung der adrenergischen Wirkung finden.

2 d) Einfluß von L(+)-Glutaminsäure auf das Leberglykogen von Pyridoxin-Mangeltieren

Männliche Albinoratten (Stamm Sprague-Dawley) wurden mit einer pyridoxinfreien Diät, bestehend aus 63% vitaminfreier Maisstärke, 18% vitaminfreiem Casein, 4% Cellulosepulver, 4% Salzgemisch nach McCollum, 11% Lebertran, ernährt; pro 100 g Diät wurden zugesetzt: 100 mg Cholinhydrochlorid, 1 mg Thiamin, 2 mg Lactoflavin, 10 mg Nicotinsäureamid, 0,2 mg Vitamin K (Synkavit). Jedes Tier erhielt täglich 180 γ Desoxypyridoxin per Schlundsonde. Nach 5—8 Tagen nahmen die Tiere nicht mehr an Gewicht zu und zeigten nach 3 Wochen alle Symptome ausgeprägten Pyridoximmangels.

Da sich herausstellte, daß derartige Ratten wegen des durch die starken Schleimhautveränderungen an Maul und Rachen bedingten Hungerzustandes außerordentlich niedrige Glykogenwerte haben, wurden die Tiere während der letzten Woche vor dem Versuch mit einer flüssigen kalorisch ausreichenden Diät (150 g mit Pepsin aufgeschlossenes vitaminfreies Casein, 500 g Glucose, 20 g Salzgemisch, 50 g Cellulosepulver, 40 mg Desoxypyridoxin, Vitaminzusatz wie oben, Wasser ad 1000 ml) 3mal täglich durch die Schlundsonde ernährt. Der einen Hälfte der so vorbehandelten Tiere wurde 2mal 10 mMol/kg Natriumglutaminat im Abstand von 2 Stdn. intraperitoneal injiziert und das Leberglykogen nach weiteren 2 Stdn. bestimmt. Die andere Hälfte der Tiere erhielt statt des Glutaminats Kochsalzlösung.

Die Glykogenwerte der behandelten und der Kontrolltiere sind aus Tab. 5 ersichtlich. Bei den mit Glutaminat behandelten Tieren beträgt der Mittelwert $a = 0{,}59$ g%, die Streuung $\sigma = \pm 0{,}196$ g%. Bei

Tab. 5. Einfluß von 2mal je 10 mMol/kg Na-Glutaminat auf das Leberglykogen von Pyridoxin-Mangeltieren.

Mit Glu behandelte Tiere			Kontrolltiere		
Tier Nr.	Leberglykogen in g	$(x-a)^2$	Tier Nr.	Leberglykogen in g%	$(x-a)^2$
1	0,44	0,0225	6	0,33	0,044
2	0,68	0,0081	7	1,34	0,64
3	0,75	0,0256	8	0,18	0,129
4	0,78	0,0361	9	0,14	0,16
5	0,29	0,09	10	0,74	0,04
$\Sigma x = 2{,}94$; $\Sigma(x-a)^2 = 0{,}1923$; $a = 0{,}59$ g%; $\sigma = \pm 0{,}196$ g%;			$\Sigma x = 2{,}73$; $\Sigma(x-a)^2 = 1{,}013$; $a = 0{,}54$ g%; $\sigma = \pm 0{,}46$ g%.		

den Kontrolltieren ist der Mittelwert $a = 0.54$ g%, die Streuung $\sigma = 0.46$ g%. Die Urteilszahl liegt bei 1.06, die Zufallsgrenze bei 4.0; eine signifikante Differenz besteht also zwischen beiden Gruppen nicht.

Diskussion

Nachdem über das Verhalten des normalen Blutzuckers nach Glutaminsäuregabe bei Mensch und Tier zahlreiche Untersuchungen zu nicht übereinstimmenden Ergebnissen geführt hatten[5–10], glaubten wir dadurch zu eindeutigeren Aussagen über den Einfluß der Glutaminsäure auf die Glykämie zu gelangen, daß wir ihn bei experimentell variiertem Blutzuckerniveau prüften. Wir untersuchten daher die Wirkung von L(+)-Glutaminsäure auf den durch Traubenzuckerbelastung oder Alloxanbehandlung erhöhten und den durch Insulinverabfolgung erniedrigten Blutzucker.

Unsere Versuche haben gezeigt, daß die alimentäre Hyperglykämie durch Glutaminsäure beim Kaninchen regelmäßig partiell unterdrückt werden kann. Den Befund von Küper[6], der einen Versuch mit dem gleichen Ergebnis an einer 23-jährigen gesunden Versuchsperson durchführte, konnten wir aber beim Menschen nicht bestätigen. Diese Diskrepanz mag ihre Ursache darin haben, daß unsere Versuchspersonen Institutsangehörige waren, die während der Versuche ihre vorwiegend sitzende Arbeit fortsetzten.

Die partielle Unterdrückung der alimentären Hyperglykämie durch Glutaminsäure scheint dafür zu sprechen, daß der Einfluß einmaliger oder stoßweiser Verabreichungen von Glutaminsäure auf den Kohlenhydratstoffwechsel nicht stets als adrenergischer Effekt[19,20] gedeutet werden kann, sondern daß die Glutaminsäure außerdem nach Transaminierung zu α-Ketoglutarsäure durch Anfachen des Citronensäurecyclus, zum anderen durch Verbrauch von Brenztraubensäure bei der Transaminierung[2] in den Stoffwechsel der Glucose eingreifen kann. Offensichtlich werden die Reaktionsprodukte α-Ketoglutarsäure und Alanin nicht zur sofortigen Resynthese von Glucose, sondern im Citronensäurecyclus verwendet.

Die Wirkung der Glutaminsäure auf das Erwecken aus dem hypoglykämischen Schock ist oft beschrieben und nachuntersucht worden[8,19]. Da neben Glutaminsäure auch andere Substanzen (*p*-Aminobenzoesäure, Glykokoll, Arginin und Eiweißhydrolysate) diese Wirkung haben, an Gehirnschnitten aber verschieden wirken, ist ein zentraler Angriffspunkt der Glutaminsäure nicht anzunehmen. Dagegen konnte Weil-Malherbe zeigen[19,20], daß es durch Injektion von nur geringen Mengen von Glutaminsäure zu einem starken Anstieg des im hypoglykämischen Schock erniedrigten Adrenalins kommt[21]. Die Folge dieses

[19] H. Weil-Malherbe, J. Ment. Sci. **95**, 930 [1949]; ders., Physiol. Rev. **30**, 549 [1950].

[20] H. Weil-Malherbe, Lancet **265**, 623 [1953].

[21] H. Weil-Malherbe u. A. D. Bone, J. Endocrinol. **11**, 285 [1954].

Adrenalinanstieges ist dann die Erweckung aus dem Schock und der folgende Blutzuckeranstieg. Dieser adrenergische Effekt, den Weil-Malherbe auch für die gelegentlich beobachteten Steigerungen des Blutzuckers nach Glutaminsäuregaben an Normglykämische als Erklärung heranzieht, ist nach unseren Versuchen auch noch dann nachzuweisen, wenn die Glutaminsäure 30 Min. vor dem Insulin gegeben wird. Er scheint also etwa so lange anzuhalten, bis der Glutaminsäurespiegel und die Verschiebungen der intermediären Stoffwechselprodukte wieder normalisiert sind. Da dieses innerhalb von 2—3 Stdn. der Fall ist, kann eine Glutaminsäuregabe 2—3 Stdn. vor dem zu erwartenden Schockeintritt diesen nicht mehr verhindern. Die Feststellung von Himwich[5], daß die Glutaminsäurewirkung auf den Stoffwechsel am nächsten Tag bereits abgeklungen ist, möchten wir dadurch weiter präzisieren, daß nur 2—3 Stdn. zur Normalisierung des Stoffwechsels notwendig sind.

Im Gegensatz zu diesen Beobachtungen stehen Befunde von Kraus und Rech[22], die eine insulinverstärkende Wirkung der Glutaminsäure nachweisen, wenn diese am Abend vor einer Insulingabe, die normalerweise keinen Schock auslöst, gegeben wird. Wir haben diese Versuche nachgearbeitet und nicht bestätigen können.

Der Anstieg des Blutzuckers nach Glutaminsäureverabfolgungen an alloxandiabetische Tiere hat nicht seine Ursache in der im Diabetes gesteigerten Glykoneogenese, sondern ist nach unseren Versuchen ebenfalls als adrenergischer Effekt zu deuten, da es gelingt, ihn mit dihydrierten Mutterkornalkaloiden zu unterdrücken.

Wir können hier also zwei verschiedene Mechanismen der Glutaminsäurewirkung auf den Blutzucker unterscheiden: einmal eine blutzuckersenkende bei alimentärer Hyperglykämie, zum anderen eine blutzuckersteigernde adrenergische bei Alloxandiabetes und Insulinschock. Beide, die glykolytische und die adrenergische, heben sich bei Glutaminsäureverabfolgungen an normglykämische Tiere meistens auf. Bei alimentärer Hyperglykämie überwiegt entsprechend der katabolischen Stoffwechselrichtung die gleichsinnig verlaufende glykolytische Wirkung, während beim Alloxandiabetes mit gestörtem Glucoseabbau diese glykolytische Wirkung offensichtlich nicht zur Geltung gekommen ist, sondern der adrenergische Effekt zu einer Freisetzung von Glucose aus der glykogenreichen Leber und damit zu einem beträchtlichen Blutzuckeranstieg geführt hat. Dieser ist um so höher, je stärker der Diabetes ist, d. h. je geringer die Fähigkeit des Organismus ist, Glucose abzubauen.

Nachdem wir zeigen konnten, daß die Wirkung der Glutaminsäure auf den Kohlenhydratstoffwechsel nicht nur auf einem adrenergischen Effekt[19,20] zu beruhen braucht, sondern daß die nachgewiesene Depression der Blutzuckerbelastungskurve in einem anderen Mechanismus ihre Erklärung finden muß, konnte der statistisch gesicherte Beweis

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[22] W. Kraus u. H. Rech, Dtsch. Gesundheitswes. 6, 992 [1953].

erbracht werden, daß genügend hohe Injektionen von L(+)Glutaminsäure zu einem fast völligen Schwund des Leberglykogens gefütterter Ratten führt. Diese Mobilisierung des Glykogens ist unabhängig von einem intakten Nebennierenmarksystem, wie wir durch dessen Ausschaltung einerseits auf pharmakologischem und andererseits auf operativem Wege anschaulich machen konnten. Schober[23] wies bei Kaninchen nach intravenöser Injektion von 6mal 0.54 g Glutaminat eine völlige Glykogenverarmung von Leber und Nebenniere mit histochemischen Methoden nach. Da er außerdem ein Verschwinden der Chromaffinität der Nebenniere fand, deutete er den Glykogenschwund als adrenergischen Effekt. Unseres Erachtens ist nach den eingehenden Untersuchungen von Weil-Malherbe[19] an einer vermehrten Ausschüttung von Adrenalin und Noradrenalin aus der Nebenniere nach Gabe von Glutaminsäure nicht zu zweifeln, jedoch befinden wir uns hier im Gegensatz zu Schober, da wir diesem adrenergischen Effekt im Hinblick auf die Mobilisierung des Leberglykogens keine wesentliche Bedeutung beimessen können.

Interessant scheint bei dieser Ausschaltung der adrenergischen Wirkung der Befund zu sein, daß es durch Glutaminsäuregabe zu einem Absinken des Blutzuckers auf hypoglykämische Werte kommt. Nach Untersuchungen von Weil-Malherbe[21] ist die Blutzuckersenkung nach Insulin eine zeitliche Folge des Adrenalinabfalls, wobei wir die Möglichkeiten der Ursache dieses Phänomens außer Betracht lassen wollen. Da wir die Adrenalinwirkung entweder pharmakologisch oder operativ unterbunden haben, ist auf diesem Wege die gleiche Stoffwechsellage mit geringer (oder fehlender) Adrenalinwirkung wie nach Insulingabe vorhanden, und dem Blutzucker ist so die Möglichkeit zum Absinken gegeben, die entweder — im Falle des Insulinschocks durch vermehrten Abbau in der Peripherie oder im Falle der Glutaminsäuregabe durch Brenztraubensäureentzug auf dem Wege der Transaminierung zu α-Ketoglutarsäure und Alanin realisiert wird. Der Adrenalinabfall scheint so eine der Voraussetzungen für den Eintritt des hypoglykämischen Schocks zu sein, deren andere ein auf irgendeine Weise zustande gekommener Glucoseabbau sein muß.

Unseren oben angenommenen Wirkungsmechanismus des Eingreifens von L(+)-Glutaminsäure in den Kohlenhydratstoffwechsel durch ihre Transaminierung zu α-Ketoglutarsäure und Alanin suchten wir durch Untersuchungen an Pyridoxin-Mangeltieren mit herabgesetzter Transaminaseaktivität zu stützen. Nach Kühnau[24] ist Vitamin B_6 für echte Transaminierungen unentbehrlich; da ein avitaminotischer Organismus aber bestrebt ist, alle Vitaminreserven für lebensnotwendige Prozesse, auch für Transaminierungen in der Leber frei zu machen, läßt sich der Vitaminmangel eines Tieres nur in besonderen Versuchsanord-

[23] R. Schober, Arzneimittel-Forsch. 4, 26 [1954].
[24] J. Kühnau, in Stepp-Kühnau-Schroeder, Die Vitamine, F. Enke-Verlag, Stuttgart 1953.

nungen als eine Beeinflussung enzymatischer Reaktionen fassen, wie besonders die Arbeiten von Awapara, Elvehjem, Meister, McHenry, Snell und ihren Mitarbeitern u. a. gezeigt haben[25]. Deswegen erhielten die Ratten für diese Versuche zusätzlich zur B_6-freien Diät Desoxypyridoxin als Antivitamin. Zwar gelang es uns trotz kohlenhydratreicher Zwangsfütterung nicht, das Leberglykogen der Tiere auf eine Durchschnittskonzentration von 2 g% zu steigern, um eine für unsere Versuche günstige Ausgangslage zu schaffen, doch zeigen unsere Ergebnisse, daß zwischen behandelten und kontrollierten kein Unterschied im Glykogengehalt der Leber besteht.

Berechnet man die signifikante Differenz zwischen den mit Glutaminat behandelten Pyridoxin-Mangeltieren und der ersten von uns angestellten Versuchsserie, so ergibt sich eine Urteilszahl $Q = 6.0$ bei einer Zufallsgrenze von 3.66. Hiermit ist also der statistische Beweis erbracht, daß ein intaktes Transaminierungssystem für die Mobilisierung von Leberglykogen durch Glutaminsäure notwendig ist.

Um einen quantitativen Anhaltspunkt dafür zu bekommen, welcher Prozentsatz der zugeführten Glutaminsäure wieder vom Organismus eliminiert wird, untersuchten wir an einer 10 Stdn. nüchternen 80 kg schweren Versuchsperson nach oraler Aufnahme von 25 g Glutaminsäure als Suspension deren Ausscheidung im Harn. Der Harn wurde in Halbstundenportionen papierchromatographisch untersucht und der Aminostickstoff nach Aufspaltung des Harnstoffs mit Urease und Adsorption des entstandenen Ammoniaks an Permutit nach van Slyke bestimmt. Die Ausscheidung der Glutaminsäure setzte 1 Std. nach der Aufnahme ein und betrug innerhalb von 3 Stdn. 0.65 g, das sind 2.6 % der aufgenommenen Menge.

Unsere Befunde stehen in einem gewissen Gegensatz zu den bekannten älteren Untersuchungen von Wilson und Lewis[26] sowie von Butts, Blunden und Dunn[27], die an 24—48 Stdn. hungernden Ratten Glutaminat durch die Schlundsonde verfütterten und signifikante Zunahmen des Leberglykogens im Vergleich zu den hungernden Kontrolltieren fanden. Wir haben deren Versuche nachgearbeitet und sie im wesentlichen bestätigen können. 10 Ratten, die 48 Stdn. nüchtern waren, erhielten 10 Stdn. lang alle 2 Stdn. 2 ml 10-proz. Natriumglutaminatlösung. Anschließend wurde das Leberglykogen im Vergleich

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[25] S. R. Ames, P. Sarma u. C. A. Elvehjem, J. biol. Chemistry **167**, 155 [1947]; J. Awapara, ebenda **200**, 537 [1953]; J. R. Beaton, R. M. Ballantyne, R. E. Lan, A. Stockley u. E. W. McHenry, ebenda **186**, 93 [1950]; J. R. Beaton, J. L. Beare, G. Beaton u. E. W. McHenry, ebenda **204**, 715 [1953]; E. F. Caldwell u. E. W. McHenry, Arch. Biochem. Biophysics **45**, 97 [1953]; A. Meister, H. A. Sober u. E. A. Peterson, J. biol. Chemistry **206**, 89 [1954]; F. Schlenk u. E. E. Snell, ebenda **157**, 425 [1949]; R. C. Wright u. E. M. Scott, ebenda **206**, 725 [1954].

[26] R. H. Wilson u. H. B. Lewis, J. biol. Chemistry **85**, 559 [1929/30].

[27] J. S. Butts, H. Blunden u. M. S. Dunn, J. biol. Chemistry **119**, 247 [1937].

zu Kontrolltieren bestimmt. Es betrug im Mittel 0,45 g% bei einer Variationsbreite von 0,15 bis 1,12 g%, bei den Kontrolltieren 0,02 bis 0,03 g%.

Wir glauben nun, daß diese Befunde nur in scheinbarem Gegensatz zu der von uns beobachteten glykogenmobilisierenden Wirkung der Glutaminsäure stehen, da die Stoffwechselausgangslage des Organismus sicher für die Richtung der ablaufenden Reaktion von Bedeutung ist, so daß es bei gefütterten Tieren mit reichlichem Glykogenangebot zu einem Abbau von Glucose kommt, während ein stark hungernder Organismus über den Citronensäurecyclus seinen Glykogenbedarf aufbaut.

Zusammenfassung

Es wurde der Einfluß der Verabfolgung von L-Glutaminsäure auf den experimentell erhöhten und erniedrigten Blutzucker bei Kaninchen und das Verhalten des Leberglykogens nach intraperitonealer Verabreichung von L(+)-Glutaminsäure als Natriumsalz bei ad libitum gefütterten Ratten untersucht.

Bei Traubenzuckerbelastungen mit 4 bzw. 8 mMol/kg Glucose führt die gleichzeitige orale Gabe von L-Glutaminsäure zu einer deutlichen Depression der Blutzuckerbelastungskurve.

Der hypoglykämische Schock nach 3 I. E. Insulin kann durch orale Glutaminsäuregaben bis zu einer Zeit von mindestens 30 Min. vor der Insulininjektion verhindert werden.

Bei alloxandiabetischen Tieren führen intravenöse und orale Glutaminsäuregaben zu Blutzuckersteigerungen zwischen 70 und 200 mg%, um so größer sind, je höher die Blutzuckerausgangswerte waren.

Der Blutzuckeranstieg bei alloxandiabetischen Kaninchen läßt sich durch vorherige subcutane Injektion von 0,5 mg/kg Hydergin unterdrücken.

Normale Ratten zeigen nach Injektion von 2- und 3mal 10 mMol/kg Natriumglutaminat signifikante Abnahmen des Leberglykogens. Die einmalige Injektion von 10 mMol/kg Glutaminat führt zu keiner signifikanten Abnahme.

Die Glykogenmobilisierung durch Glutaminsäure läßt sich durch vorherige subcutane Injektion von 0,5 mg/kg Hydergin nicht unterdrücken. Es kommt dabei zum Absinken des Blutzuckers auf hypoglykämische Werte.

Bei adrenalektomierten Ratten führen intraperitoneale Injektionen von 2mal 10 mMol/kg Glutaminat zu Glykogenmobilisierung und hypoglykämischen Krämpfen.

Pyridoxin-Mangeltiere zeigen nach Glutaminatinjektionen keinen Abfall ihres Leberglykogens, es bleibt im Vergleich mit den anderen Versuchsserien signifikant erhöht.

Die Mobilisierung von Leberglykogen durch L(+)-Glutaminsäure ist also unabhängig von einer Adrenalinwirkung, jedoch ist ein intaktes Transaminasesystem für das Ablaufen der Reaktion notwendig.

Summary

The influence of the administration of L-glutamic acid on the experimentally increased and depressed blood sugar level in rabbits was investigated. Further, the behaviour of glycogen in the liver of rats fed with an ad libitum diet was examined after intraperitoneal administration of L(+)-glutamic acid in the form of its sodium salt. In the case of loading with 4 or 8 millimoles of glucose per kgm simultaneous oral administration of L-glutamic acid produces a marked depression of the blood sugar loading curve.

The hypoglycaemic shock produced by 3 I. U. of insulin can be prevented by oral administration of glutamic acid up to a time of not less than 30 minutes before the insulin injection.

In the case of alloxan diabetic animals intravenous and oral administration of glutamic acid causes increases in the blood sugar level of between 70 and 200 mgm%. The higher the initial blood sugar values, the greater is the increase.

The increase in blood sugar in alloxan diabetic rabbits can be suppressed by previous subcutaneous injection of 0.5 mgm of „Hydergin" per kgm. Normal rats show a significant reduction of the liver glycogen after 2 or 3 injections of 10 millimoles of sodium glutamate per kgm. A single injection of 10 millimoles of glutamate per kgm causes no significant reduction.

The mobilization of glycogen by glutamic acid cannot be suppressed by previous subcutaneous injection of 0.5 mgm of „Hydergin" per kgm, this causing reduction of the blood sugar to hypoglycaemic values.

Intraperitoneal injection of 2 × 10 millimoles of glutamate per kgm in adrenalectomized rats produces mobilization of glycogen and hypoglycaemic convulsions.

After injection of glutamate in animals deficient in pyridoxin there is no reduction in their liver glycogen which remains markedly higher than in the other series of experiments.

The mobilization of liver glycogen by L(+)-glutamic acid is thus independent of any adrenaline effect although an intact transaminase system is necessary for the reaction to proceed.

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BIOCHEMICAL MEDICINE 5, 447–456 (1971)

Studies on Monosodium Glutamate Ingestion

I. Biochemical Explanation of Chinese Restaurant Syndrome[1]

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Ingestion of large amounts of monosodium glutamate (MSG) is followed by the appearance of such symptoms as headache, sweating, nausea, weakness, thirst, flushing of the face, a sensation of burning or tightness, abdominal pain, and lacrimation. Since Chinese food contains abundant amounts of MSG, these symptoms are most frequently seen in patrons of Chinese restaurants, and therefore, the condition has been termed "Chinese restaurant syndrome."

The study by Schaumburg *et al.* (1) as well as our own observations suggest that the appearance and severity of symptoms are dose related. Potential harmful effects of MSG ingestion were studied in animals by Olney (2), Olney and Sharpe (3), and Adamo and Ratner (4). The results of these studies conflict and have questionable relevance to human subjects (5, 6). In any case, no logical explanation for the clinical manifestations of MSG administration has been offered so far.

In this paper, we present evidence indicating that the ingestion of large doses of MSG causes transient increases in an acetylcholine-like substance, which is responsible for the symptoms of Chinese restaurant syndrome. This conclusion has been reached by (a) comparison of the classical signs and symptoms induced by intravenous injection of ACh with those following administration of MSG and (b) by studies of the biochemical aspects of MSG ingestion.

MATERIAL AND METHODS

Sources of Materials. The MSG used in this study is commercially available as Accent. It is claimed to be "99 + per cent" pure by its manufacturers, Accent International Division of International Minerals and Chemical Corporation, Skokie, Ill. L-Histidine (free base) was pur-

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chased from Nutritional Biochemical Corporation, Cleveland, Ohio. Sulfosalicylic acid was obtained from Fisher Scientific Corporation, New York. Cholinesterase, acetylcholine, and *m*-nitrophenol were purchased from Sigma Chemical Company, St. Louis, Mo.

Analysis of Blood Amino Acids. All blood samples taken at various intervals following administration of the loading dose were analyzed for amino acids (7). Since glutamine overlaps asparagine and occasionally threonine, separate determination for glutamine was also made (8–10). The detailed results are presented elsewhere.

Determination of Cholinesterase (ChE). ChE in the plasma from various blood samples was determined according to the procedure of Rappaport *et al.* (11). A unit of enzyme activity is defined as the amount which will hydrolyze 1 μmole of ACh in 30 minutes at 25° under the conditions of this assay.

Tests. Fourteen healthy adult members of the medical staff (10 male, 4 female) volunteered to serve in the study. Although the volunteers were informed that the study concerned Chinese restaurant syndrome, they were not aware of the identity or sequence of the test substances used. Figure 1 is a schematic representation of initial test in relation to repeat tests with different doses of MSG or histidine, with and without prostigmine or atropine. In all cases, the initial test consisted of administration of 150 mg MSG/kg body wt. The dose was dissolved in 150 ml water and administered orally after an overnight fast. All the repeat tests were performed at least 3 days after the first test.

For control of the initial test, three subjects were given histidine loading tests (tests 23, 24, and 25; 150 mg/kg body wt).

Repression of the clinical reaction was studied through reduction of the administered dose (tests 15, 16, and 17) or through the administration of the anticholinergic drug atropine (tests 21 and 22). The two subjects primed with parenteral atropine (subjects 13 and 14) had developed moderate response to the initial MSG administration and received 250 and 200 mg of MSG/kg body wt, respectively, on subsequent tests.

Three volunteers (subjects 8, 9, and 11) who showed moderate clinical reactions to the loading test were subjected to a second test with 25 mg of MSG/kg body wt. In three subjects with mild (No. 10) or moderate response (Nos. 11 and 12), the test was repeated with 250 mg/kg body wt.

Subject 13 had 0.4 mg atropine injected subcutaneously simultaneously with the ingestion of 250 mg MSG/kg body wt. The same amount of atropine was injected intramuscularly after 23 minutes, when minimal signs appeared. Since these signs persisted, another injection of the same

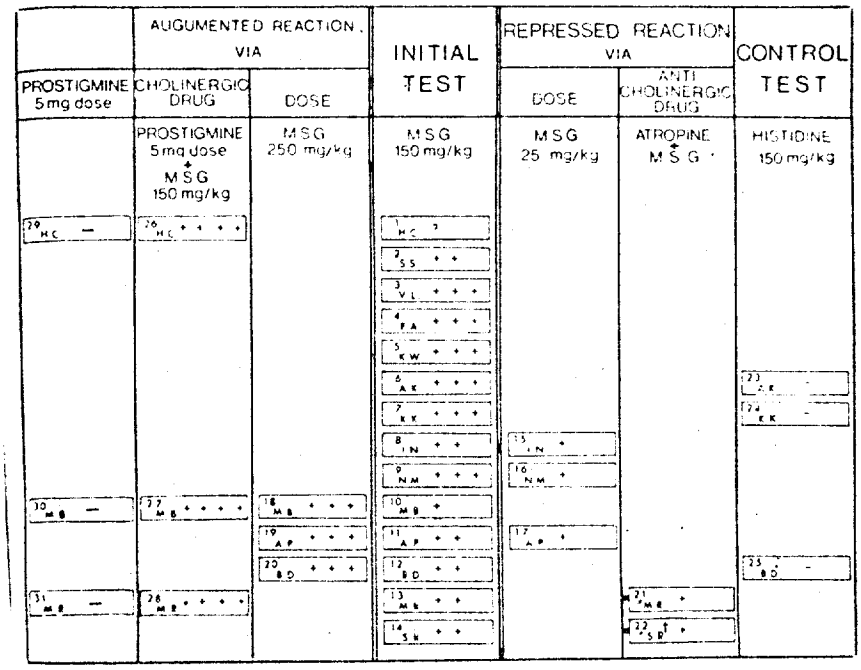

FIG. 1. MSG loading, augmentation, and repression. All volunteers initially received 150 mg of MSG/kg body wt. Repeat tests with 25 and 250 mg of MSG/kg body wt are indicated in adjacent columns. Degree of response, based on severity, number, and duration of symptoms: + minimal but definite response; ++ moderate response; +++ marked response; ++++ severe response, — no response. * Atropine was used during the test period. † Repeat test on S.R. was done with 200 mg of MSG/kg body wt.

dose was given subcutaneously 18 minutes later, at 41 minutes. Subject 14 received a subcutaneous injection of 0.6 mg of atropine 13 minutes following the ingestion of 200 mg MSG/kg body wt.

Augmentation of the clinical reaction was studied through the administration of larger doses of MSG (250 mg/kg body wt, tests 26, 27, and 28). In the three volunteers who received MSG in conjunction with prostigmine, the effect of prostigmine alone was also determined (tests 29, 30, and 31).

During all the tests, the subjects were observed closely and their complaints recorded. Clinical evaluation included observation of pulse rate, pupil size, facial color, perspiration and blood pressure; in some cases, electrocardiograms were also taken. In all loading tests, blood samples were collected in heparinized tubes at 0, 20, 40, 60, 90, and 180

minutes for various estimations. All blood samples were analyzed for glutamine and glutamic acid, but the results are not presented in this paper. Except for the samples obtained from loading tests 1, 3, 4, and 12, all the blood samples were analyzed for ChE. In addition, complete amino acid analysis was performed on all samples taken at 0 and 40 minutes. Determinations of CO_2, pH, and bicarbonate were made on all samples obtained in loading tests 19 and 20. Since the values were essentially normal, these measurements were not repeated on other samples.

RESULTS

The scores in Fig. 1 (+ to + + + +) represent an arbitrary estimate of the severity and duration of clinical response. The duration of complaints in the + category was 3–30 minutes. The complaints were generally limited to one or two of the following: numbness of the neck, "heaviness" of the eyelids, drowsiness, headache, sensation of pressure in the chest, and numbness of the legs. The complaints were definite but mild.

The reactions scored + + lasted 20–45 minutes. The symptoms were more pronounced than was the case in the first group. Moreover, there were more complaints of substernal pressure and abdominal discomfort and all the subjects manifested more than one complaint. For example, subject 11 developed nausea, abdominal discomfort, and drowsiness; this triad was also shown by subject 14, who, in addition, complained of urgency of urination.

The reactions marked + + + persisted from 1 to 12 hours. Headache and thirst were among the symptoms that continued for hours, whereas such manifestations as sense of burning or pressure, flushing, palpitation or abdominal discomfort tended to be limited to a few minutes. Subject 11, after ingesting 250 mg/kg (loading test 19) provided a characteristic example of the clinical picture seen in the + + + group. He developed colicky abdominal pain, nausea, urgency of bowel evacuation, drowsiness, retro-orbital pain and headache. These symptoms lasted 80 minutes.

The reactions scored + + + + were of a strikingly greater severity, with vomiting and relatively prolonged headache among the prominent symptoms.

All volunteers subjected to the MSG loading tests developed definite signs and symptoms. The only possible exception was subject 1, who complained of only a mild headache, the onset of which may have preceded the ingestion of MSG.

Subject 10 developed thirst 1 hour after receiving the dose in the initial test. The thirst lasted 2 hours but was considered insignificant and

unrelated to MSG. However, following a repeat test with a larger dose, he became markedly thirsty. The feeling persisted 12 hours and woke him during the night. This subject also developed "abdominal discomfort" lasting 30 minutes and flushing of the face lasting 3 minutes. In view of these findings, the reaction to the initial test was changed from "questionable" to +.

Certain complaints were not mentioned by the volunteers but were obvious to observers. For example, subject 14 was lively and talkative before taking MSG; 20 minutes after ingestion of the dose, he became markedly quiet and subdued, but never complained of his changed mood. In five subjects, intermittent EKG was obtained during the first hour of the initial test. All tracings were normal; the procedure was therefore discontinued. Similarly, no abnormal changes in blood pressure or pulse rate were noted during the 3-hour observation period following administration of the loading dose. (Subject 4 complained of a sense of palpitation but her EKG was completely normal.)

The results thus demonstrated a range of reactions to the initial test dose. Repeat tests of three subjects with 25 mg of MSG/kg body wt caused only a mild reaction. Similarly, two subjects primed with atropine (tests 21 and 22) exhibited only mild reactions, even though a larger dose of MSG was administered. On the other hand, all who received 250 mg/kg body wt of MSG (tests 18, 19, and 20) and those who received prostigmine in addition (tests 26, 27, and 28) manifested marked to severe reactions. The control loading test, carried out with histidine (150 mg/kg body wt) did not result in any observable clinical response.

Histidine values for the histidine loading test (Nos. 23, 24, and 25) are not recorded, since these did not differ appreciably from the pattern previously described (12).

Figure 2 presents the cholinesterase activity of the blood samples taken during the loading tests with 150 mg MSG/kg body wt and with 150 mg histidine/kg body wt. While no changes in the plasma levels of ChE activity were observed on histidine loading, a decrease of about 30% occurred at 60 minutes after MSG ingestion. The activity levels in the plasma, however, returned to normal at the end of 3 hours after ingestion of MSG.

Dog Experiment. To study the effect of ACh on ChE, 7.5 mg of ACh diluted in 10 ml of isotonic saline was injected intravenously over a period of 4 minutes into a healthy 44 lb dog. No medication was used to calm the animal. Blood samples were taken at 5-minute intervals before the administration of ACh. Additional samples were obtained at 1, 3, 5, 7, 13, 20, 40, 60, and 90 minutes (Fig. 3). Within 30 seconds

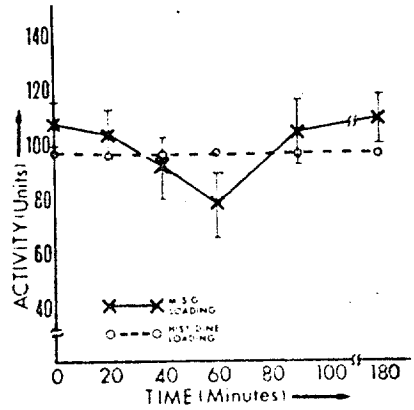

FIG. 2. Cholinesterase activity in loading tests with monosodium glutamate and histidine. The values for 10 MSG loading tests are expressed as mean ± S.D. In three histidine loading tests, ChE activity varied by less than 5%, therefore only the mean value is presented.

of the start of ACh injection (approximate rate 2 mg/min), the animal's heart rate was reduced from 104 to 60. The rhythm became irregular and respiration deepened. The animal, which had voided just prior to the experiment, urinated again on the operating table. Profuse salivation (approximately 10 ml), as well as lacrimation, were also observed. Heart and respiratory rate became normal 40 minutes after cessation of the injection. At the end of 90 minutes, the dog looked normal and eagerly consumed its food.

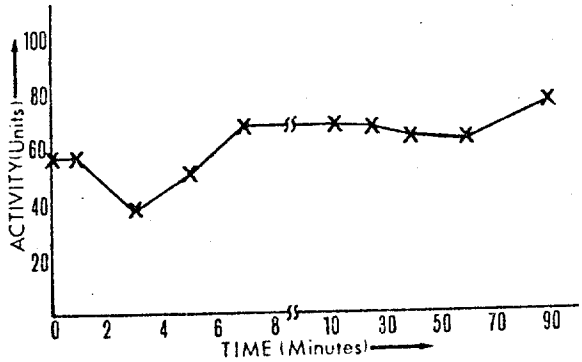

FIG. 3. Cholinesterase activity in dog's plasma. Two base line values, 5 minutes apart, were obtained before intravenous injection of ACh. 7.5 mg of ACh in normal-saline was injected over a period of 4 minutes. Other details are as given in the text.

The results of ChE determination on blood samples taken from the animal are presented in Fig. 3. The first three samples, including two taken prior to administration of ACh, show no fluctuation. A sharp dip to approximately 70% of the base line value is observed at 3 minutes, followed by an equally rapid rise. At the 7-minute mark, the base line value is significantly exceeded, and subsequent readings show a stable plateau at this level.

DISCUSSION

Ingestion of MSG on an empty stomach was followed by the appearance of signs and symptoms similar to those described as Chinese restaurant syndrome. Even in four instances where only 25 mg MSG/kg body wt was given, mild but definite symptoms appeared shortly after administration of the dose. On the other hand, loading tests with 150 mg histidine/kg body wt (Nos. 23, 24, and 25) did not cause any clinical disturbance.

The severity of the symptoms appears to be dose related. Subjects who had minimal discomfort following ingestion of 150 mg/kg body wt developed pronounced symptoms after administration of 250 mg/kg body wt. Our experience in this respect is in agreement with the observations by Schaumburg *et al.* (1). Moreover, as early as 1949, Levy *et al.* (13) suggested a correlation between the amount and rate of intravenous administration of glutamic acid and incidence of nausea and vomiting. They observed that, among several parenterally administered amino acids, only dicarboxylic acids (glutamic and aspartic acids) are responsible for undesirable side effects.

The time of appearance of the first manifestation ranged from 5 to 35 minutes after ingestion of the dose. The symptoms were all transient in nature and usually lasted less than 1 hour (in one instance, thirst lasted 12 hours; three subjects had headaches lasting up to 5 hours). The symptoms mostly consisted of an uncomfortable feeling of pressure, burning, numbness, or mild pain. In order of frequency of appearance, the signs and symptoms could be listed as follows: a sensation of tightness in the back of the neck, a feeling of pressure behind the eyes, frontal or temporal headache, sleepiness, facial flushing, sweating, nausea; a feeling of pressure on the side of the face, thirst, pressure and a burning sensation in the chest, and abdominal pain.

The mode of appearance and recovery, the shortness of duration, the diverse nature of the symptoms affecting different parts of the body are all remarkably similar to the diffuse and evanescent action of acetylcholine in humans. A classical description (Koelle, Ref. 14) of transient acetylcholinemia follows:

"Symptoms and signs observed during intranveous ACh administration are directly proportional in intensity to the size of the dose and the rapidity of injection; they include flushing of the skin, a feeling of warmth, increase in skin temperature, throbbing in the head, palpitation, bounding pulse, profuse sweating, salivation and lacrimation, a sensation of substernal constriction accompanied by inspiratory difficulty and slight cough, and nausea and vomiting when the dose is pushed to the limit of tolerance. Symptoms remain at a given intensity as long as the rate of administration is unchanged, and subside without any residual effect within a few minutes after the injection is stopped. Bradycardia does not occur. Indeed, cardiac rate is moderately accelerated as a result of sympathoadrenal discharge. Systolic and diastolic blood pressures are not lowered significantly by ACh infusion, no change occurs in cardiac output and circulatory velocity is not increased."

From a biochemical point of view, it is known that glutamate can be converted to ACh. Studies on isolated nerves of lobster (15, 16) and rabbit (17) *in vitro* and in rat brain (18) *in vivo* have suggested that glutamate provides a specially suitable substrate for ACh synthesis via the tricarboxylic acid cycle. Extracellular Na^+ has also been shown to be necessary for ACh synthesis and storage in sympathetic ganglia (19, 20) and in brain (21). It is not known whether Na^+ promotes the availability of the substrates for ACh synthesis or enhances the availability of ACh binding sites. In any case, ingestion of MSG provides both the substrate (glutamate) and the enhancing Na^+ for ACh synthesis.

Instantaneous appearance of ACh at neuromuscular junctions would permit excitatory transmission responsible for the symptoms. At this point, the degradation of ACh by the available ChE helps to terminate the clinical manifestations of transient increase in ACh. Depending on the extent of increased synthesis of ACh, a temporary depletion of ChE could be expected in the various compartments of the body, such as plasma, which do not contribute to the synthesis of the enzyme. On the other hand, tissues responsible for synthesis of ChE, such as liver, would respond to such a situation by increased formation of ChE. The temporarily decreased levels of plasma ChE would be compensated and the base line values reestablished. This expected sequence of events is confirmed by ChE determinations as presented in Fig. 2. The data show a marked decrease (28%) of ChE 60 minutes after ingestion of MSG. In this respect, it is also interesting to note that the loading tests with 250 mg MSG/kg body wt (Nos. 18, 19, and 20) showed the same pattern of ChE depletion. In contrast, loading with 25 mg MSG/kg

body wt (Fig. 2, Nos. 23, 24, and 25) failed to show any change in the base line values of ChE throughout the test period.

The conclusion that the depletion of ChE was due to increase in ACh was further confirmed by the dog experiment. In this instance, induced acetylcholinemia by direct intravenous injection of ACh (7.5 mg total) produced a pattern of ChE depletion similar to that observed after MSG ingestion in humans (see Fig. 3).

The possibility that in human loading tests decreased plasma ChE activity may be due to inhibition by MSG was examined by *in vitro* experiments with rat brains.

A 10% (w/v) homogenate of rat brain prepared in 250 m*M* sucrose was centrifuged at 15,000*g* for 20 minutes. The final concentration of the soluble supernatant was adjusted to 5% with 250 m*M* sucrose. This supernatant was used for enzyme assay. Addition of MSG in final concentrations of 0.25, 0.5, 1.0, 2.0, and 4.0 m*M* to the assay system resulted in no inhibition of ChE activity.

The hypothesis that symptoms which follow MSG ingestion are due to the parasympathominetic effects of ACh was tested by administration of anticholinergic and cholinergic drugs. Subjects 13 and 14 initially showed moderate response to 150 mg/kg MSG loading test. Much larger doses of MSG (200 and 250 mg/kg) produced only mild symptoms when the same subjects were primed with parenteral atropine.

On the other hand, subject 1, with questionable response to the initial MSG loading dose, developed severe symptoms when the same loading dose was accompanied by a subclinical amount of prostigmine. Similar results were observed with subjects 10 and 13. The repressions caused by an anticholinergic drug as well as augmentation brought about by a cholinergic drug both support the contention that a parasympathomimetic agent is responsible for the symptoms that follow MSG ingestion.

The possibility that gamma-aminobutyric acid (GABA), a decarboxylation product of glutamic acid, could cause the symptoms of MSG ingestion should be considered. However, this is unlikely since decarboxylation of glutamic acid is known to occur only in the central nervous system. Moreover, the action of GABA, unlike that of ACh, is inhibitory in nature in the central nervous system as well as in peripheral nerve.

Recent studies indicate that glutamic acid per se can act as a neuronal transmitter (22, 23). Whether or not glutamic acid in high concentration can act as parasympathominetic agent can only be conjectured at this stage.

Finally, we suggest "transient acetylcholinemia" as a name for the

reaction following MSG ingestion, in direct reference to the nature and etiology of the clinical manifestations.

SUMMARY

The signs and symptoms following the ingestion of monosodium glutamate (MSG) were found strikingly similar to those induced by acetylcholine (ACh). The effects of anticholinergic and cholinesterase (ChE) inhibitor support the hypothesis that Chinese restaurant syndrome is a "transient acetylcholinosis." The pattern of plasma ChE activity in induced acetylcholinemia was strikingly similar to that following MSG ingestion.

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THE GLUTAMATE MANUFACTURERS' TECHNICAL COMMITTEE*

THIS publication is one of a series sponsored by the Glutamate Manufacturers' Technical Committee, which is made up of research and technical service representatives appointed by the five American manufacturers of Monosodium Glutamate.

From time to time this Committee will publish other information about Glutamate* use accumulated by various researchers who have worked in the field.

Additional copies of this booklet are available from the Information Service, Glutamate Manufacturers' Technical Committee, 214 Front Street, San Francisco 11, California.

THE VALUE OF GLUTAMATE* IN PROCESSED MEAT AND POULTRY

MEAT

Introduction

It was formerly believed that Glutamate itself had a meat-like taste, but this was disproved by the work of E. C. Crocker of the Arthur D. Little, Inc., laboratories. Crocker found that purified Glutamate had a salty and sweet taste, but no odor. He also found that the characteristic flavor of cooked meat was attributable to an aroma resulting from the action of heat on meat fibre. The meaty aroma which was noticed in earlier lots of Glutamate was due to impurities resulting from protein hydrolysis and is not found in the product of leading manufacturers today.

Perhaps because of the illusion that Glutamate had a "meaty" taste, its early uses in this country were largely with meat dishes. One of its most valuable uses is still as a flavor intensifier for meats, both fresh and processed, but the information presented here will deal entirely with processed meats.

* Monosodium Glutamate

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Frozen Meats and Meat Specialties

The flavor-holding property of Glutamate during prolonged storage of certain frozen meats was established by Norton, Tressler and Farkas and described in Food Technology, 1952, Vol. VI, No. 11, Pages 405-411. Their report will be referred to as (N. T. & F.) in this publication.

All meats or specialties mentioned were treated with Monosodium Glutamate before freezing.

HAMBURGER

Beef was dusted before grinding with 0.15% of its weight in Glutamate. After freezing, Glutamate-treated samples and controls without Glutamate were stored at 0°F. By the end of one month the Glutamate-treated samples were superior in flavor and continued to be for 10 months. Results of the work on ground beef are reprinted from (N. T. & F.).

Comparison of Samples Held in Storage at 0°F.

Months Storage Time	Untreated	Glutamate Treated
1	Very Good	Very Good flavor preferred to control
6	Good	Very Good flavor preferred to control
8	Fair lacks freshness	Good much more flavorful than control
10	Poor	Fair

The level of Glutamate application for frozen ground beef is generally agreed upon as 0.15% by weight. It may be dusted on the meat before grinding or applied to the block of meat after the first rough grind, depending on the final grind for penetration to all parts of the meat.

CUBE STEAK

Results on cube steaks duplicated ground beef data in the (N. T. & F.) report. The researchers noted that again the Glutamate-treated samples held at 10°F. were juicier and more tender than those held at 0°F. In these tests the meat was dusted with Glutamate after the cubing process.

PORK SAUSAGE

There is ample information demonstrating the value of Glutamate in holding the flavor of pork sausage in frozen storage and also data indicating better color preservation in the Glutamate-treated product. Glutamate should be mixed with the other seasonings before being added to the ground pork, with a level of application of 0.15% of Glutamate to the weight of the pork.

Results of the frozen pork sausage research of (N. T. & F.) are tabulated.

Comparison of Samples Held in Storage at 0°F.

Months Storage Time	Untreated	Glutamate Treated
1	Very Good	Very Good more flavorful than control
3	Fair flat in flavor	Good more flavorful and tender
5	Poor slightly rancid	Good no rancidity
7	Very Poor rancid & color faded	Fair slightly old flavor
9	Very Poor very rancid	Fair poor flavor but not rancid

By the end of 7 months the untreated samples had lost all their fresh natural color. Fading was much less pronounced in the Glutamate-treated sausage. Samples were stored at both 10°F. and 0°F. with the latter preferred after 5 months storage.

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[illegible]

Some further research by James D. Kemp, University of Kentucky, **Food Technology**, 1955, Vol. IX, No. 5, Pages 340-1, uncovered some interesting facts on the value of Glutamate in the storage of seasoned sausage as compared with unseasoned sausage.

The researcher found that seasoned sausage did not stay as palatable as unseasoned sausage at 0°F. until Glutamate was added. The seasoned sample with Glutamate was more palatable than the unseasoned control after four months of storage and through the rest of the 12-month test.

Glutamate thus provides an answer to strong flavor changes specifically connected with storage of frozen seasoned sausage.

Further evidence of the flavor holding properties of Glutamate in frozen ground pork comes from a report by Jean Neill and Louise Page—"The Effect of Antioxidants on Frozen Ground Pork" (**Food Technology**, July 1956, Vol. 10, No. 7, pages 315-319).

The researchers, in palatability tests, found that frozen ground pork containing Glutamate was superior in flavor after storage up to 12 months to samples treated with full-fat soy bean flour, or butylated hydroxyanisole; and also superior to an untreated sample.

Some of the ground pork was cooked before freezing and some was frozen raw. In both instances the Glutamate samples were superior in the palatability tests. Application levels of Glutamate in the test were 0.10%, 0.15% and 0.30%.

The Glutamate samples scored above the others in flavor at all three levels. (NOTE: The Glutamate Manufacturers' Technical Committee recommends the 0.15% level.)

[illegible]

In 1955 researchers re[illegible] on the [illegible] Glutamate adds to frozen beef stew during low temperatur storage. This work at the University of Massachusetts b Kearns, Fagerson and Fellers showed that taste panels pr ferred beef stew with Glutamate added before freezing abo 2 to 1 over the stew without Glutamate (**Food Engineerin** April 1955, Pages 76, 77, 168). This ratio began after 4 mont of storage and continued.

The investigators tried three levels of Glutamate applicati at 0.125, 0.20, and 0.25% with the highest palatability sc at the 0.25% level. Glutamate manufacturers have reco mended an application level of 0.15% (2½ oz. per 100 lb for frozen meats, but the additional amount in this case c parently provided a flavor advantage after storage up to year.

Glutamate should be added to frozen beef stew at the ti it is cooked before freezing.

Glutamate delivers the same favorable results in fro meat pies as in beef stew.

HAM

Glutamate serves as an important flavor enhancer in h —frozen or otherwise—but it has unusual advantages in ho ing flavor in the frozen meat. Glutamate is applied in soluti in the pumping pickle so as to obtain a 0.15% level in co parison with the weight of the meat. The (N. T. & F.) resear paired hams from the same carcass so that a direct comparis could be made between an untreated ham and one from t same animal treated with Glutamate.

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Flavor results were tabulated as follows:

Comparison of Samples Held in Storage at 0°F.

Months Storage Time	Untreated	Glutamate Treated
1	Very Good	Very Good preferred to control
4	Good	Good preferred to control
9	Fair slightly stale flavor, color faded	Good still full flavored
14	Rancid	Fair still quite satisfactory

BACON

Glutamate can increase the shelf life of frozen bacon substantially, according to other results of the (N. T. & F.) frozen foods research. Glutamate was added to the salt mixture in the curing box in the ratio of 1.17 pounds of Glutamate to 24.41 pounds of salt cure mixture (this serving for 615 pounds of pork bellies). The pork bellies were paired thus giving a direct flavor comparison between Glutamate-treated and untreated parts of the same animal. The tabulated taste test results are as follows:

Comparison of Samples Held in Storage at 0°F.

Months Storage Time	Untreated	Glutamate Treated
1	Very Good	Very Good
4	Good very slightly rancid	Very Good
7	Fair slightly rancid	Good no rancidity

NOTE: Hams and bacon were stored at 0°, 10° and 34°F. The meat (both treated and untreated) stored at 34°F. was discarded as unsatisfactory at 5 months. At 7 months the treated 10° sample was slightly preferred over the treated 0° sample.

PRE-COOKED FROZEN MEALS

Studies at the Quartermaster Food and Container Institute for the Armed Forces have indicated that the addition of Glutamate to pre-cooked frozen meals prevents "warmed-over" off flavor after storage, retards development of other off flavors, and helps retain the color and freshness of food.

A full-scale research project was carried out on a frozen pre-cooked veal breakfast patty, with the Glutamate-treated sample preferred after all periods of storage from 1-13 months. The product was frozen and stored at 0°F. Testers found that the untreated samples were dry in appearance and had an "old" flavor compared with the Glutamate-treated samples. This patty was designed for reheating aloft in military aircraft.

CUTLETS and OTHER SPECIALTIES

Currently processors of frozen cutlets and other similar specialties find Glutamate necessary to intensify flavor in almost all their products. Shelf life is improved just as it is with other frozen meats which have been tested. Glutamate is applied to a breaded cutlet as part of the breading mix. One recommended level of application is 2½ ounces added to the breading mix for each 100 pounds of finished cutlets. Many processors, however, prefer to intensify the meat flavor even more by a higher Glutamate level.

FROZEN SOLID MEAT CUTS (Steaks, Chops, Etc.)

The growing market for frozen meat of this type poses a problem to processors. In general, the frozen meat sells at a higher price than fresh meat, thus making quality a "must" for the frozen meat marketer—but quality can disappear rapidly under prolonged low temperature storage.

In this situation the flavor-holding property of Glutamate can come to the aid of the processor and prolong the shelf life of solid cuts at a high quality level.

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[illegible] in fresh [illegible] every individual taste, and [illegible] the meat is pre-cooked for the consumer, as in canned meat products, Glutamate becomes even more valuable in intensifying meat flavor and preventing "warmed-over" off flavors. Its use in a number of canned meat products is described.

CORNED BEEF HASH

A variety of tests have shown conclusively that Glutamate enhances the flavor appeal of canned corned beef hash. In "flavor profile" studies of this product, made by Arthur D. Little, Inc., these comments were made by the tasters on Glutamate-treated hash: blended flavor effect; suppressed onion, salt, starch notes; suppressed starchy, fatty and "tin" notes; blends spice and pepper. Level of application in hash used by the Little researchers was 0.5%, 0.35% and 0.4%.

Michigan State College students in a taste test of canned corned beef hash, containing 0.1% Glutamate, preferred the Glutamate-treated sample 110 to 24 over the untreated sample.

A packing company test with a small "expert" panel of seven resulted in 100% preference for hash containing Glutamate. Still another using J. Walter Thompson Company Home Testers showed a ratio of 110 to 61 in favor of the Glutamate-treated hash.

The high application levels used by Arthur D. Little, Inc., are not necessary for favorable results. There are wide variations in individual tastes with corned beef hash as with all foods, however, so the Glutamate Manufacturers' Technical Committee still recommends the same level for corned beef hash as for most other meat products—0.15% or 2½ ounces per 100 pounds. Glutamate should be added at the time the hash is mixed.

[illegible] Food and Container Institute covered use of Glutamate in several canned meat products, among many other foods. The final goal of the 18 month long project was a determination of the value of Glutamate in recipes of the Army's master menu. The taste panel of 80, judging canned pork and gravy, showed a 2 to 1 preference for the sample containing Glutamate. The level of application was 0.25% or somewhat above the Glutamate Manufacturers' Technical Committee recommended level of 0.15% (2½ oz. per 100 lbs.). In canning meat and gravy, Glutamate should be added by the processor at the same time as salt and may be combined with salt in the proper proportion. Results of the Food and Container Institute tests were published in **Food Engineering**, December 1954, pages 71, 72, 182, 185.

BEEF and GRAVY

These same tests duplicated the canned pork and gravy results with canned beef and gravy. The level of application was the same—0.25%, and preference for the Glutamate-treated sample was about the same—just slightly less than 2 to 1.

BEEF STEW

Girardot and Peryam did not test canned beef stew but found a 2 to 1 preference for fresh beef stew containing Glutamate. In test results that are available on the canned product, preferences of 60% and 56% for the Glutamate-treated sample were shown. Additionally, the Arthur D. Little laboratory noted these comments: reduces "tin can" taste; increases sweet and meat notes. The recommended application level for canned beef stew is 0.15% (2½ oz. per 100 lbs.). Glutamate should be added with the salt before cooking.

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[illegible]

The Quartermaster Food and Container Institute tested Glutamate in both fresh and canned hamburger. The preference for the Glutamate-treated sample of the canned product as compared with the untreated sample was 67%. In fresh hamburger it was 65%. Since fresh hamburger has always been considered as one of the most valuable uses for Glutamate, it is apparent that the canned product can benefit even more. Application level in this test was high at 0.5%, but the Glutamate Manufacturers' Technical Committee recommends a level of 0.15% as sufficient to produce good results.

Glutamate should be added to the hamburger at the time of the grinding process as described above under frozen hamburger.

CHILI

Two tests with rather small panels (12 and 20) showed a preference for Glutamate in canned enchiladas. The preference was 60% for the Glutamate-treated enchiladas as compared with untreated. The level of application was 0.15% and 0.22%. Glutamate should be added to the sauce in enchiladas. A large consumer panel (470) showed 70% preference for Glutamate in canned tamales. The level of application was 0.15% in the gravy and 0.25% in the corn.

DEVILED HAM

A taste panel of 42 members at Michigan State College showed a 69% preference for deviled canned ham containing Glutamate compared with the untreated product. A consumer panel of 182 at Lansing, Michigan, showed a 3 to 1 preference for the Glutamate-treated product. In both tests the level of application was 0.2%.

DEVILED TONGUE

The Arthur D. Little researchers found that a 0.5% application of Glutamate to canned deviled tongue brought the following "flavor profile" comments: **intensifies salt; reduces fattiness note.**

LIVER SPREAD

The "flavor profile" comments on the use of Glutamate in this product in the Arthur D. Little tests were: **reduces liver note; less crude.**

A simplified explanation of this frequently-mentioned "flavor profile" is that the flavor of a food is composed of many notes, which together make up a "flavor profile." This profile is determined by combining the impressions of the members of a taste panel and allows a study of the effect of added materials without regard to individual preferences.

In liver spread, the Glutamate Manufacturers' Technical Committee recommends 3 oz. of Glutamate per 100 pounds of spread.

MEAT BALLS

Canned meat balls were tested with a consumer panel of 229 women. The preference was more than 2 to 1 in favor of the Glutamate-treated samples. Glutamate should be added to the meat ball mix by the processor before cooking at the rate of 3 ounces to 100 pounds of meat. Note that this level is a little higher than recommended for most meat products.

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SAUSAGE

Fresh ground pork sausage shows flavor improvement with the addition of Glutamate in the same way that frozen sausage does. One test using a panel of 39 developed an 87% preference for the Glutamate-treated sample. The Arthur D. Little laboratory found an 83% preference and noted these comments in the "flavor profile": **blends; delays saltiness; augments sweetness.**

If a variety of spices are used in the sausage, Glutamate serves a further function in blending them into a harmonious whole.

The recommended level of Glutamate application is 0.15% or 2½ oz. per 100 lbs. of meat.

Two tests have been made with other varieties of sausage. A packing company tried Glutamate in liver sausage at a level of 0.13%, and with a taste panel of 37 people found a 76% preference as compared with an untreated liver sausage sample.

The same packer tried the same level (0.13%) in bologna and found a preference of 67% as compared with an untreated sample.

Cured Meats and Sausage

HAM

Tests of Glutamate use with ham have shown positive results ranging up to 70% preference compared with untreated samples, and the flavor appeal gained by use of Glutamate warrants consideration by every packer.

A small technical panel chosen by a packing company gave positive preference for ham treated with Glutamate in the pickle solution as compared with untreated ham, and made these comments: **improved flavor; lowered saltiness; augmented sweetness and smoke taste.** The level of application was 0.15% of the ham by weight.

The table below gives the amount of Glutamate per gallon of pickle to secure three different levels of Glutamate under various pumping conditions.

	Level of Glutamate in Ham		
Pumping Schedule	**0.1%**	**0.15%**	**0.2%**
5%	2.8 oz.	4.3 oz.	5.8 oz.
10%	1.4 oz.	2.2 oz.	2.8 oz.
20%	0.7 oz.	1.0 oz.	1.4 oz.

The Glutamate Manufacturers' Technical Committee recommends a level of 0.15% for general use in ham.

BACON

The table given above for ham will also supply correct quantities for bacon. In dry cure of bacon, the measure should be one pound of Glutamate to 35-40 pounds of salt.

One taste test of 455 women consumers showed a 70% preference for bacon which had been stitched with Glutamate in the pickle when the level of application was only 0.07%.

Taste tests by "expert" panels developed these comments: **smoother better flavor; augmented sweetness and smoke; improved mildness of flavor.**

POULTRY

Introduction

Acceptance of Glutamate as a chicken flavor intensifier is of long standing. One of the early uses in the United States by processors was in canned chicken soup, followed soon by use in canned chicken specialties. Glutamate also is an extremely valuable flavor factor in fresh poultry, but information on effective methods of application has not been available to many poultry packers.

While this publication primarily is concerned with processed poultry, a section is included on use of Glutamate by the packer in fresh poultry.

Frozen Poultry and Specialties

WHOLE FROZEN CHICKEN

Norton, Tressler and Farkas in their work on frozen foods, previously referred to in connection with frozen meats, also found a decided preference for frozen chicken which had been treated with Glutamate. The Glutamate-treated samples were rated best in flavor and texture after storage at 0°F. for various periods up to 11 months. The researchers found the same preference for broilers and fowl treated with Glutamate.

In this project Glutamate was applied in three separate ways: stitched with a 5% solution of Glutamate in water; stitched with a 5% solution of Glutamate in milk; dusted in the cavity with Glutamate up to 0.25% of the weight of the bird. Samples treated in these three ways were all preferred to the control sample without Glutamate.

Stitching of fowl is not now sanctioned by the Food and Drug Administration because the addition of liquid increases weight.

Application under commercial processing conditions can be done by dusting Glutamate into the cavity at either of two times.

If birds are cooled in a cold room before freezing, Glutamate should be applied to the cavity before cooling.

If birds are cooled in an ice slush bath, Glutamate should be applied to the cavity after the cold bath.

The key to effective Glutamate application on poultry is to be certain that the Glutamate reaches an area where it can be absorbed. Glutamate is not absorbed through the skin and this is the reason for applying it in the cavity.

It may be applied by hand dusting or through a dusting apparatus under pressure from an air line.

The recommended level of application for frozen chicken is 2½ oz. of Glutamate to 100 pounds of chicken.

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FROZEN CHICKEN PARTS

Frozen chicken parts show the same flavor advantage whole fowl or broilers through use of Glutamate. Applicati may be accomplished in two ways—through cavity dusti before the bird is cut up or by dusting the parts after t bird is cut up.

In the first method, absorption of Glutamate is fast enou so that it will penetrate through the bird in a comparativ brief time and reach all the parts before cutting.

In the second method, Glutamate penetrates the individu parts through areas not covered by skin.

The application level is 2½ oz. per 100 pounds of chick

FROZEN TURKEY

When Norton, Tressler and Farkas conducted their resear on frozen turkey they cut the birds in half by sawing dov the backbone. One-half became the control without Glut mate and the other half was treated with Glutamate. Ev after five months of storage at 0°F. the Glutamate-treat sample was superior in flavor, juiciness and tenderness.

Methods of application and the level (2½ oz. per 1 pounds) are the same as for chicken.

FROZEN BONED TURKEY ("Logs")

The recently produced frozen boned turkeys, sometime called "logs," seem to require addition of Glutamate for sati factory results. This product is the meat, both white and dar of a whole turkey, pressed together and frozen. Glutamat should be dry-sprayed or dusted on the meat at a level c 2½ oz. per 100 pounds.

[illegible]

Glutamate should be added to the pie "mix" at the same time as salt and in the same way. The level is 2½ oz. of Glutamate per 100 pounds of "mix." Glutamate appears to be a "must" to hold flavor of chicken and turkey pies for any length of time under low temperature.

FROZEN CHICKEN A LA KING

Two test panels have shown a preference for frozen chicken a la king containing Glutamate over the same product without Glutamate.

At the Arthur D. Little laboratories, "flavor profile" comments were that Glutamate reduced starch and pepper notes.

In a university laboratory test, the panel preferred the Glutamate-treated chicken a la king in varying degree over the non-treated control. These were the preferences after different periods of low temperature storage:

4	months	60%
6½	months	62%
10	months	78%
12	months	60%

Glutamate should be added to the mix in the same way as salt and at a level of 2½ oz. per 100 pounds of mix.

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CANNED WHOLE CHICKEN

Glutamate is added to canned whole chicken in the same way as salt. Not quite as much is used as in frozen chicken, with the recommended level 2 oz. per 100 pounds of chicken. This is a level of 0.12%.

In a university laboratory taste panel test, various levels of Glutamate were tried in canned chicken. All were preferred by 100% of the panel over the untreated sample, but this was the order of preference for the different levels of Glutamate: 0.09, 0.18, 0.27, 0.36 and 0.45%.

CANNED BONED CHICKEN

The Quartermaster Food and Container Institute tested preference for canned boned chicken containing Glutamate and found a 75% preference over the same product without Glutamate. The results of this and other Quartermaster tests were reported by Girardot and Peryam in Food Engineering, December 1954.

The level of application was very high in these tests—0.75% of Glutamate to the weight of the boned chicken. The Glutamate Manufacturers' Technical Committee recommends a level of only 2 oz. per 100 pounds of boned chicken or 0.12%.

CANNED BONED TURKEY

The level of application is the same as for canned boned chicken—2 oz. per 100 pounds of boned turkey.

CANNED CHICKEN FRICASSEE

Glutamate should be added to the mix with the salt and the level of application is 2½ oz. per 100 pounds of mix.

CANNED CHICKEN OR TURKEY A LA KING

The level of application is 2½ oz. per 100 pounds of mix, and Glutamate should be added at the same time and in the same way as salt.

[illegible] Poultry

Adding [illegible] Glutamate to fresh poultry by the packer before the product reaches the consumer provides a flavor "plus" available in no other way. Glutamate also acts as a guard against loss of flavor during transportation and storage before the poultry reaches the consumer.

The methods of application are the same as those used for frozen poultry, varying somewhat with the set-up of the individual packer.

All tests with Glutamate-treated chicken show a preference over the untreated sample ranging near or above 70%. The packer can secure this flavor advantage only by getting Glutamate into the meat of the chicken through a surface not covered by skin. So preferred methods require application of Glutamate to the cavity.

Obviously, with this limitation, Glutamate cannot be added to New York dressed poultry by the packer. But the packer of eviscerated poultry can gain this flavor advantage by applying Glutamate in the cavity.

The cavity can be dusted by hand with Glutamate from a shaker or dusted with Glutamate under pressure from an air line.

If poultry is going to be held in a cold room, the Glutamate should be applied before cooling, while the bird is warm.

If cooling is by ice slush bath, the Glutamate application should take place after cooling. The level of Glutamate application should be 2½ oz. per 100 pounds, or 0.15% by weight.

An alternative method is to introduce the Glutamate into the cold bath. If this method of application best fits an individual processor's procedure, he can be assured that poultry will absorb Glutamate rapidly through the cavity from a water solution.

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Conclusion

The use of Glutamate as a flavor improver for meats and poultry has been well established, and much has been learned about its ability to protect the flavor of processed meats and poultry during their normal storage life.

This important property is confirmed by packers and processors who are using Glutamate in their products, and research is being continued in these directions in order to expand knowledge of the subject. As new information is accumulated, it will be disseminated to the industry as rapidly as possible.

Any questions addressed to Information Service, Glutamate Manufacturers' Technical Committee, 214 Front Street, San Francisco, California, will be answered promptly. However, if your request involves technical service help in your plant, in order to save time and secure the fastest possible service, please write directly to any of the five American manufacturers listed on Page 2 of this publication.

To the Editor: Dr. Kwok's letter of April 4 regarding the Chinese-restaurant syndrome has been read with interest. During the past year we have studied similar reactions after the ingestion of all types of Chinese food in members of the Yale Community.

In the course of investigating this strange malady, we sponsored a Chinese meal for seven people who were known to have reactions. Clinical observations were made, and a tape recorder documented their personal sequential impressions. Although the study hardly fulfils the usual "double-blind" criteria, readers of the *Journal* may be interested in these observations:

Within 17 minutes after the beginning of the ingestion of the Chinese dishes, sudden tightening of the face developed, associated with unilateral numbness, usually intensified near the zygoma. There was some weakness of the mouth, but chewing was unimpaired. Trismus was absent, and the masseters were not grossly involved. A sensation of dizziness associated with flushing and sweating soon followed. Some complained of a bandlike headache with orbital pain. Others experienced a feeling of facial anesthesia that often crossed the dermatome midline pattern. Sinus tachycardia to a rate of 100 was observed, with mild facial and cervical flushing. Nystagmus could not be elicited, and true gastrointestinal symptoms were notably absent.

In addition, a random sampling of physicians revealed that one was convinced that he was having an acute myocardial infarction, for intense, heavy aching occurred in his biceps and triceps 45 minutes after he had eaten Chinese egg rolls. In another extreme fatigue developed in the shoulder girdle, "simulating a palsy," as well as weakness of the muscles of mastication. Still another had paresthesia and weakness in the upper extremities intense enough to suggest paralysis.

All those with reactions seem to recover uneventfully within an hour and usually resume their surfeit. Although all these subjects had Chinese tea, other suspected elements such as duck sauce, mustard and Won-Ton soup were not ingested by all.

Studies of the potential role of frozen-food processing of Chinese vegetables, the elusive tannins of tea with its fermentation enzymes such as thease, as well as further clinical observations, all seem indicated and are projected. While we await greater understanding of this disorder, the words of Addison seem especially appropriate:

> When I behold a fashionable table set out in all its magnificence, I fancy that I see gouts and dropsies, fevers and lethargies, with other innumerable distempers lying in ambuscade among the dishes.

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Fluorometric Determination of Aspartate, Glutamate, and γ-Aminobutyrate in Nerve Tissue Using Enzymic Methods[1]

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Identification of chemical transmitters in the mammalian spinal cord requires specific and sensitive methods for their measurement. Interest in the distribution of Glu,[2] Asp, GABA, and Gly in cat spinal cord and roots led us to develop methods for determination of these amino acids. Preliminary reports of this work have been published (1–3).

Most of the methods available for the separation and measurement of amino acids involve the use of either paper or column chromatography (4). These procedures have two intrinsic limitations for our purposes: (*a*) either they are not sensitive enough, or (*b*) they are too time consuming to use for a large number of small samples. The use of enzyme methods offers distinct advantages over chromatographic analysis when the analysis of only a few amino acids is required (5).

In general, enzymic analyses are simple and rapid; furthermore, they do not require prior isolation or separation of specific substrates in the tissue sample because of the specificity of the enzyme reactions. In addition, if an enzyme reaction requires NAD^+ or $NADP^+$ or the reduced form of either, highly sensitive techniques have been developed for the measurement of these coenzymes.

[1] This investigation was supported in part by research grant MH-03225-07 and predoctoral fellowship 4-F4-MH-28,682-01 (BN) (to L. T. G.) from the National Institute of Mental Health, U. S. Public Health Service, and grant GB 2692 from the National Science Foundation.

[2] Abbreviations: Asp, L-aspartate; Glu, L-glutamate; GABA, γ-aminobutyrate; Gly, glycine; α-KG, α-ketoglutarate; NAD^+, nicotinamide-adenine dinucleotide; NADH, nicotinamide-adenine dinucleotide, reduced; $NADP^+$, nicotinamide-adenine dinucleotide phosphate; NADPH, nicotinamide-adenine dinucleotide phosphate, reduced; GDH, glutamic dehydrogenase; GOT, glutamic-oxaloacetic transaminase; MDH, malate dehydrogenase; GABA-T, GABA transaminase; SSADH, succinic semialdehyde dehydrogenase.

This paper describes modifications of some enzymic methods using small volumes and fluorometric measurements with which as little as 5×10^{-12} mole Asp, 1×10^{-11} mole GABA, and 1×10^{-10} mole Glu can be measured. These methods have been used to assay Asp, GABA, and Glu in several areas of the cat nervous system.

MATERIALS AND METHODS

Principles. A preliminary account of the Glu assay has recently appeared (6). We have since made some improvements in the preparation of the tissue extracts and in lowering the fluorescence blank (see below). GDH oxidized Glu to α-KG with the concomitant conversion of NAD^+ to NADH as shown in Eq. 1. The NADH produced in reaction 1

$$\text{Glu} + NAD^+ \xrightarrow{\text{GDH}} \alpha\text{-KG} + NH_4^+ + \text{NADH} \qquad (1)$$

was measured by its native fluorescence as described by Lowry *et al.* (7).

The principle of the Asp assay has been discussed by Pfleiderer (8). In the presence of α-KG, GOT converted Asp to oxaloacetate. The oxaloacetate formed was reduced to malate in the presence of NADH and a second enzyme, MDH, as shown in Eqs. 2 and 3. In our procedure

$$\alpha\text{-KGA} + \text{Asp} \xrightarrow{\text{GOT}} \text{Glu} + \text{oxaloacetate} \qquad (2)$$

$$\text{oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{malate} + NAD^+ \qquad (3)$$

the increase in NAD^+ was measured by its highly fluorescent product formed in strong alkali as described by Lowry *et al.* (7), instead of measuring the decrease in NADH by its absorption at 340 mμ.

An enzymic measurement of GABA has been used by several groups of investigators, e.g., Jakoby and Scott (9), Baxter and Roberts (10), and Hirsch and Robins (11). We now describe several modifications in the present paper which were used in this assay to measure GABA in small pieces of both gray or white spinal cord (2). Jakoby and Scott (9) described two enzymes from *Pseudomonas fluorescens* which carry out the successive reactions:

$$\text{GABA} + \alpha\text{-KG} \xrightarrow{\text{GABA-T}} \text{Glu} + \text{succinic semialdehyde} \qquad (4)$$

$$\text{succinic semialdehyde} + NADP^+ \xrightarrow{\text{SSADH}} \text{succinate} + \text{NADPH} + H^+ \qquad (5)$$

The excess $NADP^+$ is destroyed by weak alkali. The NADPH, which is stable in the weak alkali treatment, is then converted to a highly fluorescent product of $NADP^+$ in one step by the addition of strong alkali containing H_2O_2 (7). The resulting fluorescence is a measure of the GABA present.

Apparatus. Many of the techniques and pieces of equipment used in these assays have been developed by Lowry and his co-workers. Micro reaction tubes (12) are made from 4 mm o.d. glass tubing which has been cleaned by boiling in 50% concentrated nitric acid. Caps for the micro-tubes are made from short lengths of rubber tubing stopped at one end with a short length of glass rod. The fluorometer tubes are standard 3 ml Pyrex glass tubes, 10 × 75 mm (Corning No. 9820). The construction of the vacuum centrifuge evaporator (13) and "buzzer" (12) have been described. The Lang-Levy type constriction micropipetes were purchased from Bonus Laboratories. A Farrand fluorometer model A with a primary filter No. 5860 and secondary filters Nos. 4308, 5562, and 3387 was used for all three assays.

Reagents. For the Asp assay, α-KG (Calbiochem), 0.1 *M*, was prepared fresh every day by dissolving 73 mg in 4 ml sodium phosphate buffer (0.067 *M*, pH 7.2), adjusting the pH to 7.0–7.5 with 2 *N* NaOH, and bringing to a volume of 5.0 ml with water.[1] NADH (Sigma), 10 m*M*, was prepared by dissolving 36 mg in 5.0 ml of a 1:1 solution of 0.05 *M* Na_2CO_3 and 0.05 *M* $NaHCO_3$. The solution was kept at 2°C and every day before use was heated in a boiling water bath for 2 min as suggested by Lowry *et al.* (14). This procedure is used to lower the fluorescence blank. The solution was stable for at least 2 weeks (longer periods were not checked), showing no increase in the blank or change in the standard curve. Malate dehydrogenase (Boehringer) was used at a concentration of 1.25 mg/ml by diluting a 10 mg/ml solution with 2.8 *M* ammonium sulfate. Glutamic-oxaloacetic transaminase (Boehringer) was diluted to 1.25 mg/ml with 1.6 *M* ammonium sulfate. The stock enzyme solutions are stable for months at 0–4°. Stock laboratory solutions of 1 *N* HCl and 10 *N* NaOH were diluted to 0.3 *N* HCl and 9 *N* NaOH, respectively. A stock standard solution of 1 m*M* L-Asp (Cyclo Chemical Corp.) was prepared by dissolving 13.3 mg in 100 ml water; 0.3 ml portions were frozen and stored at −20°C. A single portion was removed and thawed; appropriate dilutions were made as needed for each series of determinations.

The Gly in the glycine-hydrazine buffer used in the Glu assay was purified by dissolving 3.75 gm Gly (Nutritional Biochemicals Corp.) in 30–50 ml H_2O, adding approximately 3 gm Norit-A (Fisher), shaking briefly, and filtering the mixture through a layer of Hyflo Super-Cel (Fisher) on a ground-glass filter.

The charcoal and Super-Cel were then washed on the filter with two portions of water and the washings added to the filtrate. To the com-

[1] Double-distilled water was used in the preparation and dilution of all solutions, unless otherwise stated.

bined filtrate, 5.5 ml of 24% (w/v) hydrazine hydrate (Fisher) was added; the pH was adjusted with 1 N H_2SO_4 (if necessary) to 8.6 ± 0.1 and the volume made up to 100 ml with H_2O. The Norit treatment removed a fluorescent impurity from the Gly which decreased the blank and permitted an increase in sensitivity. The buffer is stable at room temperature. NAD^+ (Sigma), 10 mM, was prepared by dissolving 33.2 mg in 5.0 ml H_2O; it is stable at 0°–4° for 2 weeks. The GDH used was Boehringer's crystalline beef liver enzyme (10 mg/ml), NH_4^+-free in 50% glycerol. It is stable for months at 0°–4°. Standard solutions of 100 mM Glu (Calbiochem) were prepared by dissolving 73.6 mg in 5.0 ml H_2O. Small portions were frozen and stored at −20°. A single portion was removed and thawed; appropriate dilutions were made as needed for each series of determinations.

Sodium pyrophosphate buffer, 0.1 M, pH 8.4, used in the GABA assay was prepared by dissolving 4.46 gm $Na_4P_2O_7 \cdot 10H_2O$ (Baker) in H_2O, adjusting the pH with 0.1 N HCl, and bringing up to a final volume of 100 ml with H_2O. The solution was treated with Norit-A and filtered to decrease the fluorescence blank as described for the Glu assay. $NADP^+$ (Sigma) was prepared as a 1 mM solution by dissolving 4.7 mg in 5 ml H_2O. A 0.06 M solution of 2-mercaptoethanol (Calbiochem) was obtained by diluting 20 µl to 5.0 ml with buffer. α-KG (Calbiochem), 0.06 M, was prepared fresh every day by dissolving 44 mg in 4 ml buffer, neutralized with 2 N NaOH, and brought up to 5.0 ml with H_2O. The phosphate solution was prepared by dissolving 15.2 gm $Na_3PO_4 \cdot 12\ H_2O$ and 5.3 gm $Na_2HPO_4 \cdot 7\ H_2O$ in 100 ml H_2O (0.4 M PO_4^{3-}, 0.2 M HPO_4^{2-}). The enzyme system was prepared from a bacterial source and carried through the acetone precipitation step. The enzyme solution, as obtained, was frozen at −100° C in small portions. A single portion was removed (stable for at least 3 months) and diluted 1:10 with buffer for use in the assay. The alkaline-peroxide solution was prepared immediately before use by diluting 100 µl of 3% H_2O_2 to 10 ml with 10 N NaOH. The 3% H_2O_2 was made from a 30% solution once a week and kept in a refrigerator. A standard 1 mM solution of GABA (Calbiochem) was prepared by dissolving 10.3 mg in 100 ml H_2O daily; appropriate dilutions were made.

Preparation of Tissue. The tissue to be assayed was weighed while frozen and then extracted with cold 75% ethanol essentially as described by Baxter and Roberts (10). Some modifications were necessary because smaller tissue samples were used. These are described below. Neither trichloroacetic acid nor perchloric acid could be used in the

* The authors wish to thank Dr. C. F. Baxter for a supply of the GABA enzymes.

extraction step because of their inhibitory effect on the enzymes used in the GABA and Asp assays. After weighing the frozen piece of tissue, it was dropped into a small precooled (0 C) ground-glass homogenizer containing cold 75% ethanol. If the tissue sample weighed less than 10 mg, 1 ml of 75% ethanol was used; for samples in the 10-30 mg range, 2 ml was used. The homogenate was poured into 2.5 ml or 5 ml glass-stoppered conical centrifuge tubes, the homogenizer was rinsed with two 0.5 ml portions of the cold ethanol and the rinses were added to the homogenate. After centrifugation at 2500 rpm in an International refrigerated centrifuge (PR-2) for 10 min, a small firm residue was obtained. The supernatant was poured into a stainless-steel centrifuge tube and the glass tube rinsed with 0.5 ml of 75% ethanol. The contents of the steel tube were evaporated to dryness in the vacuum centrifuge evaporator. At this point in the procedure, the dried steel tubes may be stored in a vacuum desiccator or the residues may be resuspended in distilled water and the assays continued. The volume of water added was directly proportional to the wet weight of the sample (1 ml/4 mg). The suspension was slightly cloudy and was cleared by centrifuging in a Model L ultracentrifuge (Spinco #40.2 or #40 head, 30,000 rpm, 30 min). The clear supernatant liquid was poured into small test tubes and used for all the assays. In the Asp and GABA assays, measured volumes, depending on the expected or predetermined concentration in the tissue, were added to the micro reaction tubes. The contents of the microtubes were then dried in the vacuum centrifuge evaporator. These samples may be stored in a vacuum desiccator until used. In the case of the Glu assay, a measured volume of the supernatant liquid was used directly in the assay. The water solution may also be frozen for storage, if necessary.

Assay Procedures. In the Glu assay, measured volumes (10 to 100 μl) of the clear supernatant were added to fluorometer tubes containing 1.0 ml of glycine-hydrazine buffer and bazzed. To this solution 20 μl of NAD was added, bazzed again, and the fluorescence measured (against quinine working standard). GDH (10 μl) was then added and the tubes allowed to incubate at room temperature for 30 min. The native fluorescence of the NADH was again measured. Under these conditions the increase in fluorescence is proportional to the Glu in the sample. Since the fluorescence of each tube is read twice and the increase obtained by difference, each tube acts as its own tissue blank. A reagent blank was obtained by adding water to one set of tubes instead of sample or Glu standard. Blanks, samples, and standards were run in duplicate or triplicate.

The incubation mixture for the Asp assay contained 2.75 ml phosphate

buffer, 25 μl NADH, 50 μl α-KG, 20 μl MDH, and 20 μl GOT. A 15 μl portion of the mixture was added to microtubes containing "dried" sample or Asp standard. Racks of the microtubes were kept in ice water (0°) during the additions. The tubes were capped and the rack transferred to a 38° water bath for 30 min. The rack of tubes was then quickly returned to ice water and 50 μl of 0.3 N HCl was added to each tube and buzzed vigorously. A 50 μl portion was removed from the microtubes and added to corresponding fluorometer tubes containing 100 μl 9 N NaOH and buzzed. The rack of fluorometer tubes was then placed in a 38° water bath for 30 min. The same results were obtained if these tubes were placed instead into a 60° bath for 10 min. The fluorometer tubes were removed, 1.0 ml of distilled water was added, buzzed, and the fluorescence read. A reagent blank was prepared by using water instead of sample or standard. Tissue blanks of each sample were obtained by adding the 50 μl of acid to a series of the microtubes before the 15 μl of incubation mixture. All Asp determinations were made in triplicate.

The incubation mixture for the GABA assay consisted of 1.0 ml pyrophosphate buffer, 0.2 ml NADP, 0.2 ml α-KG, 0.2 ml mercaptoethanol, and 0.1 ml enzyme solution. A 15 μl portion of this mixture was added to microtubes of dried sample or standard. Racks of the tubes were kept in ice water (0°) during the additions. The tubes were then capped and the racks transferred to a 38° water bath for 45 min. The rack of tubes was returned to the ice water and 50 μl of the phosphate solution added to each tube; the tubes were again buzzed vigorously. The tubes were recapped and transferred to a 60° water bath for 15 min. Then a 50 μl portion was removed from the microtubes and added to fluorometer tubes containing 100 μl of 0.03% H_2O_2 in 10 N NaOH and buzzed. The rack of fluorometer tubes was then heated at 60° for 10 min in the water bath. Distilled water (1 ml) was added, the tubes buzzed, and the fluorescence read. Blanks were treated as in the Asp assay.

RESULTS AND DISCUSSION

The standard curves for Glu, GABA, and Asp are shown in Figs. 1, 2, and 3, where each point is the mean of three determinations. These data indicate that the useful range is greater than 10-fold for Glu and GABA and 100-fold for Asp. However, during routine analysis of tissue samples, five or six standards over a 10-fold concentration range from $2\text{-}20 \times 10^{-10}$ mole for Glu and $2\text{-}20 \times 10^{-11}$ mole for Asp and GABA were run in parallel with the unknowns. Sample volumes were adjusted so that the amino acid content fell within the range of standards used.

The reproducibility of the assays is shown in Table 1. Ten determina-

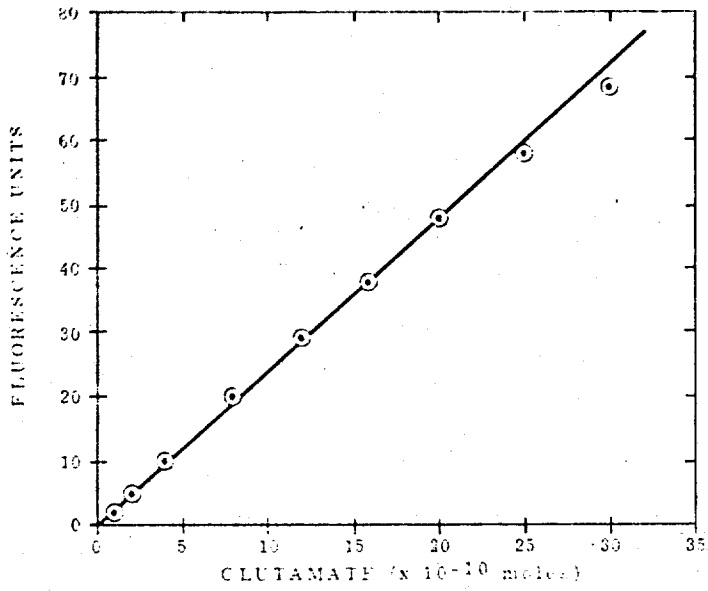

FIG. 1. Relationship between NADH fluorescence and glutamate levels in the 1×10^{-10} to 30×10^{-10} mole range. Each point is the mean of three determinations.

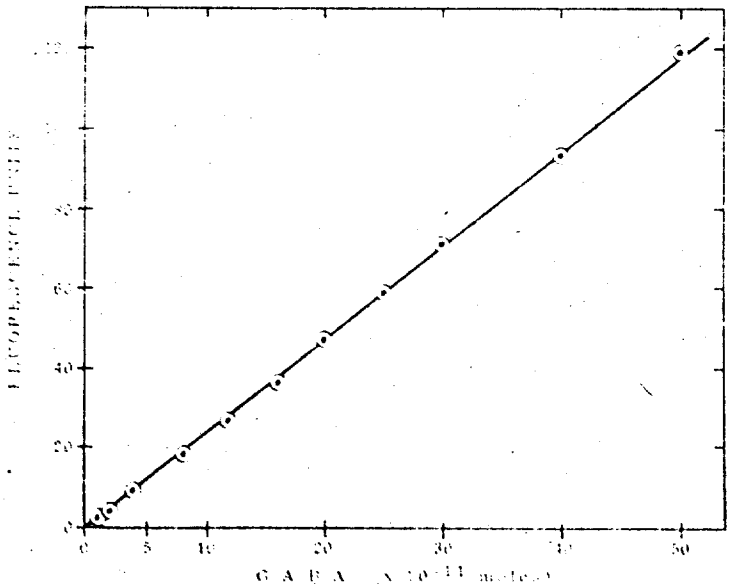

FIG. 2. Relationship between fluorescence of the NADP+ product produced from conversion of NADPH by alkaline-peroxide treatment and GABA levels in the 1×10^{-11} to 50×10^{-11} mole range. Each point is the mean of three determinations.

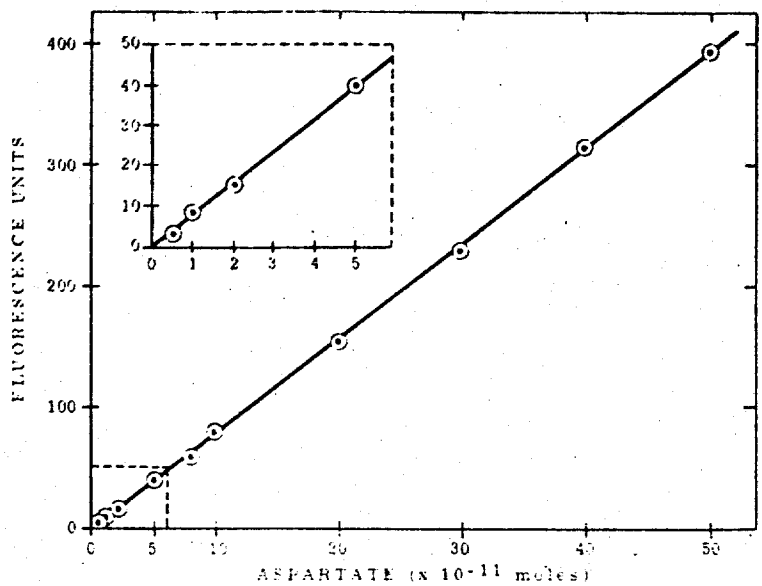

FIG. 3. Relationship between fluorescent product of NAD^+ in strong alkali and aspartate levels in the 5×10^{-11} to 5×10^{-9} mole range. Each point is the mean of three determinations.

tions were made on the same standard solution. The coefficient of variation was less than 2% for the Glu assay, 3% for the GABA assay, and 4% for the Asp assay.

The Glu assay is the least sensitive of the assays but it is the simplest. We suggested in our preliminary account of this method (6) that the sensitivity might be increased by the use of Lowry's alkaline-peroxide treatment. We have since found that the glycine-hydrazine buffer or a hydroxylamine buffer interfere with the alkaline-peroxide procedure. The sensitivity was increased 2-fold by purifying the glycine with the charcoal treatment, thereby decreasing the fluorescence blank. However, a decrease in reaction volume would be needed to further increase the sensitivity of the present method. Sample volumes used were equivalent to 100–300 μg tissue. Reaction volumes containing 4 nanomoles or more of Glu begin to show deviations from linearity. The method is specific in that D-glutamate, glutamine, and Asp are not oxidized at all. Under conditions of equal concentration, only L-norvaline is oxidized at an appreciable rate, 17% (15). The naturally occurring excess of Glu over all other amino acids in the nervous system, as well as the high dilutions used, increases the discrimination provided by use of this enzyme in the analysis of Glu in nerve tissue.

TABLE 1

REPRODUCIBILITY OF GLUTAMATE, γ-AMINOBUTYRATE AND ASPARTATE ASSAYS

Determination No.	Fluorescence reading		
	Glu (10×10^{-12} mole)	GABA (10×10^{-12} mole)	Asp (10×10^{-12} mole)
1	43	44	48
2	43	45	50
3	42	43	50
4	42	45	49
5	43	42	49
6	42	44	49
7	42	45	45
8	42	46	51
9	43	45	49
10	41	45	53
Mean	42.3	44.4	49.3
±S.D.	0.67	1.38	2.05
Coefficient of variation	1.60%	3.17%	4.17%

The Asp assay is the most sensitive of the three assays described and is linear over the entire 100-fold range tried, i.e., from 5×10^{-14} to 5×10^{-12} mole of Asp. Sample volumes used were equivalent to 10–100 μg tissue. The fluorescence blank is lowest in this assay permitting an increase in the instrument sensitivity. The equilibrium of the coupled reaction is favorable so that Asp is quantitatively converted to malate with the oxidation of a stoichiometric amount of NADH. The GOT reaction is specific for Asp. The only other reactant is the sulfo analog, cysteic acid, and it reacts much more slowly.

Sample volumes used in the GABA assay were equivalent to 20–400 μg tissue. The enzyme system used in this assay is specific. The only other compound known to give an α-KG dependent production of NADPH in this system is β-hydroxy-GABA. However, β-hydroxy-GABA reacts at a much slower rate than GABA in this assay system. The presence of β-hydroxy-GABA in nerve tissue has not been conclusively shown (16–18). The disadvantage to this assay is the preparation of the enzyme system. Hopefully, a suitable commercial preparation will become available.

Data from one animal illustrating the applicability of these procedures and methods are shown in Table 2. The tissue samples in this case weighed from 3.1 to 9.9 mg. This amount of tissue was in great excess over the amounts actually used in the assays. A paper reporting the distribution of some free amino acids in different areas of the cat spinal cord as well as in dorsal and ventral spinal roots is now in preparation.

TABLE 2

DISTRIBUTION OF GLUTAMATE, γ-AMINOBUTYRATE, AND ASPARTATE IN CAT LUMBAR SPINAL CORD (μMOLES/GM)[a]

Amino acid	Dorsal white	Dorsal gray	Ventral gray	Ventral white
Glu	4.03	5.52	4.32	3.54
GABA	0.58	3.62	1.66	0.53
Asp	1.11	1.59	2.12	1.33

[a] Values represent means of triplicate determinations on triplicate tissue samples from one animal.

SUMMARY

Procedures are described for the determination of 5×10^{-12} to 5×10^{-10} mole of aspartate, 1×10^{-11} to 5×10^{-11} mole of γ-aminobutyrate and 1×10^{-10} to 30×10^{-10} mole of glutamate using enzymic methods and fluorometric measurements of pyridine nucleotide coenzymes. After an initial ethanol extraction of 1 to 30 mg of frozen nerve tissue, samples are processed and prepared as aqueous solutions to be used in the enzyme procedures, which are described in detail. Sample volumes employed in the glutamate assay correspond to 100–300 μg tissue; in the γ-aminobutyrate assay, sample volumes are equivalent to 20–400 μg tissue, whereas in the aspartate assay volumes are equivalent to 10–100 μg tissue. The coefficient of variation was less than 2% for the glutamate assay, 3% for the γ-aminobutyrate assay, and 4% for the aspartate assay.

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that is used to keep granulated type sugars dry is used to keep Soft sugar moist. Supplementing this, storage ...dities between 60% and 70% are recommended ...oft sugars whenever extended storage periods are apt to occur.

To summarize, white sugars such as granulated and powdered products should be stored where humidities are below 60% and the temperatures are below 100° F. Brown sugars best retain their quality under damper conditions, a humidity between 60% and 70% being preferred and a temperature below 75° F.

Temperatures appreciably lower than ordinary room conditions should also be avoided. In the instance of granulated sugar, temperature differences accentuate humidities, and in the instance of Brown sugar, freezing temperatures solidify the syrup associated with the sugar crystals and the mass becomes unworkable.

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The Occurrence of Free L-Glutamic Acid in Various Foods[a]

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...nosodium glutamate has been used for many years ...y the Chinese and Japanese to make their bland foods ...ore palatable. Small amounts of this material which ...ds no flavor, color or aroma of its own, bring out or ...tensify the intrinsic flavor of meats, seafoods, vege...ables or dairy products to which it is added. Although ...e salts of many of the amino acids have a slightly ...eet or bitter taste, the monosodium salt of the di...arboxylic amino acid, glutamic acid, is the only one ...ich exhibits this particular property of accentuating ...avors.

...rocker *(1)* and Cairncross *(2)* have studied this ...articular quality of monosodium glutamate. They have ...nd that monosodium glutamate is entirely without ...or when pure. Its taste has all four components, ...eet, salt, sour and bitter and can be matched rather ...ell with 0.6 threshold of sweetness, 0.7 saltiness, 0.3 ...rness, and 0.9 bitterness. Saltiness and sourness, ...ch characterize glutamic acid and its sodium salt are ...eptional tastes among amino acids. In addition, two ...er factors not ordinarily encountered, that is, tingling ...d persistence are also present. Whereas flavor is ...ally the result of two senses, taste and smell, mono...dium glutamate brings into play a third sense since it ...s the capacity for stimulating the feeling nerves of ...mouth and throat to produce the sensation described ...rocker *(1)* as a "feeling of satisfaction."

...this country, monosodium glutamate has been used ...some time by chefs and gourmets interested in accentuating the flavors of their fine foods, but it has only been recently that the supply has been sufficient to make this material generally available to food processors and housewives. For a time, its use by food processors and canners was complicated by the fact that the provisions of the Federal Food, Drug and Cosmetic Act required that any material to which monosodium glutamate had been added must bear the label "artificially flavored with monosodium glutamate."

It is well known that glutamic acid is involved in the metabolism of plants and animals. If it could be shown that it occurred naturally in prepared foods, it seems reasonable to suppose that the term "artificially flavored" is inappropriate. Therefore, it was decided to determine whether or not glutamic acid existed as such in vegetables and meats which had *not* been hydrolyzed. This glutamic acid has been designated as "free" glutamic acid in order to differentiate it from any which would be bound in protein and liberated by hydrolysis. In foods, this free glutamic acid would undoubtedly be present as monosodium glutamate.

Determinations have been made of the amount of free glutamic acid occurring naturally in cooked fresh vegetables and meats and in several varieties of canned vegetables and seafoods of recognized commercial brands. Added monosodium glutamate has also been determined in an experimental commercial pack of boned chicken. Recently, the Food and Drug Administration ruled that the "artificial flavoring" designation is no longer required in canned foods to which monosodium glutamate has been added but its presence must be declared.

[a] Presented before the Ninth Annual I. F. T. Convention, San ...cisco, Calif., July 14, 1949.

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The superior flavor of young and of freshly harvested vegetables is well recognized. Since monosodium glutamate enhances flavor, it was of interest to us to know whether there was a larger amount of free glutamic acid present in these vegetables than in more mature ones or in those stored in the refrigerator for short periods of time before preparation.

Freshly picked sweet corn of three stages of maturity (all edible) and garden peas graded into three sizes were cooked within 6 hours of harvest and analyzed. Free glutamic acid was also determined in portions of these cooked vegetables and of raw vegetables cooked after various periods of storage in the refrigerator.

Procedure

Microbiological assay with the micro-organism *Lactobacillus Arabinosus* 17-5 has been the procedure used for determination of free glutamic acid in all the foods tested. The materials for assay were treated with 70% isopropanol according to a procedure established in our laboratory for the extraction of the non-colloidal nitrogen fraction of sugar beets.[b] Since we were interested primarily in determining the presence or absence of glutamic acid occurring naturally in prepared foods, no particular study was made to determine whether complete extraction was accomplished in the various types of foods studied.

Canned Foods: In most instances two cans of each variety of foods, purchased from a local grocery store, were analyzed. There was no indication on any of the labels that monosodium glutamate had been added. After the weights of the liquid and drained canned food had been determined, 25 g. of the drained food with its proportionate amount of liquid was mixed with 175 ml. of isopropanol and made up with distilled water to a total volume of 250 ml. This mixture was homogenized in a Waring blendor for two minutes and allowed to stand overnight and then filtered. The isopropanol from 150 ml. of the filtrate was distilled in vacuo and the residue made up to a final volume of 50 ml. for assay. Calculations were based on the 25 g. drained weight of material used.

Fresh Vegetables: 25 g. portions from a large composite of the prepared vegetables were weighed into flasks and cooked until tender in the minimum amount of distilled water. Salt was added. The mixture was cooled and then treated with isopropanol in the manner described for canned foods.

The sweet corn used for the storage experiment was a golden bantam hybrid designated as "Golden Cross" purchased from a local truck farmer a few hours after it had been picked. It was the custom of this farmer to sort the corn for sale into three grades of maturity: very young, medium, and mature. All grades were tender and had a flavor superior to most of the corn available at markets. The corn was cut from the cob of a dozen ears of each grade and thoroughly mixed. 25 g. aliquots of the raw and cooked corn were stored in closed flasks in the refrigerator. Analyses were made of the corn, raw, immediately after cooking and at 24, 48 and 72 hour intervals after storage. Duplicate determinations were made in each case and the averages of these determinations reported.

The peas were picked from an experimental plot. The duplicate samples in this case were from peas picked from different locations in the same row. They were shelled and graded roughly into very small, medium and large sizes. Aliquots were prepared and stored as in the case of the sweet corn.

COOKED MEATS. *Roast Beef:* 100 g. of beef (fat removed) from the center cut of an 8½ pound standing rib roast, salted and roasted for 3½ hours at 300° F. were minced in a Waring blendor with 100 ml. of distilled water. 175 ml. of isopropanol was added to 50 g. of the mixture and the treatment was the same as described for vegetables. 50 g. of the juice which exuded when the roast was sliced was also mixed with isopropanol and analyzed. In this case, calculations were based on the weight of the juice used.

Roast Pork and Roast Lamb: Fat-free portions of the center cut of a 6 pound pork loin roast and of a 6 pound leg of lamb roasted at 350° F. were prepared similar to roast beef.

Link and Patty Sausage: The sausages were fried over low heat until they were well done and the excess grease discarded. The skin was removed and the meat homogenized in a Waring blendor as described above.

Bologna and Frankforters: The bologna was analyzed without further cooking; the frankforters were allowed to stand for 10 minutes in water which had just come to boiling, according to the directions given on the package.

Microbiological Assay: The basal medium for assay is a modification of that of Hac, Snell and Williams.[c] The constituents of the medium and the method of preparation are given in Table 1. The total volume of medium and sample in each tube was 5 ml. The tubes containing the medium and sample were sterilized at 15 pounds pressure for 20 minutes, inoculated with a drop of a dilute saline suspension of L. arabinosus 17-5 and incubated at 34° C. for 64 hours. The acid produced during growth was titrated with 0.1 N sodium hydroxide electrometrically or using bromthymol blue as an indicator.

The standard glutamic acid curve was prepared using 0.01 mg. increments. The assay range is satisfactory between 0.02 and 0.3 mg. The glutamic acid values reported were the average of 6 determinations made in duplicate tubes at 3 levels of concentration. The procedure has proved satisfactory for the assay of most natural products although occasionally activators or inhibitors have caused difficulty.

Since L. arabinosus can utilize glutamine instead of glutamic acid for growth, this procedure can be adapted for the determination of glutamine also, provided sterilization of the sample is accomplished by some means other than heat. Heat converts glutamine into the ammonium salt of pyrrolidone-carboxylic acid which cannot be utilized. If the sample, sterilized by filtration through a Berkefeld filter is added aseptically to the sterilized basal medium, glutamine can be determined and differentiated from glutamic acid.

[b] To be published elsewhere.

TABLE 1

Basal Medium for L-Glutamic Acid Assay using L. Arabinosus 17-5

Amino Acids	Mg./L.	Purines, etc.	Mg./L.
Alanine	600	Adenine Sulfate	30
Isoleucine	400	Guanine HCl	30
Methionine	400	Xanthine	30
Norleucine	400	Uracil	30
Norvaline	400		
Phenylalanine	400	Ammonium sulfate	6 g.
Serine	400	Sodium acetate	66 g.
Threonine	400		
Valine	400	Salts B	15 ml.
Aspartic Acid	2400	$MgSO_4 \cdot 7H_2O$ 10 g.	
Arginine HCl	300	NaCl 0.5	
Cystine	300	$FeSO_4 \cdot 7H_2O$ .5	
Glycine	300	$MnSO_4 \cdot 4H_2O$ .5	
Hydroxyproline	200	Water to 250 ml.	
Histidine Mono HCl	300		
Leucine	200		
Lysine	300		
Proline	300		
Tryptophane	300		
Tyrosine	300		

Dissolve amino acids, purines, ammonium sulfate and sodium acetate by boiling in 900 ml. of distilled water; cool and add ml. of salts B; adjust pH to 6.3 with NaOH and add water to liter. This is double strength medium. Store in the refrigerator.

Vitamins	Mg.	Solution A	
Biotin	0.05	KH_2PO_4	25 g.
Calcium Pantothanate	2.5	K_2HPO_4	25 g.
Nicotinic Acid	2.5	Water to 250 ml.	
Riboflavin	2.5		
Para-aminobenzoic	3.8		
Thiamin HCl	6.3		
Choline Chloride	31.4		
Inositol	31.4		
Pyridoxine HCl	125.0		

Gently warm in 50 ml. H_2O. Store in refrigerator not longer than 2 weeks.

At the time of assay, for 100 tubes (final volume 5 ml.) mix water 245 ml. of amino acid solution, 5 ml. solution A, 2 ml. vitamin solution 10 g. glucose, and 30 mg. glutamine. If the samples to be assayed are less than 0.5 ml. in volume, add an equal volume of water to the above mixture; then add 5 ml. of diluted medium to each tube containing the sample. If samples are larger than 0.5 ml., add water to 2.5 ml. and then add ml. of the undiluted medium.

Non-Colloidal Nitrogen: In a few instances, total non-colloidal nitrogen determinations were made on the 95% ethanol extracts.[6] Micro Kjeldahl determinations were made using the Gunning and Arnold modification of the digestion procedure.

Solids: Were determined by oven drying to constant weight.

Results

The results of the assays for free L-glutamic acid in varieties of canned vegetables and 6 varieties of fresh vegetables are listed in Tables 2 and 3. Glutamine present in the vegetables would not have affected since the samples were all heat sterilized. The highest value 0.23% free glutamic acid was obtained in canned mushrooms. All other vegetables had values varying from 0.17 in fresh garden peas to none in canned yellow wax beans. No glutamic acid detectable in hominy. This might be expected since the alkali and washing treatments required in the preparation of hominy would probably remove any free glutamic acid present in the corn.

We are indebted to Mr. Basil B. Dowling for the Kjeldahl nitrogen determinations, and to Dr. A. C. Walker for the ethanol extractions.

Fresh young peas and sweet corn contain more free glutamic acid than samples of more mature vegetables from the same field (Tables 4 and 5). It may be that in vegetables, the amount of glutamic acid present is proportional to the rate of growth.

Some glutamic acid was lost during even the short cooking required for peas and corn since the raw vegetables contained a higher percentage of free glutamic acid than the cooked vegetables. However, very little loss of glutamic acid occurred in cooked vegetables stored in the refrigerator, whereas 25-35% of the glutamic acid contained in raw vegetables was lost during storage in the refrigerator. Almost all of this loss occurred during the first 24 hours.

All the canned seafoods, chicken and fresh cooked meats analyzed contained free glutamic acid in amounts ranging from 0.1% in minced clams to 0.01% in roast pork loin, pork sausage and frankforters (Tables 6 and 7). The glutamic acid content found in a commercial experimental pack of boned chicken to which monosodium glutamate had been added checked very well with the expected value.

TABLE 2

Percent Free L-Glutamic Acid in Canned Vegetables

Vegetable	Sample Identification	Total Solids	Total N	Glutamic Acid
		Percent on Drained Weight	*Percent on Drained Weight*	*Percent on Drained Weight*
Mushrooms	1	11.05	0.71	[illegible]
	2			[illegible]
Green Peas	3	16.67	.75	[illegible]
Asparagus, green	4	9.25	.59	[illegible]
	4A			[illegible]
Potatoes, whole, new	5			[illegible]
Sweet Potatoes	6			[illegible]
Sweet Corn	7			[illegible]
Red Beets	8			[illegible]
Okra	9			[illegible]
Spinach	10			[illegible]
Spinach, strained	11		.31	[illegible]
Green Lima Beans	12			[illegible]
Butter Beans	13			[illegible]
Green String Beans				
French Style	14	8.67	.28	[illegible]
Cut Style	15			[illegible]
Strained	16			[illegible]
Baked Pork and Beans	17			[illegible]
Garbanza	18			[illegible]
Yellow Wax Beans	19			[illegible]
Hominy	20			[illegible]

TABLE 3

Percent Free L-Glutamic Acid in Cooked Fresh Vegetables

Vegetable	Total Solids	Total N	Glutamic Acid	
			Sample 1	Sample 2
		Percent on Raw Weight	*Percent on Raw Weight*	*Percent on Raw Weight*
Mushrooms, wild	11.84	1.03	0.13	[illegible]
Green Peas				
Small			.19	[illegible]
Medium			.17	[illegible]
Large			.17	[illegible]
Pods			.04	[illegible]
Sweet Corn				
Very young		.21	.12	[illegible]
Medium		.33	.05	[illegible]
Mature		.30	.06	[illegible]
Potatoes, small new			.05	[illegible]
Asparagus, green	6.74	.34	.04	[illegible]
Green string beans	9.08	.28	.02	[illegible]

TABLE 4

The Occurrence of Free L-Glutamic Acid in Sweet Corn at Various Stages of Maturity and the Effect of Storage on the Glutamic Acid Content of Raw and Cooked Corn

Treatment	Stage of Maturity					
	Young		Medium		Mature	
	N	GA	N	GA	N	GA
Lot 1						
	Percent on Raw Weight		*Percent on Raw Weight*		*Percent on Raw Weight*	
Raw	0.25	0.13	0.37	0.08	0.42	0.06
Fresh cooked	.21	.12	.33	.05	.30	.05
Cooked after 24 hrs. refrigeration		.08		.04		.04
Cooked after 48 hrs. refrigeration		.07		.04		.03
Cooked after 72 hrs. refrigeration		.08		.04		.04
Fresh cooked		.12		.05		.05
Cooked and refrigerated 24 hrs.		.12		.06		.05
Cooked and refrigerated 48 hrs.		.12		.06		.04
Cooked and refrigerated 72 hrs.		.11		.06		.05
Lot 2						
Raw				.09		
Cooked on the cob				.09		
Cooked after removal from cob				.08		
Glutamine:						
Raw corn	0.03%					
Cooked corn	0.02%					

TABLE 5

The Occurrence of Free L-Glutamic Acid in Fresh Garden Peas of Various Sizes and the Effect of Storage on the Glutamic Acid Content of Raw and Cooked Peas

Treatment	Size					
	Small		Medium		Large	
	(Percent Glutamic Acid on Raw Weight)					
	Sample 1	Sample 2	Sample 1	Sample 2	Sample 1	Sample 2
Raw	0.20	0.20	0.18	0.18	0.15	0.17
Fresh cooked	.19	.19	.16	.17	.16	.16
Cooked after 24 hrs. refrigeration			.13	.12		
Cooked after 48 hrs. refrigeration			.12	.14		
Cooked after 72 hrs. refrigeration			.12	.14		
Raw after 72 hrs. refrigeration			.14	.12		
Fresh cooked			.16	.17		
Cooked and refrigerated 24 hrs.			.16	.17		
Cooked and refrigerated 48 hrs.			.17	.17		
Cooked and refrigerated 72 hrs.			.15	.16		
Pods, raw			.04	.04		

Samples 1 and 2 were picked from different portions of the same rows.

TABLE 6

The Percent Free L-Glutamic Acid in Canned Seafood and Chicken

Food	Sample Identification	Glutamic Acid	
		Percent on drained weight	
Salmon	21	0.02	0.02
Tuna Fish	22	.02	.02
King Crab	23	.06	.06
Deviled Crab	24	.03	.02
Minced Clam	25	.09	.10
Clam Chowder	26	.03	.02
Chicken, boned No. 1	27	.04	.03
Chicken No. 2	28	.02	.02
Chicken No. 2 + 0.25% MSG	29	.23	.22

TABLE 7

Percent Free L-Glutamic Acid in Cooked Fresh Meats

Meat	Cut	How Cooked	% Glutamic Acid	
			Percent on cooked weight	
Roast beef	Standing rib	Medium rare	0.04	0.05
		Juice from slicing	.07	
Roast pork	Loin	Well done	.01	.01
Roast lamb	Leg	Well done	.02	.02
Sausage No. 1	Link	Fried, grease removed	.02	.02
Sausage No. 2	Link	Fried, grease removed	.01	.02
Sausage No. 2	Patty	Fried, grease removed	.01	.01
Frankforters		Boiled, grease removed	.01	.01
Bologna		Not cooked	.03	.03

Summary

Free L-glutamic acid has been found in unhydrolyz samples of 15 varieties of canned vegetables, 6 varie of cooked fresh vegetables, 6 varieties of canned seaf canned chicken and 6 varieties of fresh cooked m in amounts ranging from 0.23% to 0.005%.

It is possible that part of the superior flavor of y and of freshly harvested vegetables may be due to i higher glutamic acid content which decreased du short storage in the refrigerator. A high glutamic content may be one of the reasons mushrooms enh the flavors of the many dishes to which they are add

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Protein Hydrolysates as a Source of Glutamate Flavors

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Author's abstract

The characteristics of a desirable protein hydrolysate to be used as a flavorin and seasoning agent include the presence of glutamic acid up to 16%, a larg representation of individual amino acids, and a low fat and carbohydrate center Wheat gluten, casein and soya flour are good sources for preparation of superi quality protein hydrolysates.

It is considered that a mixture of MSG and protein hydrolysate gives the m desirable results as to flavor and economy.

The flavoring of foods with protein hydrolysates has shown int esting and rapid progress in this country during the last decade. Si the production of protein hydrolysates, as such, has reached siza quantities and their use in the food industry attained significant volu specific information concerning their contribution to food flavor, particularly regarding their source as glutamate flavors, should more generally known.

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The purpose of the incorporation of protein hydrolysates in foods is to improve the flavor and taste of the products so as to make them more delicious. For more than 40 years, in China and Japan, it has been common practice to use protein hydrolysates from one source or another, as condiments. In the relatively short time that the American food industry has become interested in these flavors, outstanding improvements have been made in their manufacture as the result of chemical and nutritional research.

A rather voluminous literature has been developed on proteins and amino acids, and it has been exceedingly valuable in improving the manufacture of hydrolysates. A considerable number of patents have been issued, some under the title-subject "Protein Hydrolysates," and others designated "Flavoring Materials from Hydrolyzed Proteins." The numerous patents are evidence of the wide interest in these products.

Protein hydrolysates for foods consist essentially of two types: (1) complete acid-hydrolyzed proteins, and (2) acid-hydrolyzed proteins from which glutamic acid has been extracted. Both types are manufactured as liquids and commercially dry powders. This discussion is primarily concerned with the hydrolysates of Type 1, that is, the unextracted variety. It should also be stated that soya sauces and other flavoring meat sauces are produced wholly or partially by enzyme hydrolysis and that their production is of appreciable volume.

Protein hydrolysates made with sulphuric acid as the hydrolyzing agent have not been generally acceptable for food purposes, principally because of inferior or undesirable taste factors and their comparatively high cost of production.

Much has been written about meat flavor, but the subject remains far from clear. It is evident that meat flavor is due to something more than monosodium glutamate and that part of the flavor of meat is apparently due to nitrogen bases and slightly volatile organic acids. Recently, we have found that cooked fresh meat as well as cured meat contains small amounts of amino nitrogen. This indicates that meat flavor, to some extent, is tied up with amino acids. It is well known that monosodium glutamate contributes much to developing a good chicken flavor in soups and makes vegetarian food more appetizing. We also know that monosodium glutamate contributes not only to chicken flavor, but also to meat flavors in general.

Meat flavors have been discussed and advertised as amino acid flavors. This may not be entirely true, but certainly the amino acids which are present in meat protein definitely contribute to the flavor we all like in this important food. We have never had the opinion that monosodium glutamate, by itself, was entirely satisfactory for flavoring

food products. Our experiments have indicated that hydrolysates which have a combination of all the amino acids seem to give a preferred quality rating, a more satisfying flavor and a more acceptable taste than when only one of the amino acids is present. The only possible exception to this opinion is with respect to the use of monosodium glutamate in chicken soups.

We believe the "non-essential" amino acids in most instances contribute better flavor than the "essential" amino acids. Certainly, we are agreed, from experimental evidence, that several of the amino acids and their derivatives, exclusive of monosodium glutamate, have flavor significance. For example, glycine, alanine, proline, leucine, serine, phenylalanine and aspartic acid have varying sweet tastes and contribute flavor. Methionine and cysteine impart definite desirable tastes and when heated provide strong pyrogenic flavors. Valine and tyrosine are slightly bitter. These observations refer to the dextrorotatory form of the amino acids. Since the four components of taste are described as sweet, sour, salty and bitter, it is clearly obvious that protein hydrolysate containing all or most of the amino acids has the constituents present to supply full and complete taste characteristics.

In this respect, we can liken protein hydrolysate flavors to a perfume which has been developed into a pleasing, attractive and thought-provoking bouquet by the blending of various aromatic essential oils, any one of which would not be attractive to "milady," but which when blended together, would give the appealing aroma which is so alluring. The same comparison can be made of the flavors derived from the presence and the blending of amino acid derivatives in a protein hydrolysate.

Investigations relative to the improvement of protein hydrolysates have developed definite fundamental information. The importance of the protein hydrolyzed cannot be emphasized too strongly. It has been proved that the protein should be high in total nitrogen, adequate in respect to all the amino acids, and should be specifically selected on the basis of the glutamic acid content so as to give the maximum of flavor. Commercially, these proteins are wheat gluten, corn gluten, extracted soya bean flour, casein, peanut flour, yeast, dried distiller's solubles, extracted cottonseed meal and fish waste. It is to be understood that there are also other proteins, such as egg albumin, that can be and which are sometimes used.

Frequently, a combination of proteins is used and in certain instances excellent tasting hydrolysates with superior qualities are produced, such as when wheat gluten, casein and an extracted soya flour, in proper proportions are hydrolyzed with hydrochloric acid. We know of one combination of these three proteins which produces an un-

usually good, dry hydrolysate containing from 16 to 18 percent monosodium glutamate. This product has met with a ready reception from the food industry. Another combination of proteins is wheat gluten and casein which, in the correct proportions, give a hydrolysate with a high monosodium glutamate content and excellent flavor.

The protein used should contain only very small amounts, if any, of carbohydrates and fatty materials, since these constituents are frequently the source of unpalatable flavors. If the protein has appreciable quantities of carbohydrates and fatty substances, these materials should be substantially eliminated before hydrolysis.

Thus, the studies which have been applied to the utilization of protein-containing matter for the manufacturing of protein hydrolysates and amino acid flavors have shown that the type of protein and its ratio of amino acid content are fundamental concepts.

Table I gives the approximate glutamic acid content of several natural protein materials.

Table I

	Approximate Glutamic Acid Content of Proteins in percent
Wheat Gluten	36.0
Corn Gluten	24.5
Zein	36.0
Peanut Flour	19.5
Cottonseed Flour	17.6
Soya Bean Flour	21.0
Casein	22.0
Rice	24.1
Egg Albumin	16.0
Yeast	18.5

From this table it can be seen that one of the most acceptable protein materials is wheat gluten. Wheat gluten contains from 80 to 90 percent protein, is fairly adequate with respect to its amino acid content and is high in glutamic acid. Casein is also an excellent protein with good glutamic acid content. Soya bean flour and yeast are good proteins. Extracted edible cracklings are a fairly good protein, but not very high in glutamic acid content. The cost of the protein used

for hydrolysis is also important, and the proteins named are reasonably priced. The Chinese and Japanese have used many kinds of fish and fish waste, but very little of this material has been utilized in this country, for several reasons, one of which is the difficulty, even by hydrolysis, to eliminate the undesirable fish odor which seems to carry over into the finished hydrolysate.

Raw protein material containing large amounts of carbohydrates often produces bitter tasting flavors. Fatty materials present during the hydrolytic process have an effect upon the taste and keeping qualities of the hydrolysate because of oxidation and rancidity development. Since hydrocholoric acid is usually the hydrolyzing agent for food hydrolysates, the sodium chloride content of the final product is appreciable as a result of neutralizing the excess acid with soda. Therefore, only enough acid to give a slight excess for hydrolysis is needed. The salt content in a good hydrolysate powder should be 35 to 40 percent, and in a good liquid hydrolysate 12 to 18 percent. However, a majority of the products now on the market in the dry form contain 45 to 55 percent salt and in the liquid form 20 to 25 percent. A study of Tables II and III will reveal the requirements of a good protein hydrolysate and the points at which some of those produced commercially are deficient.

Commercial production of satisfactory hydrolysates is also dependent upon other factors, such as the elimination of toxic metals and metallic contamination. This requires, among other things, first class corrosion-resistant equipment. Recent trace metal research has definitely indicated that trace amounts of metals can promote flavor abberation, deterioration and destruction of these products.

With respect to the drying of liquid hydrolysates, many variables—such as temperature, time, type of drying and cooling—must be considered. Temperature and time particularly are important to consider during the hydrolysis period, for too long a hydrolysis time and too high a temperature often result in objectionable unpalatable products. Likewise, fermentation, decolorization and neutralization of excess acid after hydrolysis are all important procedures to consider in the production of hydrolysates of acceptable quality. Probably one of the most difficult production factors is to produce a protein hydrolysate powder with only slight hygroscopic properties. However, there are several hydrolysates now being manufactured which are commercially available—excellent in flavor, with high monosodium glutamate content and which are only slightly hygroscopic under normal atmospheric conditions. The scientific use of proper proteins aids materially in lessening the hygroscopicity of the completed hydrolysate powder.

Table II

Typical Analysis of a Good Protein Hydrolysate

Moisture	2.2%
Sodium Chloride	39.8%
Total Nitrogen	6.8%
Amino Nitrogen	4.7%
pH (10% solution)	5.2

Calculated Amino Acid Composition

Amino Acid	*As Is Basis Percentage*
Arginine	2.0
Histidine	1.0
Lysine	1.4
Tyrosine	1.7
Tryptophane	Trace
Phenylalanine	2.4
Cystine	0.5
Methionine	1.2
Threonine	1.3
Leucine	2.9
Isoleucine	1.7
Valine	1.8
Aspartic Acid	4.1
Glutamic Acid	12.0
Equivalent to Monosodium Glutamate	16.03

Control points to consider in selecting a good, commercially dry, protein hydrolysate are as follows:

1. Moisture should not exceed 5%
2. pH should be in the range of 4.8 to 5.4
3. Sugars should be absent
4. Taste should be neither scorched nor bitter
5. The product should be easily soluble in water
6. The salt content should not be more than 40%
7. The amino nitrogen should not be less than 4%
8. Monosodium glutamate content should not be less than 12%
9. The color should vary from light tan to caramel providing the other properties are satisfactory

Table III
Analyses of Commercial Protein Hydrolysate Powders

Sample #	Sodium Chloride %	Amino Nitrogen %	Moisture %	MSG %
1	50.25	5.65	6.25	
2	47.04	3.60	16.88	
3	58.45	3.16	2.77	2.41
4	56.19	2.50	12.88	
5	47.79	4.88	4.77	11.40
6	76.57	1.68	3.62	
7	59.66	2.25	4.53	
8	65.76	1.97	5.11	
9	39.83	4.71	2.15	16.50
10	34.05	5.50	4.00	18.20

In comparing tastes with amino nitrogen content, the higher the amino nitrogen, the better the taste.

In evaluating protein hydrolysate for foods, there should be more specific standards than there have been in the past. This would promote and eventually bring about the manufacture of the best possible products of constant and comparable quality with an acceptable minimum monosodium glutamate content.

Numerous formulations of food products using a high quality protein hydrolysate in comparison with monosodium glutamate have been made. Frequently, dependent upon the type of the food product, its taste and appearance, tests have indicated the use of a protein hydrolysate. Products which have been investigated, among others, include bouillon, soya sauces, dehydrated soup mixes, gravy powder, sausage meat, processed meat, stews, corned beef hash, bakery products and many other foods as shown in Table IV.

Table IV
Uses for Protein Hydrolysates in Food Products

Soups	Headcheese	Bread
Stews	Mincemeat	Macaroni with meat sauce
Broths	Sausage meat	Poultry stuffing and basting
Bouillons	Curing compounds	Chow mein
Bouillon cubes	Goulash	Processed meat
Fish	Biscuits & crackers	Hash
Gravies	Fruit cake	Meat sauces
Scrapple	Spice mixtures	Hors d'oeuvre pastes
Sandwich spreads	Chop suey sauce	Cheese spreads
Pickle relishes	Cheese rarebits	Mayonnaise
Baked beans	Chili sauce	Dog foods
Pancake flour	Salad dressings	

It is admitted that in chicken soups, both liquid and dehydrated and perhaps in other light colored soups, monosodium glutamate is the flavoring of choice not only because of taste factors, but because the Maillard reaction proceeds much more slowly, if at all. However, in chicken soup mixes, both liquid and dry, excellent results and stable color reactions have been observed when a 50 percent mixture of glutamate and a protein hydrolysate powder have been used, especially when sucrose replaces dextrose in the formulation—using only 60 percent as much sucrose as dextrose. The protein hydrolysate used in these experiments contained approximately 16 percent monosodium glutamate and was processed from a combination of wheat gluten and extracted soya flour. With hydrolysate products containing small amounts of monosodium glutamate, such as those produced from materials from which the glutamic acid had been commercially extracted, the results were exceedingly unsatisfactory with reference to taste, color and flavor acceptability.

In this discussion it is important to touch briefly on the economics of protein hydrolysates compared with monosodium glutamate. Tests have indicated that a comparison of monosodium glutamate and a good protein hydrolysate gives a respective flavor value in the ratio of approximately 1.25 to 2. On the basis of this ratio at 60c per pound for protein hydrolysate as against $1.50 for monosodium glutamate, the costs are respectively $1.20 and $1.87½, representing a saving of 67½c per pound when protein hydrolysate is the flavor. If these ingredients are used in sucrose-containing dehydrated chicken soup formulae on a 50-50 basis in the amount of 5%, the cost would be $5.25 per hundred pounds as against $7.50 per hundred pounds if monosodium glutamate only were used. This saving would be a very substantial one in the manufacturing cost of any product.

Further, on the basis of 60% flavoring amino acid salts in protein hydrolysates, the cost of flavor is $1.00 per pound of flavor compared with $1.50 per pound of flavor when monosodium glutamate is used. As has been mentioned, some hydrolysates are produced as a by-product from monosodium glutamate manufacture and in the dry form they contain from 3 to 5 percent of monosodium glutamate. These products are sold at prices within the range of 20 to 30 cents per pound. If they contain the apparent optimum of 5% monosodium glutamate and sell at the lower figure of 20c per pound on the monosodium glutamate content alone, their cost is 64c per pound as compared with a 60c per pound cost for the unextracted hydrolysate containing an average of 16% monosodium glutamate. Usually the unextracted hydrolysate has a better color, more delicious taste, and less hygroscopicity than the extracted hydrolysate.

Proper discrimination in selecting protein hydrolysates, or, careful compounding of hydrolysates and monosodium glutamate not only gives superior taste acceptability and flavor to your finished product but contributes desirably to a more profitable cost structure. If you have not done so, for your own satisfaction, conduct flavor tests with a good protein hydrolysate, monosodium glutamate and combinations of them in various food products; undoubtedly you will be surprised at your findings. For, unquestionably, you will find it is possible to produce products with superior taste and flavor characteristics and that you will also be able to extend and expand your use of protein hydrolysates.

From this discussion we hope sufficient confirmatory evidence has been presented to permit your agreement that protein hydrolysates scientifically made from adequate glutamic acid containing proteins are a splendid and desirable source for monosodium glutamate in the flavoring of food products.

References: (15) (16) (26) (37) (54) (57) (72) (77)

Discussion

Dr. Melnick: What procedure do you use for removing carbohydrate prior to hydrolysis?

Dr. Hall: Most of the proteins we get have very little carbohydrate present, but it is removed by treating with .5% solution of hydrochloric acid.

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Monosodium Glutamate Studies. I.

Factors Affecting Detection of and Preference for Added Glutamate in Foods

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(Manuscript received February 19, 1960)

This study of monosodium glutamate as a food flavor adjunct and as a possible flavor stabilizing agent in stored products was an attempt to resolve some of the conflicting reports and views in popular and technical literature on glutamate properties. Mosel and Kantrowitz *(10)* after discussing reasons for some of the confusion regarding taste properties of glutamate, stated that "the only thing that seems certain from existing data is that MSG can change the flavor of food, usually in the direction of increased palatability." It is generally agreed that glutamate has a "sweet salty" taste, but beyond that there is considerable uncertainty. Differences in objectives of the various studies as well as differences in methods contribute to the uncertainty. Some published reports are based on casual observation and individual opinion; others are limited to tests of threshold values or detection of differences at subthreshold concentration in water.

Interest in glutamate has a distinctly practical basis because of obvious economic implications. Potential users need assurance that by addition of glutamate they are adding something to their products that could not be accomplished more cheaply by the addition of salt. It is also important to know to what extent salt, fat, seasoning, and thickening levels of a product influence effectiveness of added glutamate, whether recommended levels must be adjusted for formula variations, and the reason for different recommended levels in various products. A level of 0.15% is recommended for most products, but levels as low as 0.10 and as high as 0.25% are recommended for a few *(1, 2)*. If glutamate has the antioxidant properties reported *(13)*, comparison with other antioxidants would be in order. Because of its wide usage in canned products, assurance that it is not lost or altered in commercial heat processing would be appreciated.

The interrelation between NaCl and glutamate has aroused the interest of a number of investigators; a few have studied the effect of fat, thickenings, and seasonings on detection of glutamate. Sjöström *et al* *(15)* reported that while glutamate has some salt value, it cannot fully replace salt in foods. They and others *(3, 6)* reported that the effect of glutamate on saltiness and other flavors varied with the concentration of added glutamate, the level of salt, and the character of the original product. Lockhart and Gainer *(9)* found no increase in the saltiness of NaCl solutions by the addition of subthreshold glutamate levels. Crocker *(4)* in tests with over a dozen amino acids, reported that saltiness was evident only in the sodium salt of L (+) glutamic acid. Fellers *(5)* stated that excessive salt covers up the glutamate flavor. Galvin *(7)* expressed the view, after studies on simple systems using a distilled water base, that particular attention should be paid to salt, pH, and sweetness to obtain maximum benefit from glutamate. He also stated that salt must be present to produce a useful glutamate taste. Mosel and Kantrowitz *(10)* found that exposure to a 5-threshold solution of glutamate did not affect taste acuity to NaCl.

Nair *(11)* reported that in the presence of thickening agents such as gums, flour, and starches, higher glutamate levels are required than in unthickened products. He stated that thickening agents act as diluents of flavor and may selectively mask certain flavors more than others. Cairncross *(3)* reported that 1% solutions of carboxymethyl cellulose suppressed the effect of low concentrations of glutamate. He also reported that addition of fat to well-seasoned nonmeat gravies thickened with cereal thickening agents suppressed flavor, and that glutamate has more influence in fat-free foods than it has when fat is present. Fellers *(5)* reported that in fish pastes and sandwich spreads as much as 1% glutamate can be used, a considerably higher level than he suggested for other fish products. The composition of the pastes and spreads was not indicated, and no reason for the higher recommended level was suggested.

The studies reported in this paper were designed to assess the relative influence of these and other factors on the detection of glutamate. They have confirmed that a number of factors have some influence on effectiveness of glutamate addition, and have demonstrated that the inherent level of glutamate in the product has a major influence on the effectiveness of added glutamate. However, in the final analysis the usefulness of adding glutamate will depend on whether it has a favorable effect on consumer preference for the food under consideration, and particularly whether supple-

* Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

ments of glutamate have a more favorable effect on consumer preference for foods than can be obtained by supplements of less expensive NaCl. Therefore, limited consumer-type tests have been conducted, the results of which should indicate the usefulness of more extensive tests under particular conditions.

METHODS

Sensory tests. Most of the studies reported here are based on detection of added glutamate, conducted with a panel of judges selected for acuity in detecting added glutamate in side-by-side comparison of products, with and without added glutamate. Relative ease of detection is considered reflected by frequency of detection. Approximately 40 members of the laboratory staff were tested for their ability to detect glutamate in various products, and the best of these (about one-third) were used, 6 to 10 per panel.

The duo-trio test was used for selection of the judges and for the experiments. In this test sample X containing no glutamate, was presented, labeled, as the control. Samples X and Y were presented, coded, in either of the possible orders: XY, YX. Each judge received the samples an equal number of times in both orders and was asked to identify the sample that was the same as the control. Since identification of X constituted detection of the added glutamate in Y, the results are reported in the table as detection of added glutamate. Ordinarily, two pairs of samples are presented in this test with the control. Based on more efficient use of the judges' time, presentation of 3 pairs at each testing period seemed desirable if it could be done without affecting the results. However, there were reports in the literature on a "persistence" of the glutamate effect that might interfere with detection of added glutamate in the second and third pairs more than in the first pair, and it was felt that judges' fatigue might operate in the same way. Therefore, the tests were conducted so that these factors could be analyzed and, if necessary, only results of the first pair would be used. The compilation of correct judgments in Table 1 showed that the judges were not affected by either a "persistence" effect of glutamate or fatigue, and that results of all pairs could be used.

TABLE 1

Equivalence of judgments for pairs of samples submitted consecutively

Product	Total judgments each pair	Correct judgments		
		First pair	Second pair	Third pair
Chicken broths, turkey a la king, sausage, beans, peas	268	198	213	214
Goose liver paste	45	30	25	
Dehydrofrozen peas	17	11	11	9
Crab and cheese sauce	72	45	40	44
Pork sausage	79	54	48	
Cream of chicken soup	80	66	64	
Chicken liver paste	81	38	42	
Total		442	443	

Because of the relatively small number of reasonably "average consumers" available at the laboratory, the consumer test was limited. The consumers were members of the staff who were not on the selected glutamate panel and had no particular knowledge of the experiment. Fifty to 100 individuals were asked to indicate which of two samples they preferred on each comparison. The order of presentation was randomized.

Although consumer tests are usually based on a preference decision between samples compared directly at the same time, such a test does not show the preference under less ideal conditions. Under practical conditions of consumption, for example, foods with and without glutamate would not be compared at the same time. They would be served in larger portions and with other foods. These interesting questions could not be adequately explored. However, an experiment comparing methods of sample presentation was conducted, with the panel selected for ability to detect glutamate at low levels, to evaluate at least partially the significance of the memory factor in flavor judgments. In addition to presenting the samples side-by-side in the duo-trio test, single samples of broth and a la king were presented alternately for detection of added glutamate. Four test samples were presented at each sitting, two of broth and two of a la king. They were presented either in a duo-trio test (side-by-side comparison of each product with and without glutamate) or in a single sample test (alternately broth and a la king). The duo-trio test was conducted as previously described, with the control sample presented for direct comparison with pairs of samples, one of which contained glutamate and the other no glutamate. For the single sample test the judges were first given a control sample of broth or a control sample of a la king. They were asked to taste it and remember its flavor for comparison with subsequent samples. The sample was removed and a control of the other product, either a la king or broth, was given with the same instructions. This control was removed. Then test samples of broth and a la king were presented singly, alternating from one product to the other; judges were asked whether they were like or unlike the controls. The days on which this test was conducted were randomly interspersed among the days of the duo-trio test. Since the preliminary tests showed it was more difficult for the judges to determine differences with the single sample test than with side-by-side comparisons, higher levels of glutamate were used in the former.

Sample preparation. Preparation of products and formulas used in Sections A and C under "Results" are as follows:

Chicken broth. A uniform lot of hens (200) was simmered with 1 part of water per 1.8 parts of chicken in covered stainless steel kettles for 3 hours. The broth obtained was separated from the fat, canned without further heat processing, frozen, and stored at −20° F. until needed. Normal salt seasonings of either 0.50 or 0.75% were used in comparisons reported in Table 2, as these levels were considered neither too bland nor too salty by the panel. The following ingredients were added to the chicken broth for items 1-9 reported in Table 3:

1. None.
2. 0.5% carboxymethylcellulose.
3. 2.9% all purpose wheat flour.
4. No. 3 + 3.8% bland vegetable fat.
5. No. 3 + 3.8% chicken fat.
6. No. 4 + 0.04% pepper.
7. No. 6 + 0.02% sage, 0.04% onion salt.
8. No. 4 + 0.04% pepper, 0.02% sage, 2.2% diced onion, 9% diced celery, 14% rice.
9. No. 8, blended.

Glutamate was added by diluting with water to a 2% solution, and pipetting the necessary amounts into the soup and a la king. Water containing no glutamate was added to the control sample to give equal dilution.

Turkey a la king. Turkeys weighing approximately 20 lb. were simmered in water and deboned. A white sauce was prepared containing turkey stock, turkey fat, milk, flour, and salt. The turkey a la king as prepared contained the following ingredients:

Ingredient	Per cent
Turkey, deboned, diced	24.10
Turkey stock	33.10
Turkey fat (separated from the stock)	5.15
Flour, all purpose wheat	1.85
Waxy rice flour (to prevent separation during freezing)	2.62
Milk	19.16
Salt	0.76
Egg yolks, hard cooked, chopped	5.90
Onion, chopped, sauteed	0.53
Green pepper, chopped, sauteed	0.99
Canned mushrooms	3.78
Pimentos, canned, diced	2.06

Pork sausage. Pork trimmings obtained from a large commercial meat packing plant were ground twice (coarse and medium) through the meat grinder attachment of a No. A-200 Hobart mixer, and mixed for approximately 3 min on speed 1 with the dough hook. The seasoning mixture containing the following ingredients, expressed as per cent of sausage weight, was added before mixing and grinding:

Salt	1.0%
Pepper	0.2%
Sage	0.26%

This lot of sausage contained 60% fat. Salt and glutamate supplements were added with uniform mixing before cooking. Sausage was brought to the desired glutamate concentration by adding glutamate solution, which raised the moisture content 1%.

Frozen vegetables were cooked following package directions with 1.5% NaCl by weight of the vegetables added to the cooking water. Vegetables were drained, mixed, and separated for butter (3%) and glutamate addition. A 2% glutamate solution was added to one part of the melted butter. An equal amount of water was added to the butter to be mixed with the control. Total water added was about 1%. These butter-water-glutamate mixtures were added to the vegetables and thoroughly mixed.

One volume of dehydrofrozen peas was added to 3 volumes of cold water containing 1.63% NaCl and amounts of glutamate determined by preliminary analyses to be needed to assure the desired glutamate level in the peas served. They were brought to a boil and cooked for 8 minutes.

Crab was prepared in two forms: crab in cheese sauce and crab cakes. For the first product two parts of crab meat were added to 3 parts of medium thick white sauce containing 21% grated sharp cheddar cheese. The crab cakes contained crab meat, bread crumbs, whole eggs, chopped sautéed onion, oleo, mustard, Worcestershire sauce, and salt. The cakes were shaped, rolled in flour, deep fat fried until brown, frozen, and reheated as needed for the panel.

Goose liver paste was prepared by combining the following ingredients:

Ingredient	Per cent
Livers, steamed, ground	59.0
Onions, chopped, sautéed, ground	9.75
Oleomargarine	29.75
Salt	1.04
Thyme	0.086
Black pepper	0.086
Paprika	0.086
Nutmeg	0.172

The a la king, sausage, crab products, and liver paste were sealed in cans and stored at −20° F until needed.

For tests of NaCl addition reported in Table 4, chicken broth and turkey a la king were the same as in Table 3 with the indicated variations in levels of NaCl.

Cream of chicken soup was prepared by combining and bringing slowly to a boil the following ingredients:

Ingredient	Per cent
Chicken broth	88.90
Dry skim milk solids	7.20
Waxy rice flour	2.90
Salt	0.80
Onion powder	0.082
Black pepper	0.025
Sage	0.020

Levels of NaCl were varied as indicated in Table 4.

Chicken liver paste was prepared as the goose liver paste previously described, with indicated NaCl levels (Table 4). For the test of added seasonings, two levels were added to cream of chicken broth, the level above and half that level. Three levels were added to pork sausage, the level used previously, a lower, and a higher level (7 times the lower level). Two levels were added to the turkey-chicken liver paste. For the test of added thickenings, the cream of chicken soup formula above was used with the two levels of thickening indicated in Table 4. For the test of level of fat, chicken liver paste was prepared with and without added bland hydrogenated vegetable fat. Fat content of the former was estimated to be approximately 30% and of the latter, approximately 4%.

Blue lake *green beans*, size 4, were obtained with ends removed, from the line of a large commercial canning plant. The beans were washed, cut into approximately one inch pieces, steam blanched at 180° F for 1½ min, and cooled in a cold water spray. They were weighed into enameled cans, 303 x 404, and divided into groups for the addition of brines containing glutamate or varying amounts of NaCl, depending upon the level desired for consumer evaluation. On the assumption that the salt and glutamate added in the brine would become evenly distributed throughout the contents during canning, these ingredients were added in quantities required to give NaCl concentrations ranging from 0.8 to 1.0% and a glutamate concentration of 0.15% in the drained beans after processing. Warburg analyses showed a glutamate content of 0.11 to 0.15% in the beans.

The base levels of NaCl used in the consumer test products were within the 0.5 to 1.0% levels used in the previous tests. Levels of glutamate that have been added to poultry products range from 0.09 to 0.75%. Levels recommended by the Glutamate Manufacturers' Technical Committee in their pamphlet, *The Value of Glutamate in Processed Meat and Poultry*, are 0.12 or 0.15% for the following products: whole frozen boned turkey, frozen chicken and turkey pies, frozen chicken a la king, canned whole chicken, canned boned chicken or turkey, canned chicken fricasee, canned chicken or turkey a la king, and fresh poultry *(1)*. The level of glutamate selected for these consumer tests was the higher of the recommended levels, 0.15%, and levels above 0.15% because of their interest if the 0.15% level showed no beneficial effect. One of each pair of samples was supplemented with glutamate, the other with 0 to 0.3% NaCl.

Determination of glutamate concentration. Glutamate concentration was determined by the enzymatic decarboxylation method of Rogers *et al (14)*. The enzyme used was prepared from *E. coli*, ATC 11246, according to Najjar *(12)*, modified to remove the glutamine, lysine, arginine, and histidine decarboxylase activities by incubating a 2% suspension of the original acetone powder in 0.3N acetate buffer (pH 5.0) at 35° C for 64 hr. Negative results on tests of the modified enzyme on these acids confirmed removal of decarboxylase activities. This suspension was frozen in 2 ml aliquots for use, since further handling greatly lowered the remaining glutamic acid decarboxylase activity.

Fifty grams of the product to be analyzed were homogenized in a blender with sufficient water (2 to 4 volumes) to permit accurate measurement. Analyses were conducted on 1.8 ml of this mixture, 0.5 ml of 0.1N acetate buffer, pH 5.1, and enzyme sufficient to complete the reaction in 30 to 45 min. The free glutamate concentration was expressed in micromoles of glutamate per gram of original sample.

RESULTS

A. Ease of detecting glutamate in foods of varying character, composition, and concentration of ingredients. Glutamate is normally used as a supplement to foods containing NaCl. Therefore, a test was designed to determine whether a supplement of monosodium glutamate was more readily detectable than a supplement of sodium chloride when these supplements were added to chicken broth containing normal amounts of NaCl. Judges compared chicken broths containing either 0.50 or 0.75% NaCl with these broths containing either added glutamate or added salt in amounts shown in Table 2. The amounts of added glutamate and added salt are nearly equimolar, and therefore contain nearly equivalent amounts of sodium ion, equalizing any taste effect of the sodium

part of added glutamate or salt. The difference between the last two columns shows that added glutamate ion was more readily detected than added chloride ion in broth containing either 0.50 or 0.75% NaCl.

Results of this test not only demonstrated the characteristic flavor of the glutamate ion compared with the chloride ion but also supported the usefulness of further tests to determine how the flavor contribution of glutamate is influenced by salt level in the product, and whether this characteristic flavor, readily detected by a selected panel, influences consumer preference for samples containing both added glutamate and NaCl.

TABLE 2

Detection of added monosodium glutamate and chloride in nearly equimolar amounts, and hence nearly equal sodium concentrations, in chicken broth[1]

Amount of salt in stock broth	Glutamate addition		Salt addition		Detection of added glutamate[2]	Detection of added salt[2]
%	%	*mM/l*	%	*mM/l*	%	%
0.50	0.05	2.7	0.016	3.0	90	73
	0.10	5.4	0.032	6.0	100	71
	0.05	2.7	0.016	3.0	86	65
0.75	0.10	5.4	0.032	6.0	94	69
	0.25	13.5	0.080	15.0	96	88

[1] The orders of presenting products for both base levels of sodium chloride and for all sodium ion supplements were randomized together.

[2] The total number of judgments for each sample was 48 (duo-trio test comparing stock broth with broth containing additive). Per cent detections reported were all statistically significant at the 5% level or higher.

Glutamate was added to foods of varying character and composition to obtain information on factors that influence its detection. The levels of glutamate added were intended to be sufficiently above threshold levels for the panel members so that the relative ease of detecting glutamate in the foods would be apparent. The percentages of correct judgments differed considerably for equal additions of glutamate (Table 3, products 1–13), and larger amounts of glutamate were required for approximately equal detection in some foods than in others (products 14–17).

Glutamate was detected most readily in chicken broth, the simplest product tested. It was detected in 95% of the possible judgments by 3 panel members at a concentration of 0.015% and by the remainder at a concentration of 0.04%. Thickening, fat, and seasonings in the amounts added decreased the ease of detection to a small extent. Glutamate was readily detected even in pork sausage in spite of its higher flavor and seasoning content. But in frozen peas, glutamate was not detected at levels corresponding to those used in the various soups, turkey a la king, pork sausage, and frozen beans. The panel detected glutamate in the crab products less frequently than in all the previous products except peas; even higher levels of glutamate did not give equivalent detection. Glutamate was least frequently detected in the goose liver paste; 0.20% added glutamate was required for significant detection. The makeup of the panels was not always identical from one product to another because

TABLE 3

Relative ease of detecting glutamate in various food products

Product	Total judgments	Added glutamate	Detection of added glutamate
		%	%
1. Chicken broth	84	0.015	95***
2. Broth + carboxymethylcellulose	84	or	89***
3. Broth + flour	84	0.04[2]	81***
4. Broth, flour, blend vegetable fat	48		77***
5. Broth, flour, chicken fat	84		77***
6. No. 5 + pepper	48		77***
7. No. 6 + sage, onion salt	84		80***
8. No. 6 + sage, dried celery, onion, rice	48		77***
9. No. 8, blended	48		71**
10. Turkey a la king	48		73**
11. Pork sausage	48		73**
12. Frozen green beans, butter	48		65*
13. Frozen green peas, butter	48		48
14. Dehydrofrozen peas	51	0.04	61
15. Crab and cheese sauce	48	0.04	57
	87	0.05	60*
	81	0.075	61*
16. Crab cakes	20	0.075	65
	32	0.15	75**
17. Goose liver paste	32	0.10	46
	32	0.15	57
	32	0.20	71*

[1] The order of presenting products as randomized for products 1–13. After these products were tested by the original panel, additional panelists were selected, and they together with members of the original panel re-evaluated five products: 1, 2, 3, 5, and 7. Since the results were similar to those of the first series, the combined results for the two tests are shown in the table. The order of presenting products containing different glutamate levels was randomized for products 15–17.

[2] Glutamate levels corresponding to the different abilities of panel members to detect glutamate (determined in preliminary tests) were used in products 1 to 13. The duo-trio test was used; pairs of samples, with and without glutamate, were compared directly with a sample containing no glutamate. Higher levels of glutamate used in products 14 to 17 were indicated from preliminary tests showing inability of panel to detect glutamate at lower levels.

[3] Three asterisks, two, and one indicate respectively 0.1, 1.0, and 5.0% levels of probability.

of absences, allergies, or dislike of some foods. Also, the total number of judgments differed. In spite of some variations, there seems little reason to doubt the generally less frequent (and therefore more difficult) detection in the crab products, peas, and goose liver paste.

The great difference in the ease with which glutamate was detected in products reported in Table 3 indicated the need for additional tests of the specific effects on glutamate detection of added NaCl, seasonings, fat, and thickenings in foods at levels at which these variables might reasonably be used. Chicken broth, turkey a la king, cream of chicken soup, and pork sausage were selected for tests of the effect of NaCl level on glutamate detection because NaCl could easily be added to them at required levels, they represent considerably different levels of over-all flavor, and they are of commercial interest. For similar reasons cream of chicken soup, pork sausage, and liver paste were selected for tests of the effect of seasoning level; cream of chicken soup for tests of thickening level; and chicken liver paste for tests of fat level. The thickening and fat levels were chosen to represent relatively high and low levels that might be used in preparing these foods.

In chicken broth and in turkey a la king containing two levels of NaCl, two to three times the amounts of glutamate needed for detection at the lower NaCl level were needed for approximately equivalent de-

tection at the higher NaCl level. However, the one level of glutamate tested in cream of chicken soup was detected equally well at two salt levels, and a relatively high level of glutamate was not detected at either of two salt levels in liver paste (Table 4).

Reducing the seasoning level in the cream of chicken soup and liver paste did not increase the ease of detection of added glutamate in these products. A 7-fold difference in seasoning level did not affect detection of glutamate in pork sausage. Increasing the thickening agent made glutamate detection easier in cream of chicken soup. A 7-fold difference in fat level did not affect glutamate detection in chicken liver paste.

The results obtained with variation in level of the thickening agent illustrate the complicated interrelationships of the factors comprising flavors. Contrary to expectations, glutamate was more difficult to detect in the thin soup than in the very thick soup. Comments of the judges indicated that other seasonings were much more noticeable in the thin soup, possibly objectionably so.

An additional test was made to verify this impression. Three soups were presented: a thick soup and a thin soup with equal seasonings (the level of the first test), and a thin soup containing half the above level of seasoning. The judges were asked to rank the 3 samples in order of intensity of seasoning. The 9 judges correctly ranked the two thin soups containing *different* seasoning levels. Eight of these 9 judges, however, ranked the thin soup higher in intensity of seasoning than the thick soup of *identical* seasoning level, indicating that the thickening did mask some of the seasoning effect. Thus, varying the thickening agent may have had its effect on glutamate detection in an indirect manner by changing the *apparent* level of seasonings that were actually at the same level. Results on pork sausage, in which a 7-fold increase in seasoning level did not interfere with glutamate detection, seem contradictory to the above theory. However, in the sausage the panel did not find even the highest seasoning level inappropriate (or objectionable) and much higher levels would probably be needed for interference with glutamate detection.

B. Relation between inherent glutamate levels in foods and detection of added glutamate. A comparison of the ease with which added glutamate is detected in foods (Tables 3 and 4) and the natural glutamate content of these foods (Table 5) indicates a striking relationship. Foods in which glutamate is readily detected, such as chicken broth, cream of chicken soup, and pork sausage, have a relatively low natural glutamate content (0.01–0.02). Those in which it is not readily detected, such as poultry liver paste and frozen peas, have a relatively high glutamate content (0.19–0.36). Natural glutamate content, therefore, appears to be the most reasonable basis yet considered for recommending levels for glutamate addition.

Lower glutamate content probably accounted for most of the improvement in detection of glutamate in the particular sample of dehydrofrozen peas used (0.05% glutamate) compared to the frozen peas used (0.34% glutamate). However, added glutamate was more difficult to detect in the dehydrofrozen peas, beans, and crab products than might be expected from knowledge of their inherent glutamate content. Lack of homogeneity and difficulty in incorporating glutamate uniformly in the discrete particles do not appear to explain the discrepancy completely, as indicated by the following: (1) added glutamate was detected as readily in broth with added diced celery and rice (Item 8, Table 3) as in the blended product (Item 9), (2) in dehydrofrozen peas containing approximately 0.19% glutamate, glutamate additions of 0.10% and 0.15% were not detected to any extent greater than that expected by chance whether the peas were served whole or as a blended puree.

The natural content of free L-glutamic acid in various foods, its variation with stage of maturity of corn, and its loss on refrigerator storage of peas were determined by Hae, Long, and Blish *(8)*. This information has only been used as the basis for the recommendation that glutamate be added to restore "flavor losses during storage prior to processing" *(1)*. However, the present study indicates that information on L-glutamic acid content of foods could be useful as a means of indicating the probable level of glutamate addition required for effectiveness. A comparison of levels of glutamate recommended by the Glutamate Manufacturers' Technical Committee *(1, 2)* and levels found in foods shows that relatively high levels (0.25 and 0.18%) are recommended not only for peas and poultry liver paste (Table 5), which have a high natural glutamate content, but also for spinach containing 0.02%; asparagus, 0.04–0.06%; and corn, 0.01–0.10% glutamate. The usually recommended level of 0.15% is recommended for products whose natural glutamate content ranges from 0.01 to 0.18%. Evidently, natural glutamate has not been the basis for recommending levels of glutamate addition. However, the fact that a wide variety of foods do have a low natural glutamate content has apparently made the same level of supplementation reasonable for many foods. Currently recommended levels of addition might well be re-examined in the light of the relationship between natural glutamate content and detection of added amounts.

C. The effect of salt on consumer preference for foods with added glutamate. The consumer preference tests comparing paired samples, all containing a base level of NaCl, one sample of each pair supplemented with glutamate (0.15 to 0.40%), the other supplemented with NaCl (0 to 0.3%), showed preferences for the glutamate-supplemented samples ranging from no preference (50%) to 72% (Table 6). In no case was a NaCl supplement preferred to a glutamate supplement. The extent of preference for the glutamate-containing sample was apparently influenced both by the product and the NaCl level. Many persons testing beans considered the samples with the higher NaCl levels too salty, while those comparing broths considered the lower NaCl levels too bland. The writers consider that the extent of preference for the glutamate-containing sample probably decreases

TABLE 4

Influence of NaCl level, thickening agent, fat content, and seasoning level on detectability of glutamate in food products

Product	Total judgments on each comparison	Added glutamate	Other additives	Per cent detection of added glutamate with each level (%) of other additives[1]		
		%				
			NaCl	0.50		0.75
Chicken broth	48	0.05		90		85
		0.10		100		94
		0.25		....		96
			NaCl	0.75		1.00
Turkey a la king	48	0.05		75		....
		0.10		77		71
		0.15		92		75
		0.25		....		75
			NaCl	0.40		0.80
Cream of chicken soup	56, 58	0.04		85		82
			NaCl	0.50		1.00
Chicken liver paste	52	0.20		50		48
			Seasonings, incl. NaCl	0.46		0.92
Cream of chicken soup	50, 56	0.04		76		82
			Seasonings, incl. NaCl	0.74		1.47
Turkey-chicken liver paste	52, 58	0.20		50		48
			Seasonings	0.12	0.46	0.82
Pork sausage	54, 52, 52	0.04		67	56	71
			Thickening	2.50		11.20
Cream of chicken soup	58, 60	0.02–0.04[2]		71		92
			Fat	4.0		27.0
Chicken liver paste	42, 48	0.20		62		60

[1] All results except those for the liver pastes show statistically significant detection of glutamate at the 1% level of probability or less.
[2] In this product a single glutamate level, either 0.02 or 0.04%, was used for each panel member depending on his ability to detect it as determined in preliminary tests. Different glutamate levels used for different products were those required for detection at significant levels in preliminary tests of the products.

TABLE 5

Comparison of glutamate levels recommended for addition and levels occurring in foods

Product	Levels industry recommends to add (8, 9)	Free L-glutamatic acid in foods: Determined by Hac, Long, and Bitsh (8)		Determined in this study	
		Canned[1]	Cooked fresh[2]	Canned[1]	Cooked fresh or frozen[1]
	%	%	%	%	%
Carrots	0.25				0.16
Peas	0.25	0.12	0.16–.17	0.15	0.34
					0.05 (dehydro frozen)
Asparagus	0.20	0.05–.06	0.04		
Mushrooms	0.20	0.21–.23	0.13–.14	0.04–.06	0.11
Spinach	0.20	0.02			0.02
Corn, whole	0.20	0.04	0.05	0.06	0.10
Poultry liver paste	0.18				0.19
Clam	0.15	0.09–.10			0.05
Crab	0.15	0.06			0.04
Lima beans	0.15	0.02			0.06
Pork sausage	0.15		0.01–.02		0.02
Snap beans	0.15	0.01	0.02	0.01	
Shrimp	0.15				0.13
Tomatoes	0.15			0.18	0.21 (raw)
Chicken, boned	0.12	0.02–.04			
Tuna	0.12	0.02		0.01	
Potatoes, new	0.10	0.04	0.05		
Tomato sauce				0.23	
Tomato catsup				0.29	
Tomato paste				0.54	
Chicken broth					0.02
Cheese sauce					0.08
Crab cakes					0.09

[1] Drained weight basis.
[2] Raw weight basis.

TABLE 6

Effect of different levels of NaCl and glutamate on preference for chicken broth, turkey a la king, pork sausage and green beans

Product	Sample A compared with samples B			Preference for sample with added glutamate
	Additions to sample A		Addition to sample B	
	NaCl	Glutamate	NaCl	
	%	%	%	%
Chicken broth	0.65	0.15	0.65	72
			0.75	62
			0.85	60
			0.95	72
	0.65	0.25	0.65	82
			0.75	65
			0.85	66
			0.95	61
	0.75	0.15	0.85	70
			0.95	70
Pork Sausage	1.0	0.15	1.0	70
			1.1	53
			1.2	50
			1.3	51
	1.0	0.25	1.0	60
			1.1	54
			1.2	74
			1.3	52
Turkey a la king	0.83	0.27	0.83	62
	0.83	0.40	0.83	64
Green beans (canned)	0.80	0.15	0.80	60
			0.85	60
			0.90	64
			1.00	72

TABLE 7

Influence of test conditions on detectability of glutamate

Product	Added NaCl	Added glutamate	Detection of samples containing added glutamate[1]	
			Duo-trio test[2]	Single-sample test[3]
	%	%	%	%
Chicken broth	0.50	0.05	90	....
		0.10	100	....
		0.25	....	68
		0.50	....	83
Chicken broth	0.75	0.05	85	....
		0.10	94	....
		0.25	96	68
		0.50	....	75
Turkey a la king	0.75	0.05	75	....
		0.10	77	....
		0.15	92	....
		0.25	....	50
		0.50	....	60
Turkey a la king	1.00	0.10	71	....
		0.15	75	....
		0.25	75	35
		0.50	....	65

[1] Per cent detection out of a possible 48 judgments. Per cent required for statistical significance: 5% level of probability, 65%; 1% level of probability, 69%; 0.1% level of probability, 75%.

[2] Pairs of samples, with and without glutamate (one product, one salt level) compared directly with control sample containing no glutamate.

[3] Single sample, alternating broth and a la king, presented for judgment on presence of glutamate after control samples of each product containing no glutamate were tasted and removed.

somewhat as the sample without glutamate approaches the optimum acceptable NaCl level.

Glutamate was detected less readily when samples were presented singly, broth alternating with a la king, than when two samples of the same food, with and without glutamate, were presented for direct comparison (Table 7). More than 10 times the amount of glutamate needed for detection in side-by-side comparisons was needed for detection in the single-sample test in three of the four product-NaCl comparisons.

Although this study was conducted with the panel selected for its ability to detect glutamate at low concentrations, it is included in this discussion of consumer tests because it seems reasonable that consumers would react in a similar manner to samples presented under the two conditions. A greater difference between samples would undoubtedly be required for a distinct preference judgment by consumer panels if samples were not presented side-by-side. This would apply whether the question involved glutamate or any other flavor difference. Consumer tests based only on side-by-side comparisons may be misleading as an index of whether the preference would remain under normal conditions of eating.

SUMMARY OF RESULTS AND CONCLUSIONS

The glutamate ion has a specific flavor effect that is more readily detected than the chloride ion when NaCl and glutamate are added at approximately equal sodium concentrations to chicken broth containing normal amounts of NaCl. The panel selected for acuity to glutamate readily (95% of possible judgments) detected glutamate added to unseasoned chicken broth at levels ranging from 0.015 to 0.01%. Addition of thickenings, fat, and seasonings interfered only slightly with detection of the above levels of glutamate. The added glutamate was less readily detected in certain foods such as peas and liver paste than in others, such as pork sausage, turkey a la king, or the thickened chicken broth. Glutamate was less readily detected in chicken broth or turkey a la king containing the higher of two levels of NaCl, but limited tests on cream of chicken broth and liver paste showed no effect of NaCl level within reasonable levels of variation. However, high levels of added thickenings, fat, or seasonings did not interfere with detection of added glutamate to any greater extent than did low levels. In fact, a high level of flour added to a highly seasoned broth made detection of added glutamate easier, probably because it masked some of the other seasonings. The case of detecting added glutamate is not greatly influenced by variation in levels of thickening agent, fat content, or seasonings that would normally be encountered in foods.

Added glutamate was much more difficult to detect in foods normally containing very high levels of glutamate than in those containing very low levels of glutamate. The glutamate content of the foods must be considered in developing recommendations for levels of added glutamate. Foods with very high glutamate content require considerably more added glutamate than foods with very low glutamate content to exhibit a demonstrable change in flavor.

The extent of consumer preference for glutamate-containing samples did not vary in a consistent manner with NaCl level, but it is suggested that the preference decreases somewhat as the optimum acceptable NaCl level is approached. This level, of

course, varies for different foods and individuals. In the foods tested, the preference for those with added glutamate either equalled or exceeded preference for those without added glutamate.

The difference in performance of a trained panel on a paired comparison and a single sample test suggests that consumer tests of preference based on samples compared directly may have limited applicability under normal eating conditions.

Acknowledgment

The contributions of members of the Glutamate Manufacturers' Technical Committee, of our colleagues, of the Langfield Company, and of Owens-Illinois are acknowledged in detail in the second part of this paper, which follows.

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Correspondence

Chinese Restaurant Syndrome

There is a major criticism that can be raised against the conclusion presented by Morselli and Garattini[1] that there is no connexion between the ingestion of monosodium glutamate and the Chinese restaurant syndrome. This is that they did not use susceptible subjects. From the very first reports of this condition it was apparent that only a relatively small proportion of normal individuals was susceptible[2], the proportion being higher among women than among men[3] (most of the subjects in the experiments reported by Morselli and Garattini were men). Obviously a study of non-susceptible subjects only will not reveal significant reactions.

My own interest in the Chinese restaurant syndrome stems from my suffering from this condition for at least five years before the original description of it by Ho Man Kwok[4] in 1968. The onset of my recognition of the symptoms occurred when I moved from England to Boston and started to eat in the many excellent Chinese restaurants in that city. Like others[3] I was able to prepare a list of "safe" and "to be avoided" Chinese restaurants. From my personal experiments (unfortunately with only one subject) I can assure the authors that a susceptible subject is capable of recognizing whether monosodium glutamate has been ingested or not: to some extent the amount ingested can also be judged from the severity of the symptoms.

Yours faithfully,

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Absorption of l-Glutamic Acid

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The possibility that the salts of l-glutamic acid have a different physiological effect than the acid has been raised by Waelsch (*1*). Pond and Pond (*2*) reported that a given dose of the acid raised the plasma level of glutamic acid less markedly but over a longer period of time than did an equivalent dose of either the sodium or potassium salt. Mayer-Gross and Walker (*3*) found that the oral administration of as much as 100 g of l-glutamic acid hydrochloride was not efficacious in arousing patients from insulin hypoglycemia, whereas the administration intravenously of 20 g of glutamic acid as sodium glutamate was.

A survey of the literature on l-glutamic acid administered either to animals or to man shows that three forms of the compound have been used: the salt of sodium or potassium, the unneutralized acid, and the hydrochloride. These three compounds differ markedly in solubility (*4*) and, thus, might be expected to be absorbed at different rates. In order to test this hypothesis, equivalent amounts of the three compounds were given to human subjects in a fasting condition. Blood samples were drawn routinely before the ingestion of the test substance and at 30, 60, 90, 120, and 180 min afterward; in four cases samples were also taken at approximately 4 and 5 hr. Plasma glutamic acid levels were determined by the method of Prescott and Waelsch (*5*). Since 16 such tolerance tests had been completed previously with 15 g of the monosodium salt of glutamic acid in tomato juice and water (*6*), the same dosage level and mode of administration were used for the other compounds.

The unneutralized glutamic acid was given twice to each of four subjects who had previously shown typical responses to the administration of sodium glutamate. In the first test of the unneutralized acid, in each of the four subjects, it was given as a sludge with tomato juice followed by water *ad libitum*. It was felt, however, that the ingestion of the acid with or immediately after food would more nearly approach the condition existing in the treatment of mentally defective children and that the presence of food might aid in the solution and absorption of the acid. Therefore, in the second test the acid was given with a liberal breakfast of hot cereal, eggs, milk, tomato juice, and toast, with water being allowed *ad libitum*. In three cases, the administration of food with the acid raised the absorption slightly but not at all to a level comparable to that of the salt. In the fourth individual no rise in blood level occurred after ingestion of the acid with or without food. In only one case was there any indication of a delayed rise, even though in two cases blood was drawn as late as 5 hr after the ingestion of the acid. The rises in these cases with unneutralized acid are similar to those reported by Bessman *et al.* (*7*) with a dose of 1 g/10 kg of body weight. One subject who

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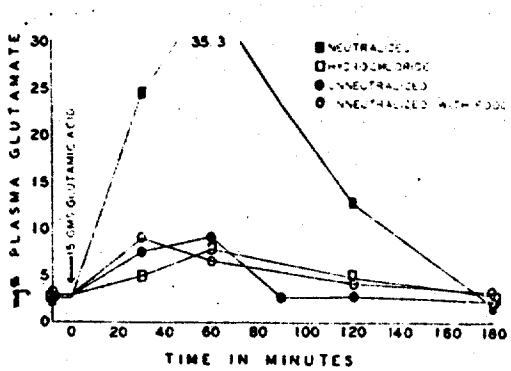

Fig. 1. 1-Glutamic acid tolerance curves on one subject with 1-glutamic acid compounds equivalent 15 g of the acid.

had nausea after taking glutamate experienced none when given the unneutralized acid with or without food.

Two of the same subjects were used for one tolerance test each with glutamic acid hydrochloride. Because of the marked fall in blood pressure and the severe stomach cramps induced by the ingestion of such a large amount of this compound, no additional tests were undertaken. The hydrochloride was given to one subject in 50 ml of water followed by tomato juice and water *ad libitum* and to the other subject in capsules followed by tomato juice, and water *ad libitum*. It is interesting that the subject who received the capsules suffered acute nausea, the first time in six tolerence tests with various glutamic-acid compounds. In the subject who received the hydrochloride in solution, the rise in plasma level was somewhat greater than for the unneutralized acid; in the subject who received capsules, the reverse was true. The tolerence curves for the sodium salt, the unneutralized acid with and without food, and the hydrochloride for one subject are shown in Fig. 1.

It seems possible that the unneutralized glutamic acid is not absorbed to any great extent and that the major part of a given dose passes through the gut without going into solution. The material would not tend to dissolve in the acid *p*H of the stomach, and, although the duodenal secretions are alkaline, the intestinal contents, with the passage of acid chyme from the stomach, tend to be acid. Even in the absence of food, the passage of a substance as markedly acid as glutamic acid into the duodenum would tend to render that area acid, unless the glutamic acid at the same time stimulated a large flow of pancreatic juice. This latter possibility does not seem likely.

It is also possible that there is an extremely slow absorption of the unneutralized acid and that the blood level is elevated slightly over a comparatively long time. Our data do not suggest such an effect, but blood samples would have to be drawn over a much longer period of time to get a conclusive answer. However, in view of the normal pattern of variation of the plasma glutamate level (*6*) it does not seem likely that such a small rise would have much physiological effect.

Some of the confusion in the literature regarding the activity of l-glutamic acid may be due in part to a failure of some investigators to specify which of the three forms of l-glutamic acid they used. We can assume that l-glutamic acid given by injection is close to *p*H 7.0 and, hence, is a salt of l-glutamic acid, probably monosodium glutamate. Material given orally in solution is probably also a salt, unless otherwise specified, since the hydrochloride in solution has such unpleasant effects; the sludge is probably the unneutralized acid, and the capsules or pills may be any one of the three. The oral ingestion of l-glutamic acid in a form that does not enter the blood stream readily cannot be expected to have any physiological effects. This situation emphasizes the fact that material placed in the gut is outside the body until it has passed the gastrointestinal mucosa. It is interesting that the majority of favorable reports of the results of administration of glutamic acid to patients or of its physiological effects in animals have been obtained with sodium glutamate or with the hydrochloride.

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Hematoencephalic Exchange as a Function of Age

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THE HEMATOENCEPHALIC EXCHANGE is a rate phenomenon which varies with the material exchanged between the blood and the brain. The exceedingly slow passage of some substances into brain has led to the concept of a blood-brain barrier. This term, however, carries the idea of an absolute barrier rather than a differential rate phenomenon. For this reason we have used the term, hematoencephalic exchange.

The change in ease with which materials enter the brain as the animal matures has excited the interest of a number of investigators. In human beings, the relative permeability of the brain of the premature infant to bilirubin has been offered as an explanation for the development of kernicterus in babies with erythroblastosis.[1] Irrespective of the blood level, kernicterus develops rarely, if at all, in the adult human being. The only other data available in the human newborn, however, do not support this thesis. Gröntoft[2] has studied the staining of the brain by dyes injected into the blood stream in human infants who died at, or shortly after, birth and in newborn rabbits. As a result of his work, he believed that some mechanism blocks the free exchange of substances between blood and brain, even in the very young, unless anoxia has injured the brain. However, the babies and animals he studied were dead before injection and the permeability of the brain might have been altered. On the other hand, Behnsen[3] found that trypan blue injected into mice colored the brain more in the young than in the older animals. Stern and Peyrot[4] traced the entrance of sodium ferricyanide from the blood into the brain in the young of several species and found that the substance penetrated into the brain freely until the animals had their eyes open, after which the ferricyanide could not be detected in the brain. Hess[5] has followed the development of the ground substance in the mouse and the guinea pig and has demonstrated that it becomes apparent histologically at about the same time that the brain is no longer stained by in vivo injection of dyes.

Several investigators have studied the entrance of radioactive phosphorus into the developing brain. Fries and Chaikoff[6] showed that more P^{32} entered the brains of newborn rats than of older animals. By the time the animals weighed 50 gm. or were approximately three or four weeks old, the rate of penetration was only slightly greater than in the adult. Bakay[7] and Manukyan[8] have both demonstrated the decreased passage of this tracer element into the brains of rabbits as they mature. They conclude that in this species free exchange does not occur after approximately 30 days of age. The gradual appearance of the relatively impermeability of the brain to certain substances seems to us to be an important facet of the growing brain which might correlate with the attainment of chemical and neurologic maturity. The studies reported here were planned to follow the function of the blood-brain barrier in the rat, using as the criterion the passage of L-glutamic acid from blood to brain. The use of this metabolite was induced by the current interest in this substance in the field of psychiatry.[9]

Glutamic acid not only occurs abundantly combined with other amino acids in brain proteins, but is also present in large amounts in the free form. During the period of rapid growth the young animal needs either to draw large amounts of this amino acid from the blood or to synthesize it rapidly to meet the needs of new nervous tissue. If the brain is capable of removing this material from the blood, that is, if the hematoencephalic exchange is not selective, a high blood level of glutamic acid may be reflected in an increase in free glutamic acid in brain.

METHOD

Four age groups of albino rats were studied: 24 hours old, 10 to 11 days old, 17 days old, and adults (six months old). The two youngest groups were injected intraperitoneally with 2 gm. per kg. of glutamic acid as sodium glutamate, a control sodium chloride solution containing an amount of sodium equivalent to that in the sodium glutamate, or 1.5 gm. per kg. of glutamic acid as ammonium glutamate. The injection of as much ammonium glutamate as sodium glutamate was lethal to most adult

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animals, so a lower level was chosen. The ammonium glutamate was omitted in the 17 day old animals, but they were given sodium glutamate and sodium chloride solutions as described above. Thirty minutes after injection, the animals were decapitated and the brains treated as described below.

In an attempt to stimulate the ingestion of increased glutamic acid in food, the test solutions were administered daily by stomach tube for nine days to male rats averaging six months of age, and weighing between 250 and 300 gm. The animals were fasted approximately eight hours during the day before they received the solutions by stomach tube. They were allowed to eat freely during the night and so maintained their body weight. On the tenth day, after the animals were starved for 24 hours, a final dose of appropriate test solution was given by stomach tube. Thirty minutes later the animals were decapitated and the brains removed.

All solutions administered either by stomach tube or by injection were adjusted to room temperature and to pH 7.0. The glutamate salts were prepared by dissolving L-glutamic acid in water and neutralizing with the appropriate base.

The brains were all analyzed by routine procedures, with certain exceptions dictated by brain size. For the 24-hour and 10-day old groups, several whole brains were combined to obtain samples large enough for analysis; in the 17-day-old group, the brains were cut at the midcollicular level and the area cephalad to the incision used. In the adult rats the same area was studied, but one hemisphere was used for glutamic acid and the other for glutamine analyses. These changes in procedure undoubtedly made for greater variations within the same experimental group, but made possible a number of analyses on the same brains.

In every case after the animal was decapitated, the brain was removed and frozen immediately in dry ice and ether. Less than 15 seconds elapsed between decapitation and complete freezing. The frozen brains were stored in aluminum foil and waxed freezer paper at −22° C. until analyzed. For the analyses, they were weighed while still frozen and homogenized in cold trichloroacetic acid. The method of Prescott and Waelsch,[10] as modified by Himwich and Petersen,[11] was used for glutamic acid. Glutamine was determined by the method of Bessman.[12] Alpha-keto glutaric acid, pyruvic acid, moisture, and total nitrogen were determined by the methods of Friedmann,[13] Graves and Himwich,[14] and Kelley,[15] respectively.

Blood levels of glutamic acid were determined on blood obtained by decapitating the animals and collecting the freely flowing blood.

RESULTS

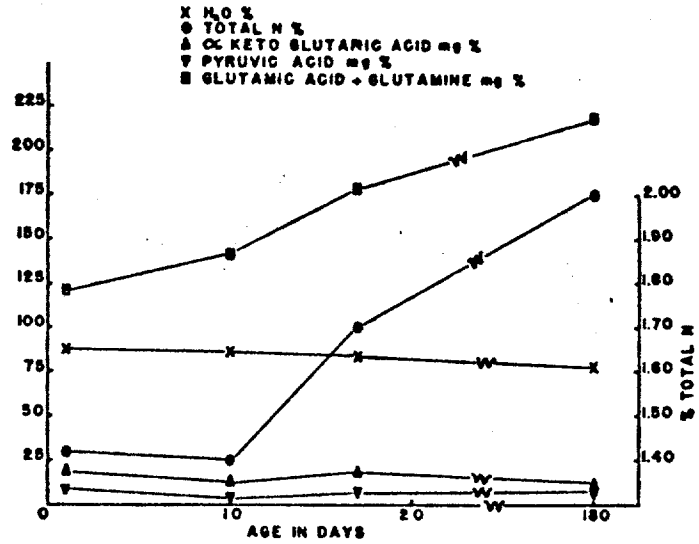
Data on the control groups of animals for free glutamic acid and glutamine in the brain show that, on a wet weight basis, these substances increase from birth to six months of age (table 1, figure 1). The increase, however, is not at a regular rate. For free glutamic acid the greatest increase occurs between the tenth and seventeeth days, with the brain containing almost the adult amount of this substance at 17 days. For glutamine, on the other hand, there is a definite fall at the tenth and at the seventeenth days, followed by a rise to the adult value. The reaction of glutamic acid + ammonia ⇆ glutamine is readily reversible and probably represents the principal route of detoxication of ammonia. Hence, it seems better to consider in the control animals the increase with age in the total of free glutamic acid plus free glutamine. Even on this basis, however, the increment per day between one and ten days is considerably less than between 10 and 17 days. During the same period of time, that is, birth to 17 days,

TABLE 1

FREE GLUTAMIC ACID AND GLUTAMINE IN RAT BRAIN AFTER ADMINISTRATION OF SODIUM GLUTAMATE OR AMMONIUM GLUTAMATE

	Control	NaGlut.[1]	$NH_4Glut.$[2]
24 HR. OLD			
Glutamic acid, mg.%	63.5	79.9**	74.3**
	(15)[3]	(17)	(18)
Glutamine, mg.%	56.8	52.4	46.8
	(16)	(14)	(14)
10 TO 11 DAY OLD			
	Control	NaGlut.[1]	$NH_4Glut.$[2]
Glutamic acid, mg.%	94.0	82.9*	76.8**
	(10)	(10)	(14)
Glutamine, mg.%	47.7	39.2**	59.3**
	(15)	(19)	(19)
17 DAY OLD			
	Control	NaGlut.[1]	$NH_4Glut.$[2]
Glutamic acid, mg.%	137.9	141.7	
	(10)	(10)	
Glutamine, mg.%	40.3	46.1	
	(10)	(10)	
6 MONTHS OLD			
	Control	NaGlut.[1]	$NH_4Glut.$[2]
Glutamic acid, mg.%	146.9	152.9	160.3
	(18)	(17)	(7)
Glutamine, mg.%	71.0	71.3	53.0
	(11)	(19)	(5)

* difference from the control is significant at 5% level.
** difference from the control is significant at 1% level.
[1] 2 gm./kg. glutamic acid as sodium glutamate.
[2] 1.5 gm/kg. glutamic acid as ammonium glutamate.
[3] Numbers in parentheses are number of animals used.



ig. 1. Water, total nitrogen, alpha-keto-glutaric acid, pyruvic acid, glutamic acid plus glutamine in developing brain.

water is decreasing and total nitrogen is increasing. The values for alpha-keto-glutaric and pyruvic acids have a tendency to remain constant, with the exception of the values for the 10 day old animals which are consistently low (figure 1).

The injection of either sodium or ammonium glutamate increased the free glutamic acid in the brains of the 24 hour old rats. The animals in both of these groups probably convulsed but, due to the inherent motor restlessness of this age group, it is impossible to be certain of the convulsions. The animals at 10 days of age receiving the glutamate salts convulsed about 15 minutes after the injection. Electroencephalograms taken from the cortex revealed large slow waves and some spikes. Glutamic acid was actually lower in these brains than in the controls for that age group. The same is true for both glutamic acid and glutamine in animals receiving sodium glutamate. In the animals given ammonium glutamate a decrease in glutamic acid was offset by the formation of additional glutamine. Since brain metabolism of both these groups of animals can be assumed to be affected by the convulsions and the overall metabolism considerably changed, no attempt was made to follow the sum of glutamic acid and of glutamine.

At 17 days only an occasional animal convulsed when given sodium glutamate. In the groups of adult animals none convulsed. In neither the 17 day old animals nor in the adult animals did the administration of glutamate or glutamine affect the brain level of glutamic acid or of glutamine significantly (table 1).

The blood glutamate was raised in each of the age groups by the administration of glutamate as shown by the average blood levels 178, 166, 210, and 92 mg. per cent at 30 minutes after the administration of sodium glutamate in the newborn, 10 day old, 17 day old, and adult animals, respectively. These rises represent increases of approximately 100 per cent, since the normal blood level is between 1 and 2 mg. per cent.[16] It is difficult in our present state of knowledge to interpret the data on alpha-keto-glutaric acid and pyruvic acid. Only the falls in pyruvic acid and in alpha-keto-glutaric acid for the newborns given glutamate were significant.

DISCUSSION

The control glutamate values obtained in the six months old animals compare favorably with those reported by Schwerin, Bessman, and Waelsch[17] and also with values given by Dawson.[18] The glutamine values reported are slightly higher than those of Waelsch and his colleagues[17] and are comparable to those reported by Dawson[18] and by Tigerman and MacVicar.[19] The sum of these two substances is in the same range as reported by Haber and Saidel.[20] To our knowledge, values are not

available in the literature for the other age groups.* It is interesting that glutamic acid approaches the adult level at 17 days, at which time Tallan reported N-acetyl-L-aspartic acid has attained the maximum value.[22] It is at this time also that postural attitudes attain full development and in the next three days the animals will show the characteristics of adult behavior.[23] The data for alpha-keto-glutaric and pyruvic acids are higher than most values reported[24] but are lower than those of Froman, Orten, and Smith.[25] Although our method of removing and treating the brains made little, if any, difference in glutamic acid and glutamine values, it is quite probable that it caused an increase in such metabolites as pyruvic acid. The total nitrogen and water content of the brains show the expected shifts as the brain develops.[26,27]

When we consider the penetration of glutamic acid from the blood to the brain in the adult, we find no significant increase irrespective of the form in which glutamic acid was given (table 1). These values suggest, as has been indicated by Waelsch and his colleagues,[17] by Dawson,[18] and by Kamin and Handler,[28] that glutamic acid does not enter the brain in the adult animal. Klotzsche[29] and Boulanger, Griffiié, and Osteux,[30] on the other hand, reported experiments which indicate that glutamic acid does penetrate into the brain. The former worker fed glutamic acid, 1.25 gm. per kg., for eight weeks to mice and found an increase in glutamic acid and glutamine resulting in a total value for the two substances of 340 mg. per cent as compared to the normal value of 160 mg. per cent. The latter investigators, using the isolated perfused head of the dog, found a significant uptake of the amino acid from the blood at plasma levels only slightly higher than normal. It is possible that the increases reported by the French workers are due to anoxia, since normal reflexes are lost by their preparation in 45 to 75 minutes. The effect of feeding glutamic acid to mice is similar to the results obtained by Tigerman and MacVicar[19] when an ammonium salt was fed with glutamic acid. These authors unfortunately determined only glutamine in the brain. When they injected approximately 1.7 gm. per kg. of glutamine or of glutamic acid with ammonia 15 minutes before sampling the brain, they obtained increases of brain glutamine only in the case of glutamine injection. Such divergent results suggest that data on the permeability of the brain in the adult animal may be dependent upon variations in the experimental conditions which are not adequately appreciated. In connection with this problem are the interesting experiments of Sacks[31] who has shown that carbon-labeled fumarate is taken from the blood by the brain and rapidly converted to carbon dioxide in man. However, the total quantities of fumarate which entered the brain were very small (0.27 mg. per cent).[32] It is always possible that the level of glutamic acid in the adult brain is maintained at such a constant level that any extra amount of amino acid taken from the blood is rapidly metabolized by being incorporated into protein, decarboxylated to form gamma-aminobutyric acid, or deaminized to alpha-keto-glutaric acid.

The data suggest immediately that free glutamic acid enters the brain easily in the 24 hour old animal, but does not penetrate readily at other age levels. In the 24 hour old animal there apparently is no advantage to the administration of glutamic acid, either as the sodium salt or as the ammonium salt. It must be remembered, however, that only three-fourths as much glutamic acid was given in ammonium glutamate as when sodium glutamate was injected. Larger quantities of ammonium glutamate resulted in marked convulsions in the adult animal, due probably to the ammonium ion. In the 17 day old animal it is also apparent that glutamic acid does not enter the brain readily. The occasional animal that convulsed probably represented the exception.

The situation in respect to the 10 day old rat is much more difficult to interpret. Animals of this age given either sodium or ammonium glutamate show convulsive seizures about 15 minutes after the injection. Large dysrhythmic electroencephalographic waves may be obtained from cortex during this time. These waves are similar to those reported by Druckman and Chao[33] in very young children with convulsions. In attempting to interpret these data it is important to keep in mind the state of the brain in the rat at 10 to 11 days of age. It has been shown by Koch and Koch[34] and others[35] that it is at this age level that the animal first begins to lay down large quantities of protein and phospholipid. The change in water content for instance between the tenth and seventeenth days is more rapid than at any other period after birth. It is at 10 days that Crain[36] first was able to obtain spontaneous electrical activity from the cortex, and Su-

*These values supersede those given by Himwich and Himwich,[7] since a systematic error was found in the calculation of those data.

gita[37] found that thickness and volume of neurons in the cortex approached the adult level. Gollan[38] reports that at this age the rat loses its infantile reaction to hypothermia. Active mitosis is still occurring in the neuroglia.[39] Many enzymes, such as glutamic acid dicarboxylase, are increasing at a rapid rate.[40] It seems quite probable that we have not been able to catch the entering glutamic acid before it has been metabolized in these animals. We have a hint as to what may happen when we consider the increase in glutamine following the injection of ammonium glutamate. It is conceivable that in this group of animals the additional ammonium is removed by glutamic acid to form glutamine.

The development of convulsions may be due to the fact that glutamic acid entered the brain. Since convulsions occurred as frequently in those given sodium glutamate as in those with ammonium glutamate, we feel justified in assuming that the seizures were due to the glutamate ion and not to the ammonium ion. The whole picture, however, is complicated by the fact that Gellhorn and Ballin[41] have shown that susceptibility to convulsions induced by electrical stimulation varies with age, younger animals convulsing more readily than older animals. It is difficult to reconcile occurrence of convulsions in the 10 day old animals with the beneficial effects of the administration of glutamic acid reported in petit mal epilepsy[42] and in audiogenic seizures in mice.[43] The results reported in these cases, as well as the increases in well-being reported in aged patients,[9] may very well not be related to the penetration of glutamic acid into the brain but to some other mechanism. These questions still await elucidation. Kittens up to the age of five or six months vomit but do not convulse after the injection of sodium glutamate, while adult cats do not vomit.[16] The observation suggests that glutamic acid may enter the brain more readily in kittens than adult cats. We have not noted convulsions even in four week old kittens. The effect of the convulsion would undoubtedly be to accelerate metabolism, and glutamate might well be used by the brain for its metabolic needs if not enough glucose were present.

Another problem which must be assessed in evaluating these data is the hyperglycemic effect of glutamate in rats,[44] as well as in other animals.[45] Although the exact mechanism of this hyperglycemia is not clear, most investigators agree that there is a mobilization of liver glycogen. Blood sugars were not determined on the rats, but it would seem probable that only in the adult rats would there be enough reserve of liver glycogen to permit a significant hyperglycemia. Undoubtedly, the results in adult animals are complicated by a rise in blood sugar which might increase the movement of glucose into the brain and conceivably block the entrance of glutamate. Dyes have been shown by Aird and his colleagues to block the entrance of cocaine into the brain of the cat.[46]

SUMMARY

The relation between the changes in glutamic acid, glutamine, pyruvic acid, alpha-keto-glutaric acid, water, and total nitrogen in the developing brain of the rat and the development of behavioral patterns are discussed. Hematoencephalic exchange of glutamic acid is relatively fast in the newborn rat, as shown by increases in glutamic acid in brain when the blood level of this amino acid is raised. In the adult animal under similar circumstances the exchange cannot be detected by the methods used in this study.

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BIOCHEMICAL OBSERVATIONS ON THE USE OF L-GLUTAMIC ACID IN THE TREATMENT OF HEPATIC COMA

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The neurological pattern of hepatic coma has been characterized by Adams and Foley (1), and their observations facilitate the early recognition of this syndrome. A similar syndrome has been produced by the administration of certain nitrogenous substances (2) or a high protein diet (3) and has been observed following portacaval shunt (4) or total hepatectomy (5). Blood ammonia levels in hepatic coma are usually elevated, but there is poor correlation with the neurological state. Walshe (6) reported in 1953 the successful treatment of hepatic coma with large amounts of l-glutamic acid. Although beneficial results have been reported with glutamic acid by some investigators (7–11), in contrast, no benefit has been observed from this treatment by others (12–14). However, independent of the clinical result the blood ammonia decreases with glutamic acid therapy (7, 12, 14).

Walshe (6) proposed that an excess of intracellular ammonia accumulates in the brain in hepatic coma because of disturbed metabolism of glutamic acid. The administration of glutamic acid might correct this defect. Bessman and Bessman (15) believed that interference with energy metabolism in the brain by removal of alpha-ketoglutarate is the essential lesion of hepatic coma. The present study characterizes some of the biochemical changes in the serum accompanying the fall in blood ammonia produced by the infusion of sodium glutamate in normal persons and patients in hepatic coma.

CLINICAL PROCEDURES AND METHODS

Glutamic acid infusions were given to two normal subjects and eight patients with cirrhosis. All were studied on the wards of the Walter Reed Army hospital. The diagnosis of pre-coma was made in such cases by serial observations of developing stupor, confusion, and flapping tremor in patients with chronic liver disease. The criteria for the definition of coma are those outlined by Webster and Davidson (13). All patients were fasting during the time of study and glucose was administered at a constant rate as far as possible. None of the patients was in shock, but patients 8, 9, and 4 during Study "C" received blood transfusion during the aftercontrol period because of continued gastrointestinal hemorrhage. Azotemia was not observed but oliguria was present in all coma patients. Pertinent clinical information for each subject is given in Table I.

Blood ammonia was determined on fresh heparinized venous blood. All determinations were made in triplicate using a microdiffusion method (16, 17). The average deviation of replicates on the same blood sample from the mean (18) was 7.6 ± 3.1 μM per L.[3] Simultaneous arterial and venous blood samples were obtained from five patients and analyzed for ammonia and alpha-ketoglutarate. The arterial blood ammonia was always higher than the venous blood ammonia, suggesting extraction of ammonia by the musculature as reported by Bessman and Bradley (19), but the alpha-ketoglutarate did not change. The impression was gained during the collection of samples that the brighter venous bloods had higher ammonia values. For this reason, the effects of *in vitro* oxygenation on the blood ammonia were studied. Venous blood was equilibrated in a tonometer with a stream of washed oxygen or nitrogen, and at the end of five minutes the original unagitated venous blood and both treated samples were analyzed for ammonia. The nitrogen equilibration was employed as a control, for the gassing process caused the loss of some ammonia. All determinations were performed in triplicate and the sequence of analysis was varied so that the oxygenated sometimes preceded and sometimes followed the other analyses. Actual ammonia analyses were started within 20 minutes of shedding of the blood and the first and last samples were invariably less than five minutes apart. The oxygenated blood assumed a bright red color in contrast to both the control venous and the nitrogenated blood; however, the oxygen tension was not directly measured. Blood from six normal persons and from four patients with cirrhosis was treated in this manner.

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[3] The designation μM per L. is used for all substances to facilitate comparisons. One μM per L. of ammonia is equivalent to 0.017 γ per ml.

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TABLE I

Pertinent clinical information on the normal subjects and patients with cirrhosis

No.	Age	Diagnosis	Duration of cirrhosis *months*†	Total bilirubin at time of study *mg./100 ml.*	Serum albumin *gms. per 100 ml.*	Factor precipitating coma	Duration of coma before glutamate infusion *hrs.*	Effect of glutamate infusion	Comment
1	21	Normal	None	0.5	5.0			None	
2	26	Normal	None	0.3	4.6			None	
3	43	*Cirrhosis	36	1.4	4.1			None	
4a	62	Cirrhosis	18	32	3.4	Hemorrhage	6	None	Died 4 days later
b							30	None	
c							54	None	
5	44	Cirrhosis	½	19	2.8	?Paraldehyde	36	Awakened	Died after 12 days
6	46	Cirrhosis	4	3.2	2.2	?NH_4Cl		None	Precoma due to NH_4Cl?
						Hemorrhage	6		Died 24 hrs. later
7a	45	Chronic pancreatitis; cirrhosis	2	28	2.2		24	None	Coma lightened
b							12	None	Coma increased; death 36 hrs. later
8	45	Cirrhosis	13	35	2.0	?Transfusion reaction	36	None	Died 24 hrs. later
9	51	Cirrhosis	66	9	1.7	Hemorrhage	8	?Convulsions	Died 6 hrs. later
10	52	Non-alcoholic cirrhosis	8	4.4	1.4	?I.V. protein hydrolysates	24	None	Died 15 days later

* No signs of coma.
† Dated from the time of the first clinical diagnosis or unequivocal historical sign of decompensation.

None of the patients is one of those described in this paper, for this phenomenon was not investigated at that time. The results are recorded in Table II. The blood saturated with oxygen from both the normal and cirrhotic patients had a higher ammonia value than blood saturated with nitrogen, and the control value was generally intermediate. The interpretation of arterial-venous ammonia differences remains difficult until the *in vitro* effects of oxygenation, recently confirmed (20), are further clarified.

Glutamine was measured by the procedure of Iber and Bruton (21), employing an enzyme prepared from the kidneys of acidotic rats. Pyruvic acid and alpha-ketoglutarate were measured by the method of Seligson, McCormick, and Sborov (22). Citric acid was measured by the method of Ettinger, Goldbaum, and Smith (23). Amino acids were measured by column chromatography using the technique of Moore and Stein[4] (24). A portion of the glutamine is hydrolyzed on the column in this method and appears as glutamic acid.

Monosodium glutamate was infused into two normal persons and eight patients with cirrhosis. All patients received intravenous glucose before and after study. Monosodium glutamate (approx. 24 gm.) was prepared in 500 cc. of 5 per cent glucose; the rate of infusion was adjusted so that glucose was given at the same rate before, during, and after the glutamate infusion. The time, amount, and duration of the glutamate infusion are given in Table III. Blood samples were taken in aqueous heparin and processed immediately for analysis. The samples for citric acid, pyruvic acid, alpha-ketoglutaric acid, glutamine, and amino acids were stored frozen until analyzed.

[4] These determinations were obtained through the cooperation of Dr. Stanley Levenson and Mr. Hyman Rosen.

RESULTS

Blood "ammonia"

The blood "ammonia" was elevated prior to therapy in the patients in coma (average value, 215 μM per L.; normals, 67.6) (Table III), but

TABLE II

*The effects of oxygen tension on blood "ammonia" (gamma per ml.)**

Normals						
Oxygenation	1.51	1.19	1.33	1.16	1.35	1.14
Control Venous	1.21	0.90	1.66	1.19	1.35	1.01
Nitrogenation	1.16	0.70	1.12	0.73	1.07	1.21
O_2-N_2 Difference	0.35	0.49	0.21	0.43	0.28	−0.07
Cirrhotics						
Oxygenation		1.03	2.08	1.42	1.67	
Control Venous		1.13	1.89	1.29	1.31	
Nitrogenation		0.91	1.54	1.31	1.23	
O_2-N_2 Difference		0.12	0.54	0.11	0.44	

* Mean difference of oxygenation—nitrogenation is 0.29/ml.
S.E. of difference is 0.062 $P < .01$.

TABLE III

Changes in blood levels before and after intravenous sodium glutamate

No.	Dose of glutamate and duration	Control and duration	Ammonia μM./L.		Glucose mg./100 ml.		Alpha-keto-glutarate μM./L.		Pyruvate μM./L.		Citrate μM./L.		Glutamine μM./L.	
			Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Normal values*			67.6 ± 10				13.0 ± 1.5		73 ± 16		79.2		550–760	
1†	24 gm. 3⅓ hrs.	Glucose 2 hrs.	166	72	88	96	12.4	20.6	115	128	70	58	229	265
2†	22 gm. 3⅓ hrs.	Glucose 1⅓ hrs.			77	82	11.3	17.8	69	71				
3‡	24 gm. 3 hrs.	Glucose 2¼ hrs.	204	161			15.6	21.5	68.5	59.6	84.3	90.6		
4a	24 gm. 2½ hrs.		140	88	90	123	29.7	144.5	156	260	73.4	122	700	1,240
b	24 gm. 4⅔ hrs.		138	107										
c	20 gm. 2 hrs.	Glucose 2 hrs.	176	126									516	410
5	32 gm. 4.5 hrs.		148	76			53.3	76.5	224.4	106.1	127	114		
6	20 gm. 5.5 hrs.	Glucose 2 hrs.	370	122	53	65	36.8	57.5	355	468	368	290		
7a	18 gm. 6¾ hrs.		165	103	124	212	33.2	47.5	56	103	201	223		
b	24 gr. 3 hrs.	Ca gluconate 2 hrs.	248	149	114	107	65.1	83.0	326	259	130	102	1,510	1,580
8	24 gm. 3 hrs.		2,440	3,550	90	69	36.2	117	506	387	154	198	1,630	1,610
9	40 gm. 3 hrs.	Glucose 3 hrs.	421	254	133	160	83.0	60.3	517	433	103	108		
10	20 gm. 3½ hrs.	Glucose 2 hrs.	191	158	208	178	23.7	18.8	123	108			925	990

* From the authors' laboratory. Glutamine range based on ten normals (21), mean citrate value taken from Ettinger, Goldbaum, and Smith (23). Pyruvate and alpha-ketoglutarate based on 17 normal subjects and the ammonia on 25 normal subjects.

† "Normal" patients.

‡ Patient with cirrhosis and no signs of coma.

the degree of elevation did not correlate with the depth of the coma. The levels tended to rise during the control infusions of glucose. In patient 8 extremely high levels of ammonia were found, 2,440 μM per L. initially, and values of similar magnitude were found in three subsequent blood specimens. The patient died a few hours after these observations. Review of the clinical information led subsequently to the suspicion that this man had septicemia with a urea splitting organism, but no blood culture was taken while the patient was alive and review of the pathology sections for bacteria did not support this possibility. The ammonia values of this case are excluded from the averages and from the figures in this paper. Sodium glutamate was effective in lowering the blood ammonia in all other patients (Figure 1). The lowering of the blood ammonia was on the average 87 μM per L. or 40 per cent of the initial value. The degree of lowering was not related in a simple fashion to either the dose given or the rate at which it was given within the ranges employed. There was no relationship between the amount of lowering and the clinical changes in

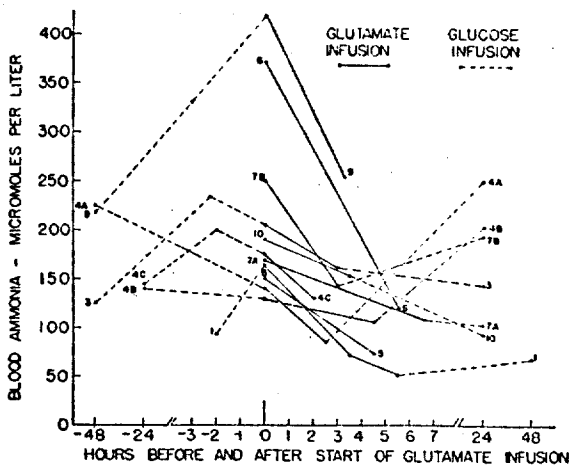

FIG. 1. THE EFFECT OF GLUTAMATE INFUSION ON THE CONCENTRATION OF BLOOD "AMMONIA"

The numbers identify the patients in Tables I and III.

the patient. Most of the ammonia values 24 hours after treatment had returned to pre-treatment levels.

Alpha-ketoglutarate

Blood alpha-ketoglutarate levels prior to the glutamic acid infusion were elevated from two to six times the normal level in the patients with liver coma. In four patients there was no striking change during the control infusions of glucose. In eight of ten infusions of glutamic acid there was a rise in ketoglutarate by from one-quarter to five times the initial value (Figure 2); in patients 9 and 10 there was a 25 per cent fall, possibly associated with a greater interval between cessation of the infusion and withdrawal of the specimen. In the study of patient 2, a normal subject, samples of blood were taken five minutes before the completion of the glutamic acid infusion, five minutes, 15 minutes, and 60 minutes after the completion. These data are presented in Table IV and Figure 3. The alpha-ketoglutarate level rises by 57 per cent and returns to the starting level within 15 minutes of the end of the infusion. The rapid changes in the levels of glutamic acid, alanine, and the glutamine-asparagine-serine complex are also to be noted.

Pyruvate, citrate, and glutamine

Blood pyruvate concentrations were elevated to two to seven times the normal in seven of eight of the comatose patients. Citrate concentration was elevated in six out of seven instances. The changes produced by glutamate infusion were variable. Glutamine was determined before and after six of the glutamate infusions. Although there was a rise in glutamine in four of the six infusions, only three of these showed a rise equal in concentration to the fall in ammonia. The

TABLE IV

Effect of sodium glutamate infusion on plasma substances *

μM./L. Plasma	Start of infusion	5 Min. before end	5 Min. after end	15 Min. after end	60 Min. after end
Threonine	79	78	73	76	
Glutamine, asparagine, serine †	168	210	215	87	
Glutamic acid	112	1130	775	400	
Glycine	82	100	89	57	
Alanine	200	305	235	182	
Ketoglutarate	11.3	17.8	16.0	11.8	13.6
Pyruvate	69.1	71.0	67.5	78.8	70.2
Citrate	80	110	127	109	193
Glucose	77	82	83	80	85

* Twenty-four gm. Na glutamate given over 205 minutes to a normal subject. Glucose given at uniform rate throughout the study.

† These came off as a group on the ion-exchange column. There may be loss of glutamine.

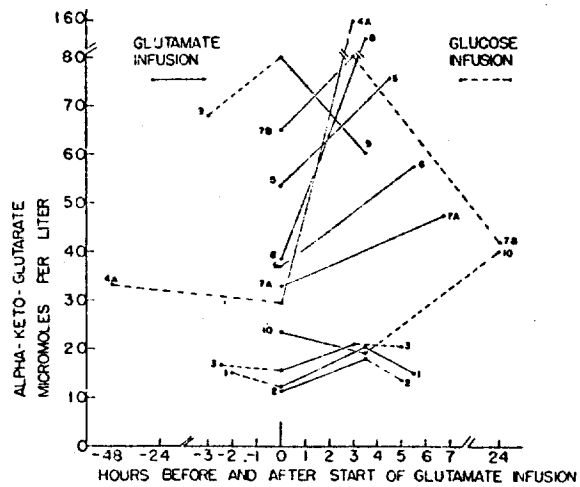

FIG. 2. THE EFFECT OF GLUTAMATE INFUSION ON THE CONCENTRATION OF ALPHA-KETOGLUTARATE

The numbers identify the patients in Tables I and III.

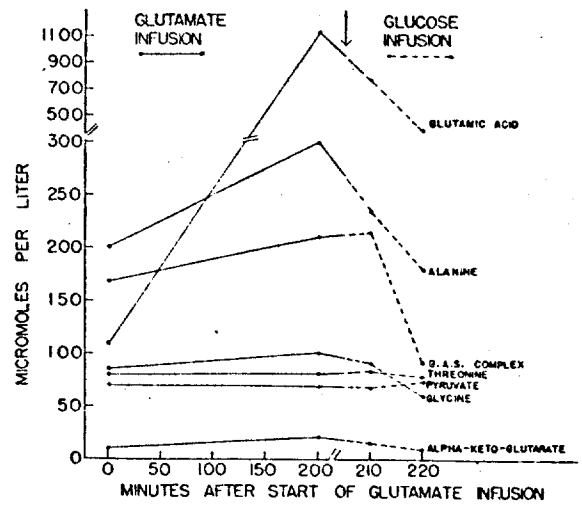

FIG. 3. CHANGES IN THE CONCENTRATIONS OF RELATED METABOLITES WITH TIME AFTER CESSATION OF GLUTAMATE INFUSION

Note the expansion of time scale after the cessation of glutamate. A 50 per cent rise and fall of ketoglutarate are minimized by the scale.

overall average change in glutamine was an increase of 96 μM per L. However, if case 4a is excluded there is essentially no change.

Clinical observations

One normal subject and the cirrhotic patient without coma tolerated the infusion well. The other normal subject had nausea, but no vomiting or other symptoms. Patient 5 is the only comatose patient who awakened with the infusion, and he remained alert for several days. The depth of coma seemed to lessen in patient 7 at the time of the first infusion but two days later the depth of coma increased during a second infusion. He died soon thereafter in coma. Patient 6 had a spontaneous remission from coma. Three days later coma recurred, and glutamic acid was given but did not produce a remission.

DISCUSSION

Glutamic acid as employed in this study was of little value in the treatment of the clinical manifestations of hepatic coma. These observations confirm the experience of others (13, 14) who found only transient beneficial effects. It must be pointed out that our group of patients all had chronic liver disease underlying their coma, and that this group is most refractory to glutamic acid treatment (7). Coma precipitated by exogenous factors such as gastrointestinal hemorrhage or ammonium chloride has been said to respond more consistently (10), but no support for this could be found in the present study. The dose of sodium glutamate, approximately 24 gm., is a smaller dose than that recommended by Walshe (7).

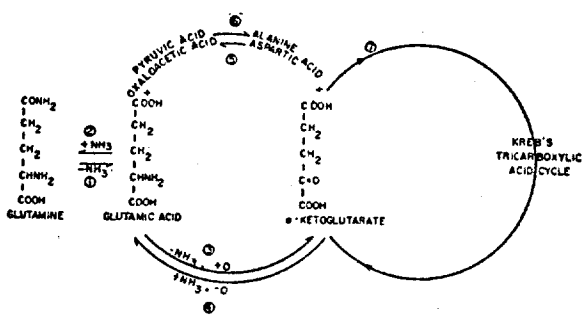

FIG. 4. SCHEMATIC INTERRELATIONS OF METABOLITES ARRANGED TO SUGGEST VARIOUS MECHANISMS BY WHICH GLUTAMATE MAY LOWER THE BLOOD "AMMONIA" CONCENTRATION

Before the biochemical changes are discussed two limitations of this study must be stated. All measurements were made on the blood, which reflects only gross changes in the body. The changes to be described for various metabolites probably occur at the intracellular level or in fluid compartments not in immediate equilibrium with the plasma. In addition, the data shown in Table IV and Figure 3 suggest that rapid changes in the blood levels at the time of cessation of the infusion may cause an appreciable variability in the data obtained from the rest of the patients.

The blood ammonia was lowered by glutamic acid in all except patient 8. Thus glutamic acid offers an effective method for lowering the blood ammonia. The possible reactions that could account for the disappearance of these quantities of blood "ammonia" (average 87 μM per L.) are outlined in Figure 4. The only direct reaction between glutamic acid and ammonia is No. 2, in which ammonia is added to glutamic acid to yield glutamine. Foster, Schoenheimer, and Rittenberg (25) have observed that the disappearance of isotopically labelled NH_3 in the normal rat is accompanied by the rapid accumulation of radioactivity in amide nitrogen, presumably glutamine. The possible value of this reaction for the removal of ammonia initially led Walshe to infuse glutamic acid (6). Glutamine rose in four of the six cases in which it was measured but in only three was the magnitude sufficient to account for the fall in ammonia. Since glutamine is not known to transfer the ammonia to other substances, its accumulation might be expected. Conversions of glutamate to glutamine cannot be the sole mechanism for ammonia removal because the glutamine actually fell in cases 4c and 8 and rose inadequately in cases 1 and 7b. Bessman (26) believes that this reasoning is incorrect because the kidney deaminates glutamine so rapidly that it cannot accumulate in the blood. However, a lack of increase in glutamine after glutamic acid administration was also demonstrated by Walshe (7) and Singh, Barclay, and Cooke (12).

The route of removal of infused glutamic acid is unknown. The dose used in this study (24 gm.) would represent 2,300 μM per L. in 55 liters, the approximate total body water of patient 2, a man weighing 82 kg. At the end of the infusion the maximum level of glutamic acid was 1,130 μM

per L. and the level fell to less than half of this value 15 minutes after cessation of the infusion (Table IV, Figure 3). At no time were there rises in the glutamine to account for disappearance of this magnitude of glutamic acid. In patient 1 approximately 5 per cent of the infused glutamate appeared in the urine as an increase in alpha amino nitrogen excretion.

The rise in ketoglutarate in eight of the ten cases seemed directly related to the infusion of glutamate (Figure 2). There are two principal routes by which glutamic acid can give rise to ketoglutarate (Figure 4), reaction No. 3 in which ammonia is released, and reaction No. 6, the transamination reaction, in which one molecule of glutamic acid reacts with the keto-acids pyruvic or oxaloacetic to yield, respectively, alanine or aspartic acid and one molecule of ketoglutarate. The latter reaction does not produce ammonia.

The observations in Table IV show that alanine increases after sodium glutamate infusion. This amino acid was measured before and after sodium glutamate infusion in one additional normal person and in three patients in hepatic coma, and it consistently increased in all after the infusion. The details of these observations are reported in a subsequent paper (27). The loss of glutamic acid and the gain of alanine by the plasma suggest that transamination (No. 6, Figure 4) is occurring. Pyruvic acid would therefore be expected to disappear at the same time. This disappearance was not observed, but glucose was being infused rapidly (approximately 700 μM per min.) in these patients and would serve to maintain the pyruvate level. The transamination reaction would also produce ketoglutarate. The ketoglutarate, once formed, may return to glutamic acid by reaction No. 4 and in this process would remove a molecule of free ammonia.

Walshe (7) presented data showing changes in ketoglutarate following glutamate infusions which are similar to these reported here, though he did not consider the rise in ketoglutarate significant. Pyruvic acid and citric acid levels were high in our study and thus confirm the observations that both are high in patients with liver disease (22, 28). However, the levels did not show consistent changes after the infusion of glutamate. Klingmüller and associates (29, 30) studied the disappearance of infused glutamic acid in rabbits. They found that the level of serum glutamic acid returned to normal in two hours after the injection of 2 mM per kg. and it disappeared on a logarithmic curve. This is approximately the dose used in the present study. Klingmüller and his co-workers also found that ketoglutarate and alanine rose in the serum and in their animals the pyruvate level fell.

From these data we believe that glutamate lowers the blood ammonia by more than one mechanism: First, the combination of glutamic acid with ammonia to form glutamine, and second, the conversion of glutamic acid to ketoglutarate by transamination and the subsequent recombination of ketoglutarate with ammonia to form glutamic acid. This conclusion is based to a large degree on our inability to demonstrate a rise in glutamine comparable in each case to the fall in blood ammonia. The glutamine method employed, though not specific for glutamine, would be expected to produce results that are correct or erroneously high, but never erroneously low. The suggestion of Bessman (26) that the kidney was rapidly removing the glutamine by the formation of urinary ammonia was not investigated.

The biochemical changes observed following an infusion of glutamic acid were similar in the normal subjects and the patients with liver disease. Additional data on this point are presented elsewhere (27).

SUMMARY

Sodium glutamate was infused intravenously a total of 13 times in two normal subjects and eight patients with cirrhosis.

1. The blood "ammonia" levels fell in 11 of 12, and the blood alpha-ketoglutarate levels rose in 8 of 10 instances, and the changes in blood pyruvate and citrate were variable.

2. Glutamine levels showed variable changes but did not rise sufficiently in all cases to account for the lowering of the blood "ammonia."

3. The pattern of response to the glutamic acid infusion was similar for normal subjects, patients with cirrhosis, and patients with hepatic coma, although the initial levels of these substances were elevated in the patients with cirrhosis.

4. Only one of seven patients in hepatic coma showed a suggestive clinical response to sodium glutamate infusion.

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Notes on the Use and Effects of Monosodium Glutamate[1]

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THERE are times in the military service when troops must subsist on a restricted diet, especially during war. Under these conditions, field tests have revealed that, in general, soldiers prefer to go hungry rather than eat food which is unacceptable to them. A great deal of effort was therefore expended in developing acceptable foods for troops so that enough nutriment would be consumed to keep them in good physical condition.

Before acceptable rations could become a reality, monotony and insipidity—the two characteristic weaknesses of limited rations—had to be eliminated. Early tests indicated that the flavor of certain ration items were improved when monosodium glutamate was added. Although this work looked promising, it was discontinued during the war because of the limited supplies of this chemical available. It was not until the cessation of hostilities that an adequate exploration of the potentialities of monosodium glutamate in Army rations could be undertaken. As a first step, a symposium sponsored by the Quartermaster Food and Container Institute for the Armed Forces and the Associates Food and Container Institute was held in Chicago (1). This symposium covered the history, production methods, uses, formulation in practice, chemical nature and relationships of this material and its pharmacology.

It is of interest to note that this product has been widely used by the Japanese in their Army rations. Dr. C. A. Fellers, in reporting his war experiences in the Pacific Theater of Operation, has stated (2), "One of the staples of the Jap soldier was canned fish products. They apparently will can any fish whatever. It was all very good, and for a long time we could not understand why. It was always packed with a sauce. What they were using was one of these hydrolyzed soy bean products. They used this gravy in all of their canned fish products, and they also used it in their very low quality beef. In Japan, the cow is used to cultivate the soil and is killed for beef when she grows old. Naturally, this beef is tough. They canned that for their Army and added this same sauce; it was cooked under pressure to make it soft. It has an excellent flavor of the same sort as these fish products. By the way, our own men liked these fish products very much. They didn't mind the old canned cow too much, either."

GLUTAMATE IN FOOD

The active form, *l*-glutamic acid, is made commercially, and the monosodium salt of this form is the one which has the unique property of enhancing flavor when added to foods. According to Crocker (3), monosodium glutamate appears to be entirely without odor when pure, and its taste has all four components: sweetness, sourness, saltiness, and bitterness. He also reports that the meaty association formerly attributed to this substance was due to other components of protein decomposition present in the crude glutamates available.

The optimum proportion used in foods must be determined experimentally, but it will probably lie between 0.1 and 1.0 per cent of the total weight of the food as eaten. The results of experimentation incorporating monosodium glutamate into various foods and food products has effectively been summarized by Cairncross (4) as follows:

> Monosodium glutamate added in small amounts had a pronounced effect on the flavor of practically all foods to which it was added, without itself being noticeable. (Exceptions: It was very noticeable in certain fruits and dairy products.)
>
> The principal effect on food flavor was a balancing, blending, and rounding out of total flavor.
>
> Flavor appeal was frequently improved, most notably in the case of meats, sea foods, stews, soups, and chowders.
>
> Cooked vegetables were markedly improved by glutamate addition, but the following were highest on preference rating: mushrooms, sweet corn, asparagus, broccoli, green and Lima beans, spinach, cauliflower, Brussels sprouts, squash, parsnips, onions, and dehydrated cream style vegetable soups. In carrots and cauliflower, the natural flavor characteristics were intensified. This result suggests application of monsodium glutamate in protecting the flavor of foods held on steam tables.
>
> Fruit, fruit juices, sweet baked goods, some dairy products, and cooked cereals were not considered to be benefited by the use of monosodium glutamate.

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It was noted that glutamate suppressed undesirable flavor factors in the following cases:

1. The sharpness in onion flavor.
2. Rawness in many vegetables and some meats.
3. The flavor of peel and earthiness in vegetables, particularly potatoes.
4. A volatile characteristic noted in boiled rice.
5. Bitter tastes in a few freshly opened canned vegetables.
6. A fishy note sometimes present in Lima beans.

Cairncross summarizes the role of monosodium glutamate in food flavoring as follows: "Glutamate accentuates sweet and salty tastes in food when they are present in less than optimal amounts. In some cases glutamate seems to suppress sourness and bitterness. Glutamate has more influence on fat-free foods than it has when fat is present." He concludes: "We believe that monosodium glutamate should be classified as a seasoning agent, since its action is more of the salt type than the condiment type. Because it can affect the balance of other seasonings, it should be added first. When used judiciously, it is not apparent in cooked foods, and its effect is not duplicated by any other seasoning agent. It can still give zest to a food after all of the recognized art has been applied."

Nair (5) points out two other factors to be considered in the addition of glutamate to foods: (a) the economics of the operation since glutamate is likely to be the most expensive seasoning added to the formula, and (b) possible changes in flavor which may develop in food under various storage conditions. Since the persistent quality of the glutamate flavor is retained indefinitely while other flavors may decrease or increase in strength on storage, the flavor of the food after prolonged storage may be notably different from that obtained initially when glutamate was added to the food.

The above work has demonstrated the many varied and interesting applications of monosodium glutamate to the food industry, and further investigations will in all likelihood lead to even more usages.

PHYSIOLOGIC EFFECT OF GLUTAMIC ACID

Glutamic acid has aroused equally as much if not greater interest in the medical field because of its use in various mental ailments. Glutamic acid is a 5 carbon dicarboxylic amino acid classed as dispensable for man in the sense that it does not need to be supplied in the dietary. It occurs as a constituent of all complete proteins and several important biologically active compounds, such as insulin, glutathione, pteroylglutamic (folic) acid, tumor tissue, tyrocidine and the drugs "Diopterin" and "Triopterin." The *l*-form is more completely and specifically utilized by the body than the *d*-form (6). It is an amino acid which can form glucose or glycogen in the body, and one which has lipotropic activity. Although the tolerance to the natural (*l*-glutamic acid) forms and racemic (*dl*-glutamic acid) forms for lower species, such as rodents, is high, intravenous administration of these forms in mixtures of synthetic amino acids causes a specific toxic effect evidenced by nausea and vomiting in dog and man (7). The glutamic acid in protein digests is also responsible for the toxic symptoms observed when parenteral infusion of the hydrolysates is too rapid.

Glutamic acid holds a unique position among the amino acids in that it is the only amino acid known to be metabolized by brain tissue slices (8). An enzyme present in the brain tissue converts the glutamic acid to glutamine by the addition of ammonia (9). This substance may be used by the brain tissue as a source of energy. Glutamic acid has also been linked with the enzyme system which synthesizes acetylcholine, a compound involved in nerve metabolism and transmission of nervous impulses (10).

The anti-convulsant property of glutamic acid may also be related to its role in brain metabolism and the functioning of the nervous system. Glutamic acid was first introduced for the treatment of epilepsy as a substitute for the unpleasant ketogenic diet, because of its acid-producing property (11).

When glutamic acid was fed to patients suffering with petit mal, urinary *pH* was significantly lowered, but no changes in the acid base balance of the blood was observed (12). It was found that not only did the frequency of the seizures decrease, but that there was an increased mental and physical alertness (13). The mental response was independent of the seizure response, both responses declining when glutamic acid therapy was discontinued. The grand mal type of epilepsy was not benefited by glutamic acid therapy. It is interesting to note that glutamic acid had no effects on chemically or electrically induced seizures in experimental animals (14, 15). At present, glutamic acid has been replaced by more effective drugs in the treatment of petit mal.

The effectiveness of glutamic acid in increasing the physical and mental alertness has also been demonstrated in other types of mental deficiencies not associated with epilepsy and also in normal animals (16, 17). Rats fed a normal diet plus glutamic acid exhibited greater speed in maze learning than those fed the normal diet alone (17). Similarly, rats receiving the glutamic acid were able to learn more complex mazes than those on the control diet alone (16). Recent reports, however, indicate that these findings could not be confirmed by other workers (18–20). Both the speed and the level of the complexity of the learning were similar for the rats fed the normal diet and for those fed the normal diet plus additional glutamic acid. A possible explanation offered by the authors for the discrepancy in results is the differences in strain of the animals used, the Sherman strain responding to the glutamic acid (20). Further work is, therefore, needed to elucidate the role of glutamic acid in learning.

Mentally-retarded patients, both children and

adults, showed significant increases in intelligence quotient as a result of glutamic acid therapy (21–23). When placebos were substituted for the glutamic acid tablets, the I.Q. declined and was again raised when the glutamic acid therapy was resumed. In these patients there was improvement in personality and verbal ability and performance. The evaluation of such studies depends on the validity and reliability of the intelligence measurements available. It is indicated by Pfeiffer (6) that "before a general program of glutamic acid therapy can be introduced for the mentally deficient individuals or the public at large, the answers to the following questions should be known: (a) Is the increase in I.Q. real or due to better application and cooperation during the test? (b) Are subjects with normal intelligence benefited by glutamic acid therapy? (c) Will nervous disorders be increased as indicated by the side actions of restlessness and insomnia? (d) What is the effect of large and continued daily dosage of glutamic acid on longevity? (e) Is the glutamate effect modified by the other amino acids in casein? (The recent work of Christensen *et al.* [13] indicates that individual amino acids compete with each other for concentration into body cells from body fluids.) (f) Are cancerous growths or cell rests matured by glutamic acid?"

On the basis of the effectiveness of glutamic acid in increasing learning ability in rats and raising the mental age of retarded patients, there has been much speculation on the possibility of increasing the intelligence of already normal persons. In this connection glutamic acid has been referred to as a "brain food" in some of the popular periodicals (24, 25). Although glutamic acid may some day be proved to have a role in producing mental giants, at present, its action is generally interpreted as resulting not in new intellectual tools but in improving existing intellectual facilities which have been dulled or damaged from the normal state by injury or disease or inhibited by emotional mechanisms (11).

In conclusion glutamic acid has been shown to have some effect in raising the intellectual and physical output of certain mentally retarded individuals. Its ability to increase the speed and level of complexity of learning by rats needs further elucidation before such a role can definitely be established. The establishment of the specificity of glutamic acid in performing these functions will take long and tedious research. The mechanism in which glutamic acid functions is still unknown, except that it is in some way involved in brain metabolism and intimately connected with the functioning of the nervous system. Whether the glutamic acid acts as a drug or as a result of the interaction with other amino acids or related compounds is also unsolved as yet. The work thus far has opened up new fields of research in that it has shown that brain function may be improved by biochemical means in addition to social and educational methods (11).

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Convulsions induced in 10-day-old rats by intraperitional injection of monosodium glutamate and related excitant amino acids

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THERE HAS been considerable controversy concerning the safety of monosodium glutamate as a food additive, due largely to the findings of degenerative lesions in the retina and hypothalamus of infant mice following subcutaneous injection of this acidic amino acid salt.[1] L-Glutamate, like many other acidic amino acids, directly excites mammalian central neurones when applied by microelectrophoresis.[2] The acidic amino acid, β-*N*-oxalyl-L-α,β-diaminopropionic acid (ODAP), is a more powerful excitant of central neurones than is L-glutamate;[3] ODAP occurs in the seeds of *Lathyrus sativus* and has been implicated as the agent responsible for the crippling disease, neurolathyrism, which sometimes results from the consumption of these seeds.[4] Several excitant amino acids, including L-glutamate and ODAP, produce convulsions on suitable injection into mammals, providing that the blood–brain barrier is relatively inefficient as in immature or acidotic animals,[5] or by-passed by the route of administration.[3,6,7] In the present investigation a comparison was made of the convulsant activity of L-glutamate, ODAP and other known excitant amino acids in 10-day-old rats following intraperitoneal injection.

The amino acids were administered to unanaesthetized 10-day-old rats (*Rattus norvegicus*), weighing between 16 and 23 g, by intraperitoneal injection of neutral aqueous solutions (up to 1 M, neutralized where necessary with sodium hydroxide). Adult rats (13-weeks-old, 180–200 g) were similarly injected. The highest dose used was 20 mmoles/kg body wt. Control injections of sodium chloride solutions of the appropriate molarity were used. *N*-Methyl-D- and L-aspartate were prepared by Dr. J. C. Watkins.[8] β-*N*-Oxalyl-L-α,β-diaminopropionic acid and *N*-oxalyl-β-alanine were gifts from the late Prof. P. S. Sarma (Bangalore), and ibotenic acid was a gift from Prof. C. H. Eugster (Zürich). The other amino acids were purchased from commercial suppliers.

All of the excitant amino acids tested produced convulsions in the 10-day-old rats, as listed in Table 1. These convulsions were characterized by repeated tonic seizures, involving all limbs, head and tail, and which were of several minutes duration. The time of onset of these seizures was dose-dependent, and was used to compare the relative potencies of these excitant amino acids.

ODAP was comparable with DL-homocysteate as a convulsant, and weaker than *N*-methyl-D-aspartate and ibotenate, at doses of 1 mmole/kg of these amino acids. L-Aspartate, D- and L-glutamate

TABLE 1. CONVULSIONS INDUCED IN 10-DAY-OLD RATS BY INTRAPERITONEAL INJECTION OF AMINO ACIDS

Amino acid	Dose (mmoles/kg)	Time of onset of convulsions (min)
(a) Amino acids known to directly excite central neurones:		
N-Methyl-D-aspartate	0·5	9·3 ± 0·7
N-Methyl-D-aspartate	1	4·8 ± 0·9
Ibotenate	1	7·8 ± 0·5
β-*N*-Oxalyl-L-α,β-diaminopropionate	1	13·5 ± 1·8
β-*N*-Oxalyl-L-α,β-diaminopropionate	2	4·5 ± 1·2
DL-Homocysteate	0·5	34·2 ± 3·0
DL-Homocysteate	1	14·3 ± 0·7
DL-Homocysteate	2	5·0 ± 0·9
DL-Homocysteate	5	3·7 ± 0·1
DL-Homocysteate	10	3·8 ± 0·7
DL-Homocysteate	20	3·3 ± 0·6
N-Methyl-L-aspartate	1	18·3 ± 0·6
L-Glutamate	10	*
L-Glutamate	20	30·1 ± 8·6
D-Glutamate	10	*
D-Glutamate	20	33·3 ± 1·5
L-Aspartate	10	*
L-Aspartate	20	36·3 ± 7·6
(b) Amino acids known to directly depress central neurones:		
γ-Aminobutyric acid	20	†
Glycine	20	†
DL-C-Allylglycine	2	57·5 ± 5·4
(c) Amino acids with no direct action on central neurones:		
DL-Methionine-DL-sulphoximine	2	290 ± 17
N-Oxalyl-β-alanine	20	*

Values are means ±S.E. of results from 4 animals. * No apparent effects up to 3 hr after injection, † Generalized depression after ca. 5 min.

were inactive at this dose, these amino acids producing convulsions only after some 30 min at the highest dose tested of 20 mmoles/kg.

As excitants of feline central neurones, ODAP is more potent than DL-homocysteate and only slightly less active than *N*-methyl-D-aspartate, the strongest amino acid excitant yet reported.[3] Ibotenate is comparable with DL-homocysteate in potency,[9] whereas L-aspartate, D- and L-glutamate are weaker excitants.[2] Ibotenate, and the related isoxazole, tricholomate, which is also a neuronal excitant,[10] have flavours twenty times more intense than monosodium glutamate.[11] The possible use of these isoxazoles in food formulation has been discussed.[12] It seems possible that the flavour and taste enhancing properties of these amino acids involves depolarization of peripheral taste receptors, analogous with the depolarization of central neurones by excitant amino acids.

While ODAP is thus somewhat weaker as a convulsant than might be expected from its potency as an excitant of feline neurones, these results are compatible with the view that the convulsant and excitant activities of these acidic amino acids have the same basic mechanism, i.e. the direct depolarization of central neurones. Injection of amino acids (γ-aminobutyric acid and glycine) which have a direct hyperpolarizing action on neurones[13] produced a generalized depression of the activity of the 10-day-old rats.

DL-C-Allylglycine, which is a weak depressant of neuronal firing,[14] produced seizures in the 10-day-old rats which were very similar to those produced by the excitant amino acids, but of longer latency. Both the dose required and the time of onset of these seizures are comparable with those reported for adult rats.[15,16] DL-Methionine-DL-sulphoximine also produced convulsions in the 10-day-old rats similar to those reported in adult rats with respect to dose and time of onset.[17] The convulsant actions of DL-C-allylglycine and DL-methionine-DL-sulphoximine have been related to inhibition of glutamate-metabolizing enzymes in adult animals.[17–19] In the 10-day-old rats, some of the excitant amino acids appeared to be more powerful convulsants than DL-C-allylglycine and DL-methionine-DL-sulphoximine, but none of the excitant amino acids produced convulsions in the adult rats used in the present experiments. The adult rats also appeared to be unaffected by injections of γ-aminobutyric acid and glycine. These observations may be interpreted on the basis of blood–brain barrier(s) protecting the adult animals from certain amino acids in the blood stream, or on the basis of other factors such as the levels of pyridoxal 5′-phosphate and the activity of various metabolizing enzymes[20] and transport systems.

A number of excitant amino acids in addition to L-glutamate are now known to produce neuronal damage in the hypothalamus of infant mice following subcutaneous injection.[21] Olney and his colleagues[21] have pointed out that "the close correspondance in molecular specificity associated with neurotoxic and neuroexcitatory properties of simple amino acids suggest the two phenomena may be governed by similar mechanisms of action". The present study indicates that the neuroexcitatory properties are also directly related to the ability of these amino acids to produce convulsions in 10-day-old rats. It is suggested that any excitant amino acid should be suspect as a food additive.

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TOXICOLOGICAL EVALUATION OF SOME EXTRACTION SOLVENTS AND CERTAIN OTHER SUBSTANCES

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MONOSODIUM GLUTAMATE

Biological data

Biochemical aspects

L-glutamic acid occurs as a common constituent of proteins and protein hydrolysates and can be synthesized by the rat and rabbit from acetate fragments. Human plasma contains 4.4 - 4.5 mg/l of free glutamic acid and 0.9 mg/100 ml of bound glutamic acid. Human urine contains 2.1 - 3.9 µg/mg creatinins of free glutamic acid and 200 µg/mg creatinins of bound glutamic acid (Peters et al., 1969). Human spinal fluid contains 0.34-1.64 (mean 1.03 mg/l) free glutamic acid (Dickinson & Hamilton, 1966). Human milk contains 1.2% protein of which 20% is bound glutamic acid which is equivalent to 3g/l calculated as sodium glutamate. The free glutamic acid concentration is 300 mg/l. In contrast cows milk contains 3.5% protein equivalent to 8.8 g/l calculated as MSG, but only 30 mg/l free glutamic acid (Maeda et al., 1958; 1961). Strained infant foods provide 80 Cal/100g with wide variations depending on the recipe, while human milk provides 70 Cal/g (Dept. Health & Soc. Sec., 1970).

Infant food may contain up to 0.4% added MSG, the natural content depending on the basic constituents. Carrots contribute 0.32% free glutamic acid calculated as MSG, tomatoes, 0.45% and cheese 0.7%. Substitution of some unprepared foods in equal weights for prepared baby foods containing 0.3% added monosodium glutamate would also result in an ingestion of greater amounts of glutamate than is provided by mothers' milk on a calorie for calorie basis. The level of 0.3% in prepared foods appears to be an upper level since higher concentrations impart an unpleasant flavour.

Infants aged 3 days and weighing 3kg consume 480 g mothers' milk/day. This is equivalent to a daily intake of 1.104 g bound glutamic acid, and 0.115 g free glutamic acid corresponding to 0.408 g/kg body-weight of glutamic acid/day. One-month old infants, weighing 3.8 kg consume 600 g mothers' milk/day. This is equivalent to a daily intake of 1.37 g of bound glutamic acid and 0.144 g free glutamic acid corresponding to 0.405 g/kg body-weight of glutamic acid/day.

Infants aged 5-6 months, weighing 7.5 kg, consume 500 g cows milk and 2 jars of baby food/day. The respective daily intake of bound glutamic acid amounts to 3.5 g and 0.5 g. The corresponding free glutamic acid intake is 0.015 g and 0.060 g/day, which is equivalent to 0.62 g/kg body-weight of glutamic acid/day. If the 2 jars (200g/jar) contain 0.3% MSG, this increases the total intake of free glutamic acid from 0.06 to 0.60 g. In a seven day survey of children aged 9 to 12 months the intake of baby foods has been observed to range from zero (in 20% of the surveyed cases) to a maximum of 250 g daily in which up to 12 different preparations may be represented and not all of which have monosodium glutamate added (Berry 1970).

There is evidence of rapid absorption of dietary glutamate since in rats the glutamic acid level in portal blood rose within 1/2-3/4 hour to 250 per cent. in adults and 150 per cent. in young animals over the testing level (Wheeler & Morgan, 1958). L-glutamic acid absorption by the dog failed to increase noticeably the amino-N_2 of the peripheral blood but increased that of portal blood, possibly because of increased uptake by tissue (Christensen et al., 1948).

Groups of 8 rats were given by gavage 200 mg/kg body-weight of MSG alone or with 2000 mg/kg raw veal. Blood samples were taken at 10 minute intervals and after 30 minutes the animals were killed and free glutamic acid determined in blood and brain. Plasma glutamic acid rose rapidly to a peak in 20 minutes if monosodium glutamate was given alone and more slowly to a peak in 30 minutes if given with veal. Using 100, 500 and 2500 mg/kg orally produced a dose-related increase in plasma level only at the two higher test levels and more if monosodium glutamate was given alone. There was no effect on the brain glutamate acid levels. Using s.c. 500 mg/kg body-weight produced the same plasma levels as oral feeding. There was no adaptation. Brain levels were not affected. 100 mg/kg monosodium glutamate was the threshold dose before plasma levels rose. There was great variability in the response (McLaughlan et al., 1970). Continuous infusion of dogs with glutamic acid (05-4 mg/kg/hr) did not result in entry of glutamic acid into liver and muscle cells, cerebrospinal fluid or brain. Kidney cells appeared to be freely permeable. Metabolism of the infused glutamic acid was limited (Kamin & Handler, 1950).

The intact rat as well as rat liver and rat tissues metabolize glutamate by oxidative deamination (von Euler et al., 1938) or transamination to oxaloacetic or pyruvic acid (Cohen, 1949) via alpha-ketoglutarate to succinate (Meister, 1965). This was shown by the use of 2-C^{14}-labelled DL-glutamic acid given i.p. and resulting in the production of aspartin acid labelled in the -COOH radical and glutaric acid labelled in position 1-C and 2-C. Intracaecally administered 2-C^{14}-labelled DL-glutamic acid is rapidly converted to acetate, labelled in the methyl group, by the messaconate and citramalate cycle. After gastric intubation of 2C^{14}-labelled DL-glutamic acid part is absorbed and metabolized to succinate, the rest to methyl-labelled acetate (Wilson & Koeppe, 1959). Rat tissue has only a poor ability to oxidise D-glutamate. After i.p. or s.c. administration conversion to D-pyrrolidone carboxylic acid occurs. Rat liver and rat kidney also convert enzymatically D-glutamic acid to D-pyrrolidone carboxylic acid (Wilson & Koeppe, 1961). The specific ensyme was isolated from the liver and kidney of mice, rats and man (Meister et al., 1963). Oral administration of C-monosodium L-glutamate (2g/kg) to weanling rats caused a marked increase in the specific activity of liver carbamyl phosphate synthesase. Prolonged administration resulted in a return to control values, indicating an adaptation to the administered substrate (Hutchinson & Labby, 1965). Biochemical aspects are summarized in recent reports (Ajinomoto Co., 1970).

I.v. injection of C^{14}-labelled glutamic acid into intact rats and mice showed it to enter rapidly the brain, liver, kidney and muscle as such (Lajtha et al., 1959). Glutamic acid was shown to be distributed among more than one metabolic pool as animals mature (Berl, 1965). Compartmentation of glutamate metabolism in the mouse brain has been demonstrated by examining the time course of C^{14} incorporation into glutamine and glutamate (Van den Berg, et al. 1968).

I.v. injection of sodium P-glutamate produced a prolonged increase in the amino-nitrogen content of blood and prolonged urinary excretion in rabbits (Yamamura, 1960). Other effects observed were a decrease in tryptophan and tyrosine metabolism of the liver following daily injection of 1-4 g/kg glutamic acid into rats (Fumiwake, 1957) a reduction in the activity of liver catalase in mice after single injections of D-glutamic acid at 1.5 mg/g reverting to normal after 4 days and not observed with L-glutamic acid (Ando, 1958), enhanced oxygen consumption by rats after injection of 1 mg/kg sodium glutamate at low pO_2, not observed at normal pO_2 (Genkin & Udintsev, 1957) and hyperglycaemia after i.p. glutamate in rats due to conversion to glucose and

additional stimulation of gluconeogenesis (Marcus & Reaven, 1967). The effect on cerebral metabolism was studied by intraventricular injection of L-glutamic acid into mice, when 150 mg produced convulsions or only inco-ordinated grooming or circling of the cage (Crawford, 1963). Two per cent. intra-arterial sodium glutamate increased epileptic fits and intracisternal L-glutamic acid caused tonic-clonic convulsions in animals and man. High parenteral dosage of L-glutamic acid caused EEG changes only in dogs with previous cerebral damage and no rise was detected in the CSF level of glutamate (Herbst et al., 1966). L-glutamic acid is oxidized by the brain to alpha-ketoglutaric acid, NH_3 and later CO_2 and H_2O and is the only amino acid that on its own can maintain brain slice respiration (Weil-Malherbe, 1936). Decarboxylation to γ-aminobutyric acid is significant in the mammalian brain (Roberts, 1951; Perrault & Dry, 1961).

I.v. injection of large doses of glutamic acid in rabbits, caused ECG changes that could be interpreted as symptoms of myocardial lesions. Arterial hypertension induced by glutamic acid preparations was demonstrated to be of central origin. Studies with isolated cat heart showed that large doses of glutamic acid slowed heart action, increased systolic amplitude and constricted coronary vessels. Very large doses stopped cardiac action (Mazurowa, et al., 1962).

Nutritional studies in the rat have shown glutamic acid to be a non-essential amino acid replaceable by others and to be required in substantial amounts to ensure high growth rates in rats (Hepburn et al., 1960). Some interconversion between glutamic acid and arginine can occur to cover minor dietary deficiencies (Hepburn & Bradley, 1964). Gouty patients have raised levels of plasma glutamate compared with normals and following a protein meal glutamate reaches excessive levels (Pagliari & Goodman, 1969). Premature and full term infants hydrolyse any given protein in the stomach to very similar extents (Berfenstam et al., 1955). Hepatic glutamate dehydrogenase appears at 12 weeks of human foetal life, is present in rat foetal liver on day 17 and reaches its maximum within 2 weeks after birth (Francesconi & Villee, 1968).

Fifty patients with circulatory hypoxia received orally 1 g three times/day of glutamic acid for 1 week. All patients showed less blood lactic acid, a better alkali reserve and clinical improvement (Gorbunova et al., 1960). 15 g equivalents of unneutralized L-glutamic acid, L-glutamic acid-HCl and monosodium glutamate were given orally to man. There was little absorption of the poorly soluble L-glutamic acid with very slight elevation of blood levels within one hour from 20 to 80 mg/l. Little absorption occurred with L-glutamic and acid-HCl, but monosodium glutamate was well absorbed, the blood level rising in one hour from 20 to 350 mg/l (Himwich, 1954). 6-10 male healthy children were given 1, 2 and 4 g sodium glutamate. Total creatinine excretion was not affected but the amino acid/creatinine ratio increased much more than the glucuronic acid/creatinine ratio. Values returned to normal within 36 hours after 1-2 g and within 69 hours after 4 g (Inoue, 1960). Sodium glutamate has been used therapeutically in uraemia to reduce blood levels of ammonia. 8 g of i.v. glutamic acid caused nausea and vomiting in 11 from 17 individuals (Smyth et al., 1947).

Introduction of 0.15 per cent. glutamate solution into the small intestine of the dog did not cause a rise in glutamate concentration in the blood draining the intestinal loop. Only at 0.5 per cent. did the venous blood contain extra glutamate. However, alanine appears in high concentration in the portal blood. When this mechanism is overwhelmed then glutamate appears also over and above the arterial blood level (Neame & Wiseman, 1957). In the

cat and rabbit in vivo the same phenomenon occurs (Neame & Wiseman, 1958) and in the rat in vitro (Matthews & Wiseman, 1953) and in vivo (Peraino & Harper, 1962). Further removal of excess portal glutamate and alinine occurs in the liver. In man only 2 out of 4 subjects given 0.1 g/kg glutamic acid as a 7 per cent. solution orally showed an appreciable rise of free glutamic acid in plasma. Hence a similar mechanism may operate.

Additional glutamic acid, e.g. 10-20 g, if given to man, may raise the amount of glutamic acid absorbed from ingested protein. (Bessmann et al., 1948). Bound glutamate from proteins and polypeptides is realeased gradually during digestion and would be absorbed as alanine into the portal blood (Wiseman, 1970). L-glutamic acid and DL-glutamic acid are absorbed orally by the rat to nearly the same extent, L- being a little better absorbed (Aroskar & Berg, 1962). The foetal circulation has a higher amino acid concentration than the maternal in the rhesus monkey (Kerr & Waisman, 1967).

Monosodium glutamate (8g/kg body-weight) was administered orally to pregnant Wistar-Imamichi rats on day 19 of gestation. Plasma glutamic acid was determined in mothers and foetuses, at 30, 60 and 120 min. after dosing. In the mothers' plasma, glutamic acid increased from approx. 100 µg/ml to 1650 µg/ml, in the first 30 minutes. At the end of the test period the level was 1000 µg/ml. No significant changes occurred in the plasma glutamic acid of foetuses during this period (approx. 50 µg/ml) (O'hara et al., 1970a).

Rats (Wistar-Imamichi), male adults (11-14 weeks of age) and male neonates (2-3 days of age), were dosed orally with monosodium glutamate (0.5-8 g/kg body-weight for adults, and 0.5-4 g/kg body-weight for neonates). Plasma glutamic acid was measured over a 4 hour period. For adult rats, the highest level tested showed maximum plasma glutamic acid, 1650 µg/ml, after 30 minutes. At the other dose levels there were no appreciable changes in plasma glutamic acid. In the case of neonates, levels rose to a maximum of 350 µg/ml after 90 minutes at the 2 g/kg dose level, and 1850 µg/ml after 90 minutes at the 4.0 g/kg dose level. In a similar study with mice (4CS strain) plasma glutamic acid of adults rose to a maximum of 530 µg/ml after 30 minutes, at the 2 g/kg dose level, and 1030 µg/ml at the 4 g/kg dose level. Neonate mice showed maximum level of plasma glutamic, 700 µg/ml 30 minutes after treatment at the 2 g/kg dose level, and 2300 µg/ml, 2 hours after treatment at the 4 g/kg dose level, (Ichimura et al., 1970c). Another study showed that there was a marked correlation between liver SOT, SPT and plasma glutamic acid of rats and mice dosed orally with 1 g/kg body weight monosodium glutamate. Measurements were made during the period 1-100 days of age. (Hashimoto et al., 1970).

When monosodium glutamate (1 g/kg body-weight) or monosodium glutamate (1 g/kg body-weight) plus powdered milk (1.5 g/kg body-weight) or powdered milk (1.5 g/kg body-weight) was administered orally to 10 day old rats, the maximum levels of plasma glutamic acid were 425 µg/ml, 160 µg/ml and 105 µg/ml respectively. These levels occurred 30 minutes after dosing (O'hara et al., 1970b).

Acute toxicity

Animal	Route	LD_{50} mg/kg body-weight	References
Mouse	i.p.	6900	Yanagisawa et al., 1961
	p.o.	12961	Izeki, 1964
	p.o.	16200 (14200-18400)	Ichimura & Kirimura, 1968
	i.v.	30000	Ajinomoto Co., 1970
Rat	p.o.	19900 (L MSG)	International Minerals & Chem. Corp., 1969
	p.o.	10000 (DL MSG)	International Minerals & Chem. Corp., 1969
	p.o.	> 30000 (L-GA)	International Minerals & Chem. Corp., 1969
Guinea-pig	i.p.	15000	Ajinomoto Co., 1970
Rabbit	p.o.	> 2300 (L-GA)	International Minerals & Chem. Corp., 1969
Cat	s.c.	8000	Ajinomoto Co., 1970

Mouse. Mice aged 2 to 9 days were killed 1 to 48 hours after single subcutaneous injection of monosodium glutamate at doses from 0.5-4 µg/kg, lesions seen in the preoptic and arcuate nuclei of the hypothalamic region on the roof and floor of the third ventricle and in scattered neurons in the nuclei tuberales. No pituitary lesions were seen but sub-commissural and subfornical organs exhibited intracellular oedema and neuronal necrosis. Adult mice given subcutaneously 5-7 µg/kg monosodium L-glutamate showed similar lesions. Similar lesions were seen in another strain of mouse and in neonatal rats (Olney, 1969b).

After a single subcutaneous injection of monosodium glutamate at 4 g/kg into neonatal mice aged 9-10 days, the animals were killed from 30 minutes to 48 hours. The retinas showed an acute lesion on electron microscopy with swelling dendrites and early neuronal changes leading to necrosis followed by phagocytosis (Olney, 1969a).

Sixty-five neonatal mice aged 10-12 days received single oral very high loads of monosodium glutamate at 0.5, 0.75, 1.0 and 2.0 g/kg body-weight by gavage. 10 were controls and 54 mice received other amounts. After 3-6 hours all treated animals were killed by perfusion. Brain damage as evidenced by necrotic neurons was evident in arcuate nuclei of 51 animals. 62 per cent. at 0.5 g/kg, 81 per cent. at 0.75 g/kg, 100 per cent. at 1 g/kg and 100 per cent. at 2 g/kg. The lesions were identical both by light and electron microscopy to s.c. produced lesions. The number of necratic neurons rose approximately with dose. Four animals tested with glutamic acid also developed the same lesions at 1 g/kg body-weight. The effect was additive with aspartate (Olney, 1970b).

Groups of five 3-day and 12-day old mice receiving subcutaneously or orally a single acute dose (1g/kg) monosodium glutamate, monopotassium glutamate, sodium chloride, sodium gluconate or distilled water, were sacrificed 3 hours and 24 hours after treatment. Preliminary light microscopic studies of the large mid brain area, showed similar non-specific scattered tissue changes in all treatment groups. (Oser et al., 1970). In another study, mice, 5-9 days old, received a single dose of monosodium glutamate (4 g/kg in phosphate buffer), either subcutaneously or orally.

Animals were sacrificed at 24 hours. Light microscopy of the hypothalmic area of the brain, indicated abnormal neuronal cells in 12/30 of the mice receiving a subcutaneous injection of the test substance. Only 5/35 mice receiving the oral dose showed some change. (Coulston et al., 1970). Six, nine to ten day old mice, dosed orally with 10% monosodium glutamate (2 gm/kg), showed characteristic brain lesions (Geil, 1970).

Monosodium glutamate caused reversible blockage of beta wave in the electroretinogram in immature mice and rats indicating retinotoxicity (Potts et al., 1960). The timing of treatment of mice was quite critical. After 10 to 11 days postnatal age, it was difficult to produce significant lesions of the retina (Olney, 1969a). A study of the glutamate metabolizing enzymes of the retina of the glutamate treated rat indicated a decrease in glutaminase activity, an increase in glutamic aspartate transaminase, and no change in glutamyl synthetase and glutamotransference. The effects appear to be due to repression and induction of enzyme synthesis (Freedman & Potts, 1962; Freedman & Potts, 1963). Glutamate uptake by retina, brain and plasma decreases with age and is slower at 12 days when compared with 50 day old animals (Freedman & Potts, 1963).

Obesity and acute irreversible degeneration in liver and retina of neonatal mice has been seen following parenteral administration of monosodium glutamate (Cohen, 1967). S.c. injection of L-monosodium glutamate at 4-8 g/kg into mice caused retinal damage with ganglion cell necrosis within a few hours. In very young animals there was extensive damage to the inner layers (Lucas & Newhouse, 1957).

Rat. Groups each of 20, ten day old rats, Charles River Strain, (10 male, 10 female) were dosed orally with 0.2 ml of either strained baby food containing no monosodium glutamate, strained baby food containing monosodium glutamate up to 0.4%, or strained baby food containing monosodium glutamate equal to a dosage level of 0.5 mg/kg, additional to that found in normal commercially distributed baby food (390 mg per jar). The rats were mated and half of the offspring were removed from parental females, and sacrificed after 5 hours. Histological studies were made of brain in the area of the hypothalmus at the roof and the floor of the third ventricle. The remaining rats were returned to parental females and allowed to grow to maturity (90 days post weaning), then sacrificed, and histological studies made of the brain. No lesions were observed in the brain of animals sacrificed at either 5 hour post treatment, or after reaching maturity. Animals which were reared to maturity showed normal growth and food consumption (Geil, 1970).

Groups of 5 3- and 12-day old rats receiving subcutaneously or orally a single acute dose (1g/kg) monosodium glutamate, mono potassium glutamate, sodium chloride, sodium gluconate or distilled water, were sacrificed 24 hours after treatment. Preliminary light microscopic studies of the large midbrain area showed similar non-specific scattered tissue changes in all treated groups (Oser et al., 1970).

Dog. Intravenous casein hydrolysate or synthetic amino acid mixture caused nausea and vomiting in dogs (Madden et al., 1944). Groups of 3 dogs at 3 days or 35 days of age received subcutaneously or orally a single acute dose (1 g/kg) monosodium glutamate, monopotassium glutamate, sodium chloride, sodium gluconate or distilled water; and were sacrificed 3 hours and 24 hours after treatment. Preliminary light microscopic studies of the large midbrain area showed similar non-specific scattered tissue changes in all treated groups (Oser et al., 1970).

Monkey. A newborn (8 hours old) rhesus infant, probably somewhat preature was given subcutaneously 2.2 g/kg body-weight monosodium glutamate. After 3 hours (no abnormal behaviour noted) the monkey was killed and the brain perfused in situ for 20 minutes. A lesion was seen in the periventricular arcuate region of the hypothalamus identical to those seen in mice given similar treatment. Electron microscopic pathological changes were seen in dendrites and neuron cells but not in glia or vascular components (Olney & Sharpe, 1969). Monkeys, 4 day old, received a single dose of monosodium glutamate (4 g/kg in phosphate buffer), either subcutaneously or orally. Animals receiving subcutaneous injections were sacrificed at 3, 24 and 72 hours, the one receiving an oral dose at 24 hours. No brain lesions were observed. (Coulston et al., 1970).

Man

Pharmacological effects were studied in 56 men given 1-12 g monosodium L-glutamate orally on an empty stomach. Burning of the face and trunk, facial pressure and chest pain were noted as well as headache, the last sometimes as the only symptom. Amounts of 3 g or less were effective in all. Similar effects were obtained by 3-5 g of monopotassium glutamate, L-glutamic acid and DL-glutamic acid but no effects were seen with monosodium D-glutamate or other L-aminoacids. Thirteen subjects received i.v. 25-125 mg sodium glutamate with symptoms occurring within 20 seconds. The burning sensation is due to a peripheral mechanism and no genetic predisposition was noted (Schaumburg et al., 1969). A survey was made of 912 Japanese individuals to determine if any of these symptoms were noted after eating a prepared Oriental Type Noodle, containing 0.61-1.36 g monosodium glutamate/serving. In no case were any of the characteristic symptoms reported (Ichimura et al., 1970a). In another study, the effect of monosodium glutamate on 61 healthy men was determined by the double blind method. The doses of monosodium glutamate administered were 2.2 g, 4.4 g or 8.7 g. Intake was either on a non-empty stomach (30 minutes after meal) or an empty stomach (overnight fast). In experiments on the non-empty stomach conditions the number of persons showing some symptoms were the same for the Placebo and the others. In the case of the empty stomach conditions a number of the test subjects on the highest level of monosodium glutamate experienced two of the typical symptoms at the same time. No individual experienced three of the symptoms. The effect of monosodium glutamate intake (2.2 g, 4.4 g or 8.7 g) on changes in blood pressure, pulse rate, ECG, and sodium and glutamate levels in blood, was measured in 5 persons who had not experienced any symptoms, and 9 who had experienced some symptoms. There were no differences in increase in glutamic acid in the blood in either group. Sodium content of the blood and all other parameters measured showed no changes in either group (Ichimura et al., 1970b).

The occurrence of nausea and vomiting following the i.v. administration of various preparations in a series of 57 human subjects was found to parallel the free glutamic acid content of the mixture. There was a direct relationship between free serum glutamic acid and the occurrence of toxic effects, following i.v. administration. When the serum glutamic acid reached 12 to 15 mg/100 ml, nausea and vomiting occurred in half the subjects. Other amino acids appear to potentiate the effect (Levey et al., 1949).

Intravenous glutamic acid (100 mg/kg) produces vomiting (Madden et al., 1945).

Intravenous solutions of 2.9 per cent. monosodium glutamate in 5 per cent. dextrose are given in hepatic coma but too rapid injection causes salivation, flushing and vomiting; afterwards oral doses of 5-20 g are given

daily. High doses (3 g) are said to produce Kwok's disease, pain in the chest, tingling sensations or temporary numbness of back and arms, weakness and palpitation in susceptible people (Kwok, 1968). 25 g have no effect in non-sensitives (Schaumburg & Byck, 1968).

Arginine glutamate may be used in the treatment of ammonia intoxication. It is given by intravenous infusion in doses of 25 to 50 g every 8 hours for 3-5 days in dextrose and infused at a rate of not more than 25 g of arginine glutamate over 1-2 hours. More rapid infusions may cause vomiting (Martindale, 1967).

Single and double blind studies were done with single oral doses of monosodium glutamate in human male volunteers on a fasting stomach (18 hours after last meal). 98 received 5 grams of monosodium glutamate in single blind studies, 6 received 8 grams and 5 received 12 grams in double blind studies. Physical examinations were done on all subjects. Complaints were registered in all groups ranging from 23-80%. There was a low incidence of most complaints except for lightheadedness and tightness in the face. No subject reported or was observed to have experienced the complete triad of symptoms as described in the original Chinese-Restaurant syndrome (Kwok's disease). In the double blind studies where clinical chemistry, blood pressure and pulse were measured in addition to clinical examination, no significant differences between monosodium glutamate and sodium chloride were detected (Rosemblum et al., 1969).

Short-term studies

Mouse. 38 neonate mice were observed for 9 months. 20 received subcutaneous monosodium glutamate daily for 1 to 10 days in doses of 0.5 g/kg to 4 g/kg. 18 were controls. Although treated animals remained skeletally stunted and both males and females gained more weight than controls from 30 to 150 days yet treated animals consumed less food than controls. Test animals were lethargic, females failed to conceive but male fertility was not affected. At autopsy of test animals massive fat accumulation was seen in test mice, fatty livers, thin uteri and adenohypophysis had overall fewer cells in the adenohypophysis.

10 test neonates received a single subcutaneous injection of 3 g/kg monosodium glutamate 2 days after birth, 13 neonates were controls. Again test animals were heavier than controls after 9 months but less so than mice given repeated injection treatment. It was postulated that an endocrine disturbance would lead to skeletal stunting, adiposity and female sterility. Lesions differed from those due to gold thioglucose or bipiperidyl mustard which affect the ventro-medial nucleus and cause hyperphagia (Olney, 1969b).

Rat. Natural monosodium L-glutamate, synthetic monosodium L-glutamate, and synthetic monosodium D-glutamate in amounts of 20, 200 and 2000 mg/kg body-weight were given orally to groups of 5 male rats each once a day for a period of 90 days. No effects on body-weight, growth, volume and weight of cerebrum, cerebellum, heart, stomach, liver, spleen and kidneys in comparison with the control group were observed. No histological changes in internal organs were found by macroscopic and microscopic examination (Hara et al., 1962).

Nine groups of 20 rats were given 0.5 per cent. and 6 per cent. of calcium glutamate in their diet. No effect was noted on maze learning or recovery from ECT shock (Porter & Griffin, 1950). Two groups of 14 rats received 200 mg L-monosodium glutamate per animal for 35 days. No difference

in their learning ability for maze trails was noted (Stella & McElroy, 1948). 8 male rats fed 5% dietary DL-glutamic acid in a low protein diet (6% protein) showed little or no depression of growth, when compared to low protein controls. There was a 50% increase in the free glutamic acid in the plasma (Sauberlich, 1961).

Man. Monosodium glutamate has been used in the treatment of mentally retarded children in doses up to 48 g daily but on average 10-15 g was given.

150 children aged 4-15 years were treated with glutamic acid for six months and compared with 30 controls. There was a rise in verbal intelligence quotient but was not statistically significant. 64 per cent. showed improvement of behavioural traits (Zimmerman & Burgemeister, 1950).

17 patients received up to 15 g monosodium glutamate three times a day but showed a raised blood level for 12 hours only. No effect on BMR, EEG, ECG, BP, heart rate, respiration rate, temperature and weight was noted over 11 months (Himwich et al., 1954a). 15 g then 30 g monosodium glutamate were given per dose for one week each, followed by 45 g for 12 weeks to 53 patients without any effect on basal plasma levels of glutamic acid (Himwich et al., 1954b).

DL-glutamic acid HCl was given in doses of 12, 16 and 20 g to 8 patients with petit mal and psychomotor epilepsy without adverse effects (Price et al., 1943). Five episodes of hepatic coma in 3 patients were treated with i.v. 23 g of monosodium glutamate with improvement (Walshe, 1953). 10-12 g of L-glutamic acid given to epileptics and mental defectives appeared to improve 9 out of 20 cases (Waelsch, 1949).

Long-term studies

Mouse. 1 control group of 200 male mice and 6 test groups of 100 male mice received 0 per cent., 1 per cent. or 4 per cent. in their diet of either L-glutamic acid, monosodium L-glutamate or DL-monosodium glutamate. No malignant tumours appeared after 2 years that could be related to the administration of test material. Growth and haematology were normal, histopathology showed no abnormalities in the test animals (Little, 1953a).

Rat. Groups of 75 male and 75 female rats received for 2 years dietary levels of 0, 0.1 per cent. or 0.4 per cent. either monosodium L-glutamate, monosodium DL-glutamate or L-glutamic acid respectively. No adverse effects were noted on body-weight, growth, food intake, haematology, general behaviour, survival rate, gross and histopathology or tumour incidence (Little, 1953b).

Special studies

Mouse. The 4CS strain and Swiss white strains were studied. Groups of 6 mice (3 male, 3 female), were maintained on diets containing 0 per cent., 2% (=4 g/kg/day) or 4 per cent. (= 8 g/kg/day) monosodium glutamate. Mice were mated after 2 to 4 weeks on the test diet. Offspring (F_1) were weaned at age 25 days, and fed the same diet as parents. At age 90 days, selected (F_1) male and female mice from each group were allowed to produce a single litter (F_2). Parent mice were maintained on test diets, for 100 days after delivery and F_1 mice for 130 days of age. F_2 mice were reared until 20 days of age. No effects were observed on growth, feed intake, estrous cycle, date of sexual maturation (F_1 generation), organ weight, litter size and body-weight of offspring, and histopathology of major organs (including brain and eyes) of parent and F_1 generation. Day of eye opening, general

appearance and roentgenographic skeletal examination of F_2 generation showed no abnormalities (Yonetani et al., 1970).

Rat. 6 groups of 5-8 male and 5-10 female rats received by oral intubation daily 25 mg/kg or 125 mg/kg body-weight of glutamic acid monohydrochloride. Males and females received the compound during days 5-19 of one month, days 20-31 of the following month and days 1-10 during the third month. No adverse effects were noted on weight gain, feed intake or sexual cycles of females. No organ weight changes were seen in females but males on the higher dose level had enlarged spleens. Animals were mated at the end of the experiment and pups were normal (Furuya, 1967).

Rats were given thalidomide combined with 2 per cent. L-glutamic acid and showed essentially the same defects in the pups as groups treated with thalidomide alone. A group receiving L-glutamic acid alone was no different from controls (McColl et al., 1965).

Four females and 1 male fed for 7 months on either 0 per cent., 0.1%, 0.4% of monosodium L-glutamate, monosodium DL-glutamate or L-glutamic acid were mated and number of pups per litter was similar in all groups. Only 15-20 per cent. survived because of cannibalism. No abnormalities regarding fertility were seen on mating other groups of 4 females and 1 male at nine and eleven months. The F_1 generation was mated at 10 months and an F_2 generation produced in most groups but only the groups as 0.1 and the 0.4% L-glutamic acid produced an F_3 and F_4 generation. No impairment of fertility was noted (Little, 1953).

Monosodium glutamate, was administered orally in doses up to 7 g/kg/day to pregnant rats on 6-15 or 15-17 days following conception, it produced no adverse effect in the progeny up to the period of weaning. Further physical development to maturity was also normal except that the progeny obtained from gravida treated on the 15-17 days during gestation showed impaired ability to reproduce (Khera et al., 1970).

Two female rats received 4 g/kg body weight of monosodium glutamate commencing at day one of pregnancy. There was no effect on pregnancy or lactation. Pups were divided into 3 groups. Two groups were nursed by parents receiving monosodium glutamate, and one group by untreated parent. At weaning (day 20), one group of pups that had been nursed by a parent receiving monosodium glutamate received approx. 5 g/kg monosodium glutamate daily for 220 days. Parents received 4 g/kg monosodium glutamate for 336 days. No effects were observed on growth or oestrus cycle. All pups developed normally, and no abnormalities were noted in growth rate, time of sexual maturity, oestrus cycle and fertility. (Suzuki & Tagahashi, 1970). For histological studies, brain, hypophysis and eye were fixed in 10% neutral buffered formalin. Sections were stained with Hematoxylin-Eosin and Luxol fast blue-cresyl echt violet. No differences were observed between arcuate nuclei, medium eminence of hypothalmus and retina of control and monosodium glutamate treated groups. (Shimizu & Aibara, 1970).

Rabbit. In one group of 10 female and 4 male rabbits only the females received orally 25 mg/kg body-weight of glutamic acid for 27 days. Two of the females were pregnant and the others were not pregnant. A second group of 4 female and 2 males received orally 25 mg/kg glutamic acid with 25 mg/kg vitamin B_6. A third group of 6 females and 2 males received orally 25 mg/kg glutamic acid alone. A fourth group of 20 females and 8 males served as controls. The test substance was given by gavage. The first group showed two animals with delayed pregnancy, the uterus containing degenerate foetuses.

Two others had abortions of malformed foetuses. Two animals delivered at the normal time but the pups had various limb malformations. Four animals did not conceive. The pups did not become pregnant during seven months and showed limb deformities, decreased growth and development compared with controls. The histopathology showed scattered atrophy or hypertrophy of different organs. The second group produced 2 pregnant females which delivered malformed pups. These died soon after birth and showed bony deformities as well as atrophic changes in various organs. The third group produced 3 pregnant females which delivered pups with limb deformities. All 3 groups showed testicular atrophy in parents and multiple changes in the pups (Turgrul, 1965).

4 groups of rabbits (24 females and 16 males) received either 0, 0.1 per cent., 0.825 per cent. or 8.25 per cent. of monosodium glutamate in their diet for 2-3 weeks before mating. A positive control group of 22 pregnant females received 100 mg/kg thalidomide from day 8 to 16 of pregnancy. All does were sacrificed on day 29 or 30 of gestation and the uteri and uterine contents were examined. All males were sacrificed and the gonads and any abnormal organs examined. No significant effect on body-weight gain or food consumption was seen, nor on general appearance and behaviour. Gross and histopathology revealed no toxic effects on embryos, resorption and pups and all litter data were comparable among test animals and negative controls (Hazleton Laboratories, 1966). The brains of 5 female and 5 male pups at the 8.25 per cent. level were subsequently checked for neuronal necrosis compared with controls, but none was found (Hazleton, 1969a). Similar investigations on 5 male and 5 female pups at the 0.1 and 0.825 per cent. levels were also negative (Hazleton, 1969b).

In another experiment on rabbits, these animals received 2.5 mg/kg body-weight, 25 mg/kg and 250 mg/kg of L-glutamic acid hydrochloride at 70 hours post coition and 192 hours post coition. Operative removal of foetuses was performed on the 11th, 17th and 30th day post coition in 3 different series. The corpora lutea, the resorbed, implanted, normal and deformed foetuses were examined. No significant effects due to L-glutamic acid were noted with respect to teratogenesis (Gottschewski, 1968).

Glutamic acid hydrochloride in a dose of 25 mg/kg body-weight was given orally to 15 pregnant rabbits once a day for a period of 15 days after copulation, monosodium glutamate in the same dose and for the same period of time to 9 pregnant rabbits and saline solution to 11 pregnant rabbits which served as control group. No differences were noted between the treated groups and the controls as to rate of conception, mean litter size, and nursing rate. The average body-weight of the young in the treated groups was slightly lower as compared with the control group, but the weights of testes, ovaries and adrenal glands in the young and ovaries, adrenal glands, liver, kidneys and spleen in the mothers were not different from those in the controls. In the young, no external and skeletal malformations were observed. There were some abnormal changes in gestation such as abortion or resorption of foetuses, but these observations were made in all groups, with an incidence of 21 per cent. in the glutamic acid hydrochloride group, of 25 per cent. after administration of monosodium glutamate, and of 27 per cent. in the controls. There were no external and skeletal malformations in the aborted foetuses (Yonetani, 1967).

Chick embryo. Fertilised hen eggs were incubated after a single injection of 0.01-0.1 mg glutamic acid into the yolk sac. The mortality of embryos was raised compared with controls (53 per cent. against 24 per cent.) and there was a higher incidence of developmental defects (24 per cent.

against 3 per cent.) especially depression of development of the spine, pelvis and lower limbs (Aleksandrov et al., 1965). In another study many variables were studied such as route of injection, dose and time of injection. No obvious toxicity or teratogenicity was observed (US Food & Drug Administration, 1969).

Tests on tissue cultures

Cells (kangaroo-rats cell line) were exposed continuously for 72 hours at 0.1% monosodium glutamate without showing any toxic effect (US Food & Drug Administration, 1969).

Comments

Glutamic acid is a component of proteins and comprises some 20 per cent. of ingested protein. Much is known about its metabolism in various animal species. During gastrointestinal absorption transamination to alanine occurs. As a consequence there is only a slight rise in glutamate levels in the portal blood. A similar mechanism probably also occurs in man. However, if the capacity of this mechanism and the further conversion of glutamate in the liver is overwhelmed, or if monosodium glutamate is administered parenterally in large doses, it is possible to obtain significantly high blood levels. For primates and man it has been demonstrated that blood levels of glutamate are higher in the foetus compared with the mother, particularly during the early phases of foetal development. Recent data show that, after glutamate loading of the mother, the full term prenatal rat foetus has less glutamate in its circulation than exists in the maternal circulation.

Numerous reproduction studies in mice, rats and rabbits revealed no deleterious effects on the offspring if the parent generation was fed glutamate in high doses, suggesting that an earlier claim of teratogenic effects in the rabbits was not related to glutamate administration. There is evidence that glutamate administered parenterally or orally is retinotoxic but only during a brief period of neonatal life and not in utero or after weaning.

Work using subcutaneous injection suggests a vulnerability of the developing mouse, rat and primate central nervous system to high levels of glutamate in addition to other amino acids. Attempts at reproducing these effects after oral administration were successful only in mouse by the use of high doses.

Acute reactions reported after ingestion of glutamate as food additive are probably due to the rapid absorption of large amounts of the substance. These occur fairly frequently, and particularly sensitive persons develop Kwok's disease.

Evaluation

On the data provided it is possible to arrive at a formal acceptable daily intake making allowance for the fact that glutamate is a normal constituent of protein. In arriving at the ADI the acute reactions due to rapid absorption have been taken into consideration. In view of the uncertainty regarding the possible susceptibility of the very early human neonate to high oral intakes of glutamate, it would be prudent not to add monosodium glutamate to foods specifically intended for infants under one year of age. When the further work on this aspect has become available, it may be possible to arrive at an acceptable daily intake for these infants as well.

Level causing no toxicological effect in the mouse

4 per cent. in the diet equivalent to 6000 mg/kg body-weight

Estimate of acceptable daily intake in man

	mg/kg body-weight
Unconditional acceptance*	0-120

*Except for infants under one year. This figure is additional to intake from all dietary sources.

Further work required

1. Oral no-effect level of monosodium glutamate in neonatal mice.

2. Determination of the period of susceptibility to monosodium glutamate in neonatal mammals.

3. Age correlation between neonatal experimental animals and the human infants.

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