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Teratogenic action of intermediate products of thalidomide synthesis on chick embryo

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The use of thalidomide as sedative by pregnant women has led to the birth of children with developmental defects, the commonest of which were amelia, phocomelia, and defects of the digits and intestine. Less frequent abnormalities were defects of the nose, upper lip, eyes, heart and urogenital system (McBride; Pfeiffer and Kosenow; Taussig; Ward).

Experiments on chick embryos have shown that thalidomide causes developmental defects in 20% of embryos. It has also been found that thalidomide depresses primordia of both mesodermal (mainly) and ectodermal origin, thereby exhibiting relative teratogenic specificity (Aleksandrov et al.).

Considerable interest has been shown in the study of the teratogenic properties of the intermediate products of thalidomide synthesis: glutamic acid, Nα-phthaloylglutamic anhydride (I-50) and Nα-phthaloylglutamine (I-51).[1] At the same time an attempt has been made to determine the relationship between the chemical structure and the teratogenic action of the compounds. The results of those investigations are described in the present paper.

Experiments were carried out on fertilized hens' eggs. Before incubation, a single injection of the test compounds were given into the yolk sac in doses of 0.1–0.001 mg in a volume of 0.1 ml of physiological saline. The eggs were incubated at 38C and at a relative humidity of 60%.

TABLE 1. Teratogenic action of intermediate products of thalidomide synthesis in 5- to 14-day chick embryos

Result of treatment	Preparation and dose				
	Control	Physiological saline (0.1 ml)	Glutamic acid (0.1–0.01 mg)	I-50 (0.01–0.001 mg)	I-51 (0.01–0.001 mg)
Total no. of embryos	70 (100%)	57 (100%)	43 (100%)	48 (100%)	50 (100%)
Total no. which died	17 (24%)	36 (61%)	23 (53%)	39 (81%)	40 (80%)
Embryo with defects	2 (3%)	4 (7%)	11 (25%)	11 (24%)	10 (20%)
Defects of brain	1	1	7	6	2
of eyes	0	1	7	6	8
of beak	1	1	4	7	6
of internal organs	0	2	4	6	2
of limbs	0	2	5	6	1
of spine	1	1	4	10	5
Total no. of defects	3	8	31	41	24
Coefficient	1.5	2.0	2.8	3.7	2.4

The results of these experiments are given in Table 1. By the 14th day of incubation the mortality in the control series was 24%, and 3% of the embryos had developmental defects. After injection of physiological saline, these indices were 61 and 7%, respectively. Under the influence of glutamic acid the mortality of the embryos reached 53%, and 25% of the embryos showed developmental defects. After injection of compound I-50 the mortality was 81%, and developmental defects were observed in 24%

[1] The authors are grateful to A. L. Ivanov for making these compounds available for study.

FIG. 1. Effect of intermediate products of thalidomide synthesis on development of chick embryos. 1—Control (9-day embryo); 2—after injection of compound I-51.

of embryos. The corresponding values for I-51 were 80 and 20%.

Developmental defects were observed in the brain, beak, internal organs, spine and limbs. The developmental defects of the brain took the form of various irregular swellings of different portions of the brain. Developmental defects of the eyes were in the form of absence of one or both eyes, reduction in their size, or the formation of cysts in the cornea. Defects of the beak consisted either of curvature or reduction in size of the whole beak, or of retarded development of the upper or lower parts. The limbs showed developmental defects in the form of curvature and shortening. Failure of the abdominal wall to close and herniation of the abdominal and thoracic organs were occasionally observed. The impaired development of the spine was accompanied by depression of its growth, as shown by curvature and shortening of the body of the embryo and a decrease in the size of the sacrum and coccyx.

Some of the developmental defects arising under the influence of intermediate products of thalidomide synthesis are shown in Figs. 1–3. Injection of glutamic acid is accompanied by depression of the development of the spine, the pelvis and the lower limbs. Injection of compound I-50 leads to a marked disturbance of the development of the brain, absence of the eyes, defects of the beak, herniation of the abdominal and thoracic organs, and curvature and shortening of the spine and limbs. These disturbances of development are rare. Under the influence of compound I-51, embryos show impaired development of the brain, absence of one or both eyes, shortening of the upper part of the beak and depression of the development of the spine. The developmental defects observed in the brain, beak and eyes demonstrate depression of the anlagen of ectodermal origin. The defects of the spine and limbs indicate impaired development of structures of mesodermal origin.

The incidence and total number of these developmental defects, and also the number of embryos

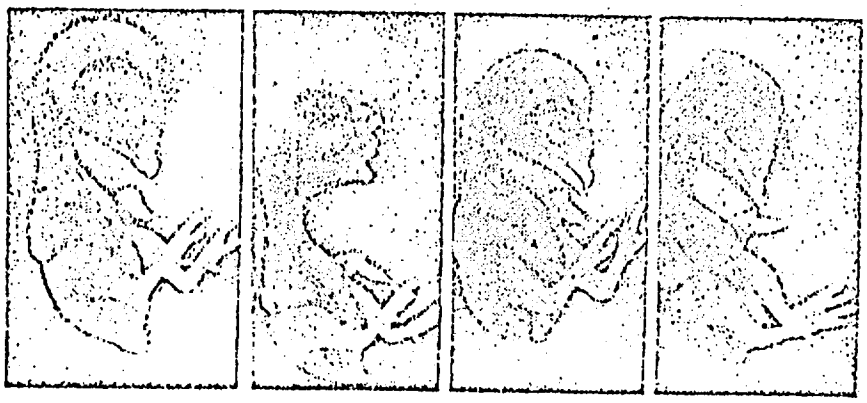

FIG. 2. Effect of intermediate products of thalidomide synthesis on development of chick embryos. 1—Control (11-day embryo); 2—after injection of compound I-50; 3 and 4—after injection of compound I-51.

FIG. 3. Effect of intermediate products of thalidomide synthesis on development of chick embryos. 1—Control (13-day embryo); 2—after injection of glutamic acid; 3—after injection of compound I-50.

with defects are shown in Table 1. The ratio between the first number and the second is expressed by a coefficient characterizing the degree of disturbance per embryo. The higher the coefficient, the greater the disturbance of development. It follows from this table that the coefficient rose from 1.5 in the control series to 2.8 in the case of glutamic acid and 3.7 in the case of compound I-50. Table 1 also shows that, under the influence of intermediate products of thalidomide synthesis, depression of primordia of both mesodermal and ectodermal origin may be observed. It may also be concluded from these investigations that a definite relationship exists between the chemical structure of the compound and its teratogenic action. For instance, all the intermediate products of thalidomide synthesis had a comparatively uniform action on primordia of ectodermal origin but a varied action on those of mesodermal origin. This difference did not apply to the nature of the developmental defect, but to its incidence. In order of increasing intensity of the depressant action of the intermediate products of thalidomide synthesis on the mesodermal primordia they may be arranged as follows: compound I-51, glutamic acid, compound I-50. In other words, whereas glutamic acid possesses the power to depress mesodermal primordia in embryos, this power is intensified during synthesis in compound I-50 but weakened in compound I-51. Earlier investigations by the authors showed that this property is again intensified in thalidomide, the final product of synthesis.

CONCLUSIONS

1. The intermediate products of thalidomide synthesis (glutamic acid, Nα-phthaloylglutamic anhydride, Nα-phthaloylglutamine) produced developmental defects in the developing chick embryo.
2. These compounds act comparatively uniformly on primordia of ectodermal origin but differently on primordia of mesodermal origin.
3. Nα-phthaloylglutamic anhydride exhibits greatest activity with respect to depression of mesodermal primordia.

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The Effect of Monosodium Glutamate on GDH, GOT, and GPT Levels in Mice[1] (37521)

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Olney (1) reported that a single subcutaneous injection (0.5–4.0 mg/g body weight) of monosodium glutamate (MSG) in newborn mice (2–9 days old) induced acute necrosis in several regions of developing brain including the hypothalamus. The newborn mice injected daily over long periods of time showed, as adults, stunted development, marked obesity, and female sterility. Olney (1) also reported pathological changes in several organs associated with endocrine function. He postulated that the adult syndrome suggested a complicated neuroendocrine disturbance. Similar findings have been reported by Redding and Schally (2). Adamo and Rather (3), following the experimental procedures of Olney (1), found that injections of MSG produced no changes in the brain or the reproductive function in rats. Olney and Sharpe (4) have reported acute brain damage in a newborn rhesus monkey following a single injection of MSG. Recently, Olney and Ho (5) have found that both MSG and monosodium aspartate (MSA) administered orally to infant mice (10–12 days old) caused necrosis of hypothalamic neurones. They also reported that the free amino acids, L-glutamate, L-aspartate, and L-cysteine produced the same effect. Bazzano *et al.* (6) reported that large amounts of glutamic acid incorporated in a formula diet and fed to adult humans and gerbils appeared to cause no clinical pathological changes.

The injection of large doses of monosodium glutamate by Olney (1) and others (2–4) has raised the question of the effect of MSG on the three important glutamate metabolizing enzymes, glutamic dehydrogenase (GDH, EC 1.4.1.3), aspartate aminotransferase (GOT, EC 2.6.1.1), and alanine aminotransferase (GPT, EC 2.6.1.2). The present study was designed to determine the effect of MSG on the levels of GDH, GOT, and GPT in the brains and livers of newborn and weanling mice.

Materials and Methods. Animals and treatments. Male weanling mice (25 days old) of the Charles Rivers CE-1 strain received daily subcutaneous injections of monosodium glutamate in saline (1.0 mg/g body weight) or monosodium aspartate in saline (0.92 mg/g body weight). Two control groups were used; one received saline and the other no injection. The MSG concentration used was twice that which Olney (1) has reported as a cause of brain lesions in newborn mice. The animals were sacrificed and the brains and livers excised 24 hr after the completion of daily injections given for 1, 3, 7, 13, and 21 days. Thirty days following the completion of the original experiment, the study was repeated, again using weanling mice. At a later date, the original experimental design was used to study the effects on newborn mice (1 day of age).

Both brains and livers were homogenized in 0.025 *M* phosphate buffer pH 7.4 in the proportion of 1 g tissue to 9 ml of buffer. The tissue was sonified for two minutes with a Heat Systems Model W-1855 sonifier at an

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output setting of 8. A magnesium chloride ice bath was used to prevent heating of the homogenate. The sonified tissue was centrifuged at 21,000*g* for 30 min at 0°. The enzyme assays were made on the supernatant. Glutamic dehydrogenase was assayed according to the method of Olson and Anfinsen (7, 8). The assay mixture contained 2.1 ml of 0.025 *M* phosphate buffer (pH 7.6), 0.1 ml of 3 *M* NH_4Cl, 0.5 ml of 0.47 m*M* NADH, 0.2 ml of 0.167 *M* K-α-ketoglutarate and 0.1 ml of the appropriate tissue supernatant. Aspartate aminotransferase activity was determined according to the method of Karmen (9). The assay mixture contained 0.5 ml of 0.2 *M* L-aspartate, 0.2 ml of 1.41 m*M* NADH, 0.1 ml of malic dehydrogenase (2000 units/ml), 0.2 ml of 0.1 *M* K-α-ketoglutarate, 0.8 ml of water and 0.2 ml of the appropriate tissue supernatant. The method of Wroblewski and La Due (10) was used to determine alanine aminotransferase activity. The assay mixture contained 0.5 ml of 0.2 *M* L-alanine, 0.2 ml of 1.41 m*M* NADH, 0.1 ml of lactic dehydrogenase (8000 units/ml), 0.2 ml of 0.1 *M* K-α-ketoglutarate, 1.8 ml of water and 0.2 ml of the appropriate tissue supernatant. The aminotransferases and glutamic dehydrogenase were assayed by measuring nucleotide oxidation at 340 nm in a recording spectrophotometer. All enzyme assays were performed at 25° in 3 ml, 1 cm cuvettes. A linear relationship was obtained between the enzyme activity and varied quantities of the tissue supernatant for each enzyme assayed.

Tissue protein was determined by the method of Lowry *et al.* (11) utilizing crystalline bovine serum albumin as the standard.

Results. The injections of monosodium glutamate, monosodium aspartate, and saline for 21 days had no significant effect on the levels of GDH, GOT or GTP in the brains and livers of weanling mice. The combined data for both experiments using weanling mice are presented in Table I. The enzyme activities reported are the mean values of the five injection periods. As can be seen, there were no significant differences in enzyme activities between treatments at the $p < 0.05$ level (Duncan's New Multiple Range Test). It was also found that there were no differences in enzyme activities between treatments after 1, 3, 7, 13 or 21 injections. Body, liver or brain weights were not significantly different between treatments.

The data for the experiments using newborn mice are presented in Tables II and III. These data represent the mean enzyme activity values for livers and brains after one injection (Table II) and after 21 injections (Table III). Enzyme activities were compared at the 0.05 level using Duncan's New Multiple Range Test.

Since newborn mice (one day of age) were used in this experiment, it was considered essential that one group of animals receive no treatment. This determined any enzymatic changes due to postnatal development and any changes caused by the stress of handling and administering the subcutaneous injections.

There were no significant differences ($p < 0.05$) in the enzymes studied between the nontreated controls and the saline-treated controls after either one or 21 injections of

TABLE I. Effect of Monosodium Glutamate on Enzyme Levels in Weanling Mice.

Enzyme system	Tissue	Treatment			
		None	Saline	MSA	MSG
GDH	Brain	3.25 ± 0.27[a,b]	3.19 ± 0.29	2.80 ± 0.25	2.84 ± 0.26
GDH	Liver	11.41 ± 0.56	11.09 ± 0.49	11.09 ± 0.43	11.23 ± 0.60
GPT	Brain	5.09 ± 0.28	4.95 ± 0.29	4.54 ± 0.22	4.57 ± 0.22
GPT	Liver	16.09 ± 0.76	16.31 ± 0.69	15.42 ± 0.59	16.15 ± 0.68
GOT	Brain	45.92 ± 2.18	45.56 ± 1.92	43.04 ± 2.52	43.16 ± 2.02
GOT	Liver	37.85 ± 1.65	38.11 ± 1.47	37.64 ± 1.78	38.00 ± 1.30

[a] Values are means ± SE, $n = 6$.

[b] Specific activity, micromoles NADH oxidized per hour per milligram protein.

TABLE II. Effect of Monosodium Glutamate on Enzyme Levels in Newborn Mice After One Injection.

Enzyme system	Tissue	Treatment			
		None	Saline	MSA	MSG
GDH	Brain	0.68 ± 0.04[a,b]	1.18 ± 0.05	0.57 ± 0.07	1.18 ± 0.06
GDH	Liver	7.71 ± 0.33	7.92 ± 0.09	9.35 ± 0.14	9.54 ± 0.30
GPT	Brain	1.24 ± 0.10	1.27 ± 0.16	2.97 ± 0.29	2.47 ± 0.06
GPT	Liver	12.12 ± 0.07	11.19 ± 0.22	14.77 ± 0.46	16.48 ± 0.79
GOT	Brain	8.47 ± 0.63	7.45 ± 0.46	14.21 ± 0.22	13.08 ± 0.37
GOT	Liver	33.10 ± 0.15	34.05 ± 0.26	41.32 ± 0.83	46.84 ± 3.03

[a] Values are means ± SE, $n = 6$.
[b] Specific activity, micromoles NADH oxidized per hour per milligram protein.

the saline except for GDH in the brain after one injection. The same difference is observed between the MSA and MSG treated mice after one injection. After three injections, the GDH level was 1.15 ± 0.10, 1.23 ± 0.06, 1.50 ± 0.03, and 1.17 ± 0.01 μmoles NADH oxidized per hour per mg protein, respectively, for the non-treated controls, saline-treated controls, MSA-treated and MSG-treated mice. The authors feel that since after three injections there were no differences found in GDH levels between treatments, that the differences found after one injection were due to the variability in GDH levels in the developing brain of one-day-old mice and/or stress on the animals. It can be seen that these differences only occurred in the brain. The data shows that the largest increase in enzyme activity in the control groups occurred in the brain indicating a very rapid change in this tissue. Brain GDH, GOT and GPT levels in both the saline-treated controls and the non-treated controls increased significantly with time. The increase in enzyme activity closely parallels the increase in glutamic acid, aspartic acid, and α-aminobutyric acid concentrations reported by Agrawal *et al.* (12) in developing mouse brain. Also, the increase in enzyme activity is similar to that found by Wang (13) in newly hatched chicks.

After one injection, the brain GDH level was significantly lower in the nontreated controls and MSA-treated animals than in the MSG-treated animals, and the saline-treated controls. GDH activity in the liver after one injection was significantly higher in the MSG-treated animals than in the nontreated controls, but was not different from either of the other treatments. The GOT and GPT levels in both the brains and livers were significantly lower in the saline-treated controls and the nontreated controls than in the MSA and MSG-treated animals. MSA and MSG-treated animals were not significantly different

TABLE III. Effect of Monosodium Glutamate on Enzyme Levels in Newborn Mice After Twenty-one Injections.

Enzyme system	Tissue	Treatment			
		None	Saline	MSA	MSG
GDH	Brain	2.52 ± 0.18[a,b]	3.08 ± 0.29	4.89 ± 0.70	5.32 ± 0.28
GDH	Liver	11.90 ± 0.14	10.24 ± 1.17	17.86 ± 0.34	17.43 ± 0.44
GPT	Brain	3.37 ± 0.56	2.86 ± 0.24	7.14 ± 0.71	7.25 ± 0.08
GPT	Liver	12.59 ± 0.52	8.96 ± 0.36	34.87 ± 1.59	31.25 ± 1.01
GOT	Brain	26.62 ± 0.85	27.97 ± 0.48	45.04 ± 0.22	48.28 ± 0.64
GOT	Liver	34.42 ± 0.54	31.08 ± 1.05	71.79 ± 0.96	65.61 ± 0.79

[a] Values are means ± SE, $n = 6$.
[b] Specific activity, micromoles NADH oxidized per hour per milligram protein.

from each other in GOT and GPT activities in either brains or livers. After 21 injections, enzyme activities (GDH, GPT or GOT) in the brains and livers of MSA and MSG-treated animals were not significantly different from each other. However, MSA and MSG-treated animals had significantly higher enzyme activities (GDH, GPT, and GOT) in both brains and livers than the saline-treated controls and the nontreated controls.

The GDH, GPT, and GOT activities in the brains and livers were affected by both MSA and MSG. In both cases, there was a 2–3-fold increase in activity of the three enzymes studied. The GDH, GPT, and GOT activities following injections for 7 and 13 days also showed an increase with MSA and MSG treatment, but of a lesser degree. As in the weanling mice, no differences were observed in body, brain, and liver weights.

Discussion. These data indicate that monosodium glutamate (1 mg/g body weight) does not affect the enzyme activity of GDH, GOT, or GPT in weanling mice. These results appear to indicate that the weanling mouse has the biochemical capacity to metabolize large doses of monosodium glutamate. Although no differences in enzyme activities were found 24 hr after 1, 3, 7, 12, or 21 injections, there may be an effect at shorter time periods. Olney (1) found brain lesions three hours after monosodium glutamate injections. Larger doses of monosodium glutamate may also have an effect on GDH, GOT, and GPT activities.

As previously stated, both monosodium glutamate and monosodium aspartate caused a 2–3-fold increase in GDH, GOT, and GPT activities in newborn mice. These results indicate that newborn mice may not have the capacity to metabolize large doses of monosodium glutamate or monosodium aspartate and, therefore, must adapt to the higher exogenous levels that were administered. Kraus (14) has shown that L-glutamate (1 mg/g body weight) caused induction of L-glutamate decarboxylase (EC 4.1.1.15) in mice brains. Therefore, it appears that MSG and MSA may also cause an induction of GDH, GOT and GPT in mouse brain and liver in a similar manner.

Summary. Monosodium glutamate and monosodium aspartate were injected subcutaneously in newborn and weanling mice. The brains and livers were excised and GDH, GOT, and GPT levels were measured. Monosodium glutamate or monosodium aspartate administration resulted in 2–3-fold increases in activities in both the brains and livers of newborn mice. However, the injection of either monosodium glutamate or monosodium aspartate, resulted in no significant effect on the enzymes measured in the brains and livers of weanling mice.

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Monosodium Glutamate: Feeding of Large Amounts in Man and Gerbils

Abstract. *Oral administration of large amounts of glutamic acid to adult humans and animals in a formula diet appeared to cause no clinical pathological changes. The only biochemically demonstrable effect was a decrease in serum cholesterol and associated beta lipoproteins.*

Interest in the toxicity of monosodium glutamate (MSG) as a food additive has increased greatly in the past year because of the association of this compound with both the Chinese-restaurant syndrome in humans (*1*) and a lesion of the hypothalamus in newborn mice and monkeys (*2, 3*). The burning sensation and, most probably, the headache in the Chinese-restaurant syndrome appear to be of vascular origin and represent a chemical idiosyncrasy (*1*). A discrete brain lesion is unlikely in this syndrome since the symptoms are transient and since sensitive individuals need only avoid exceeding their tolerance threshold in order to prevent a recurrence. No permanent neurological lesions have been observed. On the other hand, the brain lesions induced in newborn mice and monkeys by parenteral or oral administration of glutamate are characterized by a degeneration of dendritic processes and cell bodies of hypothalamic neurons (*3, 4*). Associated with these lesions is a complex syndrome of metabolic, nutritional, and reproductive abnormalities visible in newborn mice for a 9-month period. These discoveries have given rise to concern about the safety of sodium glutamate when added to foods ingested by pregnant women and by infants. We report the harmless effects of large amounts of monosodium glutamate given to both animals and man by mouth. This project had its origin in a study of the hypocholesterolemic effects of glutamic acid when it was fed as a sole source of nonessential nitrogen in amino acid formula (AAF) diets (*5*). Subsequently, related studies were carried out in gerbils (*6*), in which a similar hypocholesterolemic effect was noted.

Table 1. Effects of glutamate feeding in human subjects. Δ-Cholesterol represents the difference in concentrations of plasma cholesterol from those in subjects on the control diet (AAF). None of the subjects showed any clinical effects.

Subject	Time (days)	Dose (g/day)	Δ-Cholesterol (mg/100 ml)
J.B.	34	147	−27
R.S.	28	88	−82
R.M.	27	147	−16
J.M.	21	137	−46
P.B.	21	137	−40
P.K.	21	147	−55
J.A.*	14	25	−16
	14	50	− 7
	21	100	+ 3
S.S.†	22	131	− 5
R.M.	21	133	−23
M.E.†	21	126	−20
M.E.	21	68	−41
	21	137	−42
G.R.	28	137	−29
W.B.	42	137	−75
D.M.	21	147	−40

* Monosodium glutamate was added to a house diet. † Monosodium glutamate was added to a low protein (25 g) diet.

A chemically defined diet [amino acid formula with glutamate (AAFG)] supplying all of the essential amino acids in optimum amounts, 16.0 g of total nitrogen including 137 g of glutamic acid as the sole source of nonessential nitrogen, and calories to maintain body weight has been fed to 11 adult male subjects for periods ranging from 14 to 42 days. Lesser amounts were fed to three other subjects. All subjects tolerated the regimen well, showed no changes in neurologic or hepatic function, and demonstrated a reduction in serum cholesterol and β-lipoproteins of 25 to 40 percent of control levels. None developed the Chinese-restaurant syndrome. Substitution of glycine plus ammonium ion for glutamate (AAF) abolished the hypocholesterolemic effect. Supplements of glutamate of as much as 100 g/day to an ordinary diet were ineffective in lowering the serum lipids but showed no toxic manifestations. Table 1 shows the effects of feeding from 25 to 147 g of MSG per day to adult human subjects. No changes in weight, irritability, appetite, or mentation were noted. The average fall in plasma cholesterol was 42 mg (± 5.8 standard error of the mean) per 100 ml.

When these same diets (*6*) were fed to gerbils, with animals fed a 30 percent casein diet and a chow diet as controls, it was observed that AAFG was less effective in maintaining body weight than the casein diet but more effective than AAF. Nevertheless, casein, AAF, and AAFG did not differ in their effects upon hair coat and general neurological status. When AAFG was replaced by food control diet (FCD), changes in body weight, hair appearance, and serum cholesterol concentration were significantly, if not completely, reversed within 1 week. No increase or decrease in neurological activity was noted during the feeding of AAFG. More specifically, there was no evidence of alteration of visual acuity or change in frequency of convulsion. A summary of these results is shown in Table 2. Animals on the glutamate diet were more tranquil than those on casein or chow diets. The oral doses employed in the study of gerbils (30 g per kilogram of body weight per day) should approximate the effect of the parenteral doses

Table 2. Effects of various diets on mature male Mongolian gerbils. Observations on test diets were recorded at the end of 1 week. Weight is reported as the mean ± standard error (S.E.) of the mean. Food control diet (FCD) was Purina laboratory chow for small animals; 20 of the gerbils received FCD with 1 percent (by weight) of cholesterol added. See text for casein diet; 26 of the gerbils on this diet received 30 percent casein with 1 percent (by weight) of cholesterol added. (Δ-Cholesterol represents the difference in concentration of cholesterol from that in animals on the preceding diet ± S.E. of the difference.) Amino acid formula (AAF) was fed to 56 gerbils, 28 of which received AAF with 1 percent (by weight) of cholesterol added. For animals fed amino acid formula with glutamate (AAFG), the statistical evaluation is limited to the AAF-AAFG interaction ($P < .005$). (Δ-Cholesterol represents the difference in concentration of cholesterol from that in animals on the preceding diet ± S.E. of the difference.) Twenty-eight of these gerbils received AAFG with 1 percent (by weight) of cholesterol added.

Diet	Animals (No.)	Weight (g)	Δ-Cholesterol (mg/100 ml)	Observations
FCD	110	72 ± 1.0		Hair is smooth; occasional convulsions during handling
Casein (30%)	49	72 ± 1.1	−8 ± 7.8	Hair loss; increased number of convulsions; increased mortality rate
AAF control	56	60 ± 1.2		Hair ruffled with occasional hair loss; rare hyperactivity, ataxia without additional convulsions
AAFG experimental	56	65 ± 1.0	−53 ± 14.0	As above

employed by others in animal studies [Lucas and Newhouse (*7*), Potts *et al.* (*8*), Freedman and Potts (*9*), Olney (*2*), and Olney and Sharpe (*3*)], who administered MSG in the range of 2 to 10 g per kilogram of body weight. In their latest report, Olney and Sharpe (*4*) reported effects of 1 g/kg, by feeding tube, in newborn mice.

Mayer-Gross and Walker (*10*) demonstrated a transient toxic effect (vomiting) after intravenous administration of 20 g of sodium glutamate to normal adults, while this dose produced a favorable metabolic effect (arousal) in comatose, hypoglycemic patients. To our knowledge, then, no permanent neurotoxic effect for glutamate has been definitely reported for adult humans.

Rapid utilization of MSG would prevent the accumulation of free amino acid in the plasma and would reduce the danger of damage to susceptible tissue. Oral MSG is well utilized by human subjects, as indicated by no significant alterations in concentrations of plasma glutamic acid plus glutamine 12 hours after thrice daily feeding of glutamate formulas. The subjects also were at constant body weight and in nitrogen balance (*11*).

Clearly, glutamate produced metabolic effects consisting of decrease in serum cholesterol, phospholipids, and β-lipoproteins without alterations of body weight, nitrogen balance, liver function, or neurological activity (*12*, *13*).

The question of MSG toxicity appears, therefore, to center around the permeability of the blood-brain barrier to glutamic acid. How much of the fed or injected glutamate does actually reach the brain? It is obvious that significant amounts reach the brain in newborn animals and possibly infants. Himwich *et al.* (*14*) have reported ready access of glutamate to the brain at birth but exclusion of it with establishment of a blood-brain barrier by the 10th day. In view of this finding, it is likely that even with parenteral administration of large doses of MSG only small amounts of it would reach the adult brain. In newborn mice and monkeys prior to the establishment of this barrier, the brain could be susceptible to the toxic effects of MSG described by Olney (*2*, *3*).

We conclude from our studies that very high oral doses of glutamate (147 g/day) are tolerated, with no neurological changes, by adult gerbils and humans. The problem of determining the age of transition from susceptibility to tolerance for MSG in human subjects remains open.

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Control of Glutamate Oxidation in Brain and Liver Mitochondrial Systems

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1. Glutamate oxidation in brain and liver mitochondrial systems proceeds mainly through transamination with oxaloacetate followed by oxidation of the α-oxoglutarate formed. Both in the presence and absence of dinitrophenol in liver mitochondria this pathway accounted for almost 80% of the uptake of glutamate. In brain preparations the transamination pathway accounted for about 90% of the glutamate uptake. 2. The oxidation of [1-^{14}C]- and [5-^{14}C]-glutamate in brain preparations is compatible with utilization through the tricarboxylic acid cycle, either after the formation of α-oxoglutarate or after decarboxylation to form γ-aminobutyrate. There is no indication of γ-decarboxylation of glutamate. 3. The high respiratory control ratio obtained with glutamate as substrate in brain mitochondrial preparations is due to the low respiration rate in the absence of ADP: this results from the low rate of formation of oxaloacetate under these conditions. When oxaloacetate is made available by the addition of malate or of NAD$^+$, the respiration rate is increased to the level obtained with other substrates. 4. When the transamination pathway of glutamate oxidation was blocked with malonate, the uptake of glutamate was inhibited in the presence of ADP or ADP plus dinitrophenol by about 70 and 80% respectively in brain mitochondrial systems, whereas the inhibition was only about 50% in dinitrophenol-stimulated liver preparations. In unstimulated liver mitochondria in the presence of malonate there was a sixfold increase in the oxidation of glutamate by the glutamate-dehydrogenase pathway. Thus the operating activity of glutamate dehydrogenase is much less than the 'free' (non-latent) activity. 5. The following explanation is put forward for the control of glutamate metabolism in liver and brain mitochondrial preparations. The oxidation of glutamate by either pathway yields α-oxoglutarate, which is further metabolized. Since aspartate aminotransferase is present in great excess compared with the respiration rate, the oxaloacetate formed is continuously removed by the transamination reaction. Thus α-oxoglutarate is formed independently of glutamate dehydrogenation, and the question is how the dehydrogenation of glutamate is influenced by the continuous formation of α-oxoglutarate. The results indicate that a competition takes place between the α-oxoglutarate-dehydrogenase complex and glutamate dehydrogenase, probably for NAD$^+$, resulting in preferential oxidation of α-oxoglutarate.

Several alternative pathways are available for the oxidation of glutamate in mammalian tissues. Until recently it has been assumed that glutamate is mainly oxidized by a pathway involving glutamate dehydrogenase (reaction 1):

$$\text{Glutamate} + \tfrac{1}{2}O_2 \rightarrow \alpha\text{-oxoglutarate} + NH_3$$
$$\alpha\text{-Oxoglutarate} + \tfrac{1}{2}O_2 \rightarrow \text{succinate} + CO_2$$
$$\text{Sum: Glutamate} + O_2 \rightarrow \text{succinate} + NH_3 + CO_2 \quad (1)$$

However, the activity of glutamate dehydrogenase is very low in some tissues that nevertheless oxidize glutamate at a high rate (Jones & Gutfreund, 1961; Borst, 1962). Recent experiments *in vitro* from different Laboratories have indicated that in most tissues glutamate oxidation proceeds mainly through transamination with oxaloacetate followed by oxidation of the α-oxoglutarate formed (reaction 2) (cf. Krebs & Bellamy, 1960; Jones & Gutfreund, 1961; Borst, 1962; Haslam & Krebs, 1963*b*; Chappell, 1964*b*):

$$\text{Glutamate} + \text{oxaloacetate} \rightarrow \alpha\text{-oxoglutarate} + \text{aspartate}$$
$$\alpha\text{-Oxoglutarate} + 1\tfrac{1}{2}O_2 \rightarrow \text{oxaloacetate} + CO_2$$
$$\text{Sum: Glutamate} + 1\tfrac{1}{2}O_2 \rightarrow \text{aspartate} + CO_2 \quad (2)$$

Besides these routes glutamate can also be oxidized through transamination with other α-oxo acids, and in brain there is a by-pass of the tricarboxylic acid cycle, the γ-aminobutyrate shunt, which involves glutamate as an intermediate (Roberts, 1956). There are a few quantitative data published for the contribution of these different pathways of glutamate oxidation, but the data are not comparable owing to the different experimental conditions used (e.g. Krebs & Bellamy, 1960; Borst, 1962). In the present work the different routes of glutamate oxidation were measured in brain and liver mitochondrial preparations in the resting and active states of mitochondrial respiration, and the effect of pyruvate on the oxidation of glutamate has also been investigated (Balázs, 1965). Factors that might control the relative contribution of the different pathways were also studied. A part of this investigation has already been reported in a preliminary form (Balázs, 1963).

EXPERIMENTAL

Mitochondrial preparations. Rats lightly narcotized with ether were decapitated. The two hemispheres of the brain were homogenized in 0·3M-sucrose containing EDTA (sodium salt), pH 7·2 (0·5mM), with an Aldridge homogenizer (Aldridge, Emery & Street, 1960), and a 10% suspension was prepared. The nuclei and cell debris were separated at 1000*g* for 10min. in a Spinco model L centrifuge. The low-speed fraction was resuspended in the sucrose solution and sedimented at the same speed and time. The combined supernatants were again spun at 1000*g* for 10min. The mitochondrial fraction was separated by centrifugation at 18000*g* for 10min. The fluffy layer was removed as far as possible by gentle swirling and this treatment was repeated at the following two washings. Finally the mitochondrial fraction was suspended in the sucrose solution (about 1g. wet wt./ml.).

Liver mitochondrial fractions were prepared by the method of Aldridge (1958).

Manometric techniques and incubations. Conventional Warburg technique was used (Umbreit, Burris & Stauffer,

Table 1. *Effect of different concentrations of mitochondria on the oxidation of glutamate*

The incubation mixture contained basic medium (see the Experimental section), ADP (2mM) and 2,4-dinitrophenol (40μM). The concentration of glutamate was 9·6mM for liver mitochondria and 9·1mM for brain mitochondria. The incubation volume was 3·0ml. Metabolic changes are given in μmoles/hr./vessel.

	Liver mitochondria			Brain mitochondria		
Vessel no....	1	2	3	1	2	3
Mitochondrial protein (mg.)...	1·28	2·56	5·12	1·52	3·05	6·10
Metabolic changes:						
O_2	−5·30	−12·3	−25·7	−5·88	−13·1	−24·9
Glutamate	−3·52	−8·20	−17·3	−4·40	−8·57	−16·1
Aspartate	+2·68	+7·09	+14·0	+3·56	+7·61	+13·1
Conversion of glutamate into aspartate: $\frac{\text{Aspartate}}{\text{Glutamate}} \times 100$	76	86	81	81	89	81

Table 2. *Effect of glutamate concentration on the utilization of glutamate*

The incubation mixture contained basic medium (see the Experimental section), ADP (2mM) and dinitrophenol (40μM). Liver and brain mitochondrial fractions contained 2·56 and 3·05mg. of protein respectively. The incubation volume was 3ml. except in vessels 4 (6ml.). Metabolic changes are given in μmoles/hr./vessel.

	Liver				Brain			
Vessel no....	1	2	3	4	1	2	3	4
Glutamate concn. (mM):								
Initial...	9·6	7·7	5·8	3·8	9·1	7·2	5·4	3·6
Final...	6·9	5·1	3·3	2·7	6·2	4·8	3·5	2·7
Metabolic changes:								
O_2	−12·3	−11·4	−11·3	−10·6	−13·1	−11·7	−10·0	−9·64
Glutamate	−8·20	−7·89	−7·52	−6·70	−8·57	−7·28	−5·62	−5·50
Aspartate	+7·09	+6·70	+6·54	+5·28	+7·61	+5·70	+4·85	+3·92
Conversion of glutamate into aspartate: $\frac{\text{Aspartate}}{\text{Glutamate}} \times 100$	86	85	87	79	89	78	86	71

1945). The basic medium contained $MgCl_2$ (6mM), potassium phosphate buffer, pH 7·2 (15mM), tris-HCl buffer, pH 7·2 (20mM), EDTA (sodium salt), pH 7·2 (2mM), sucrose (20–50mM) introduced with the mitochondrial suspension, and KCl to give an osmolarity of 0·3 after the addition of all constituents. The pH of substrates was adjusted to 7·2 and the substrates were used in 7–10mM final concentration. When yeast hexokinase was added (type III, Sigma Chemical Co., St Louis, Mo., U.S.A., or Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) the inorganic phosphate concentration was increased to 40mM. The centre well of the flasks contained 0·2ml. of 2N-NaOH. The incubation was carried out at 37·5° in an O_2 atmosphere for 40–60min., and readings of O_2 uptake were taken after a temperature equilibration of 7–10min. The reaction was stopped by tipping 2N-HCl from the side arm (final concn. 0·4N). The samples were kept in the deep-freeze until analysed, except when oxaloacetate was estimated, a procedure which was carried out without delay.

Since the rate of glutamate oxidation is much lower in the absence than in the presence of ADP the experimental conditions had to be adjusted in a way to enable the measurement of metabolic changes under the different experimental conditions. Unless otherwise stated, the vessels contained in the absence of ADP 4–10mg. of mitochondrial protein and the incubation volume was 1·5ml. In the presence of ADP (2mM) plus 2,4-dinitrophenol (40–50μM) the incubation volume was 3ml. and less mitochondrial suspension was added (1·5–4mg. of protein). When only ADP was included in the medium the volume was 2–3ml. and 3–8mg. of mitochondrial protein was added. The initial concentration of glutamate was the same in all cases and the procedure described made it possible to keep the final concentration of substrate within the same range under the different experimental conditions. This procedure was justified by the observations given below. The concentration of mitochondria within the limits investigated in Table 1 had only a small effect on the metabolism of glutamate. Further, the conversion of glutamate into aspartate was only affected when the concentration of glutamate was lower than about 3·5mM (Table 2). In the results given in Tables 3 and 7, only those experiments were included in which the final concentration of substrate was higher than 3·5mM. The time-course of glutamate oxidation was also investigated and did not indicate any marked change in the pattern of glutamate metabolism during 1hr. incubation (Fig. 1). de Haan, Tager & Slater (1964) observed a small increase in the amount of glutamate utilized through the glutamate-dehydrogenase pathway in the second 20min. of incubation at 37°; this was not apparent in the present experiments.

Analytical methods. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) and the protein content of the mitochondrial fraction was expressed as mg. of albumin. Aspartate and glutamate plus glutamine were determined by the method of Krebs & Bellamy (1960) by using *Nocardia globerula* and *Clostridium welchii* respectively (kindly supplied by Mr A. Tuffrey, Laboratory Animal Centre, Carshalton, Surrey) for the decarboxylation of the amino acids. As glutamate and glutamine were never assayed individually the values for glutamate removal may include endogenous glutamine and refer to glutamate metabolized by pathways other than glutamine synthesis. The following enzymic determinations were carried out by spectrophotometric methods with neutralized portions of the samples (Bergmeyer, 1962). α-Oxoglutarate was measured by recording the oxidation of NADH in the presence of glutamate dehydrogenase and ammonia. Pyruvate and oxaloacetate were determined by measuring the oxidation of NADH on the addition of lactate dehydrogenase and malate dehydrogenase respectively. Malate was determined by the reduction of NAD^+ on the addition of malate dehydrogenase. The extinction change due to NADH was determined with a Unicam SP.700

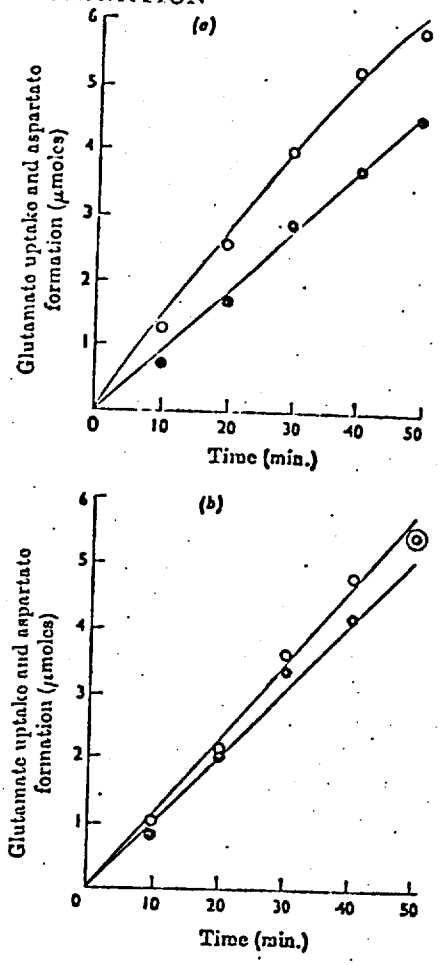

Fig. 1. Time-course of glutamate oxidation in mitochondrial systems of liver (*a*) and brain (*b*). The incubation was carried out in a conical flask in a Dubnoff metabolic shaking incubator at 37·5° in O_2 atmosphere. The total volume was 8·0ml. containing the basic medium (see the Experimental section), ADP (2mM), dinitrophenol (40μM) and L-glutamate (10mM). The reaction was started by the addition of 0·8ml. of cold mitochondrial suspension (20 and 24mg. of protein for liver and brain mitochondria respectively). Then 1·2ml. portions of the medium were withdrawn at the time-intervals indicated and were acidified and analysed. The glutamate and aspartate contents found in the samples after the first 10min. incubations served as the control. ○, Glutamate uptake; ●, aspartate formation.

recording spectrophotometer at 340mμ by using micro-cells containing 0·4ml. total volume and having a light-path of 1cm.

Chromatography of amino acids. A portion of the HCl extract of the incubated samples was evaporated in a desiccator over NaOH. The dry residue was taken up in 75% (v/v) ethanol and centrifuged. Two-dimensional chromatography was done by the method of Gaitonde (1961). Alternatively, in a few experiments the amino acids were determined from neutralized perchloric acid filtrates by the modified technique of Gaitonde (1964).

Radioactive tracer techniques. The general techniques used were those described by Haslam & Krebs (1963*a*, *b*). The respiratory $^{14}CO_2$ was absorbed in 0·2ml. of 2N-NaOH placed in the centre well of the Warburg flasks. The flasks were incubated for approx. 1hr. after the medium had been acidified. The $^{14}CO_2$ trapped in the alkali was converted into $BaCO_3$ and plated on filter-paper disks (3cm. diam.). The planchets were assayed for radioactivity with a Nuclear-Chicago gas-flow counter. The counts were corrected for background, dead-time and self-absorption. The specific activities of [1-^{14}C]glutamate and [4-^{14}C]aspartate were determined by assay of the $^{14}CO_2$ liberated by decarboxylation with *C. welchii* and *N. globcrula* respectively. The specific activity of [5-^{14}C]glutamate, kindly given by Dr R. J. Haslam, was determined by direct plating and counting at infinite thinness, and by measuring the amount of glutamate by decarboxylation with *C. welchii*. Values for the relative specific activity of residual substrates are defined as:

$$\frac{\text{Sp. activity of residual [}^{14}\text{C]substrate}}{\text{Sp. activity of added [}^{14}\text{C]substrate}}$$

Enzymic assays. Aspartate aminotransferase (EC 2.6.1.1) was assayed by the method of Gutfreund, Ebner & Mendiola (1961). The reaction mixture contained L-glutamate (40mM), oxaloacetate (1mM), potassium phosphate buffer, pH 7·66 (0·1M), NADPH (0·17mM), NH_4Cl (0·15M) and 100μg. of crystalline glutamate dehydrogenase. The rate of the reaction increased in the first 5–6min. after the addition of oxaloacetate, but remained constant for another 5min. The enzyme was also assayed in the reverse direction by the method of Karmen (1955) as modified by Boyd (1961). The observed rate was higher in the latter direction, mainly in liver (Table 10), than in the other direction under the conditions of the assays.

Glutamate dehydrogenase (EC 1.4.1.3) was assayed in both directions by the method of Olson & Anfinsen (1952) in the presence of 1mM-ADP (Frieden, 1959; Mildvan & Creville, 1962). When the activity was measured in the direction of glutamate + NAD^+ + H_2O to α-oxoglutarate + NH_3 + NADH + H^+ the reaction mixture contained potassium phosphate buffer, pH 7·66 (0·1M), L-glutamate (13·3mM), NAD^+ (0·77mM) and ADP (1mM). The composition of the medium for the reverse direction was potassium phosphate buffer, pH 7·66 (0·1M), α-oxoglutarate (10mM), NH_4Cl (0·15M), NADH (0·17mM) and ADP (1mM).

The mitochondrial preparations were diluted in Triton X-100 (0·1% final concn.; Charles Lenning and Co. Ltd., London, W.C.1) before the assay. The change of extinction given by NADH was followed at 340mμ with a Unicam SP.700 recording spectrophotometer.

Chemicals. DL-[1-^{14}C]Glutamate (obtained from The Radiochemical Centre, Amersham, Bucks.) was dissolved in unlabelled L-glutamate solution to decrease the proportion of D-glutamate to a negligible value (about 1%). The final specific activity of the L-glutamate was 10μc/m-mole. 2,4-Dinitrophenol was recrystallized before use. The following chemicals were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany: ADP (sodium salt), NAD^+, NADH, malate dehydrogenase, lactate dehydrogenase and glutamate dehydrogenase. The other chemicals were commercially available analytical-grade preparations. Ordinary glass-distilled water was passed through an Elgastat deionizer.

RESULTS

Glutamate oxidation in brain mitochondrial systems. The fate of glutamate in brain mitochondrial systems under different experimental conditions is shown in Table 3. Glutamate uptake and $^{14}CO_2$ formation were comparable, indicating that the dilution by endogenous glutamate was small. The glutamate content of ten mitochondrial preparations was only 7·7 ± 1·62μmoles/100mg. of protein. Further, glutamate was not formed in appreciable amounts during the incubations, as shown by the observation that the relative specific activity of the glutamate remaining after incubation was near to 1·0 (0·94–1·01 in five experiments). Aspartate formation accounted for a large fraction of the glutamate removed (last line of Table 3). Paper chromatography confirmed that the amino acid product of glutamate oxidation was almost exclusively aspartate. Alanine was not detected and in a few experiments only trace amounts of γ-aminobutyrate were found. Since the accumulation of α-oxoglutarate was very small (0·04–0·1mM final concn.) the glutamate removed was apparently oxidized beyond this α-oxo acid. Therefore the contribution of the transamination pathway to the oxidation of glutamate can be calculated on the basis of reaction (2), and under the conditions tested the percentage value of the conversion of glutamate into aspartate can be used for assessing the magnitude of this pathway.

If all the glutamate consumed were oxidized through the transamination pathway the expected ratio of oxygen consumed to glutamate removed would be 1·5 (reaction 2). In fact this ratio was much higher in the absence of ADP (1·93) and the values were also greater in the active state (last two columns of Table 3). This finding is explicable by the observation that a portion of the disappeared glutamate was not converted into aspartate and thus was probably oxidized by glutamate dehydrogenase. Since oxaloacetate accumulation was very low under all conditions tested (less than 0·01mM final concn.) it is probable that a part of oxaloacetate which did not react by transamination was further metabolized. This was shown to occur in experiments in which the mitochondrial suspension was incubated with [5-^{14}C]glutamate as substrate (Table 4). The oxaloacetate derived from [5-^{14}C]glutamate will be

Table 3. *Glutamate oxidation in rat-brain mitochondrial systems*

Rat-brain mitochondrial preparations were incubated in the basic medium (see the Experimental section) with L-glutamate (7–10mM) (in some experiments [1-^{14}C]glutamate, 10μc/m-mole, was used), and also ADP (2mM), 2,4-dinitrophenol (50μM) and 0·5mg. of yeast hexokinase (Sigma type III) plus glucose (10mM) when added. When the hexokinase–glucose trapping system was used the concentration of inorganic phosphate was 40mM. Incubation was for 40–60min. at 37·5° in O_2 atmosphere. The metabolic changes are given in μmoles/hr./100mg. of protein (± S.E.M.; no. of experiments in parentheses). The mean of the ratio of O_2 uptake to glutamate uptake and the mean of the percentage conversion of glutamate into aspartate are given with the range in square brackets.

Additions...	None	ADP	ADP+dinitrophenol	ADP+hexokinase
Metabolic changes:				
O_2	−45·8 ±1·50 (5)	−207 ±11·1 (8)	−474 ±19·7 (7)	−474 ±19·9 (3)
$^{14}CO_2$	+23·6 ±1·14 (5)	+117 ±20·6 (6)	+278 ±12·2 (5)	+271 (2)
Glutamate	−24·5 ±1·16 (4)	−139 ± 8·5 (8)	−283 ± 9·2 (7)	−272 ±16·9 (3)
Aspartate	+19·0 ±2·26 (4)	+121 ± 6·3 (8)	+249 ± 8·9 (7)	+258 ±20·9 (3)
α-Oxoglutarate	+ 0·31±0·04 (4)	3·29± 0·49 (7)	3·18± 0·79 (6)	5·09 (2)
$\Delta O_2/\Delta$glutamate:	1·93 [1·58–2·09]	1·49 [1·33–1·65]	1·68 [1·53–1·92]	1·73 [1·64–1·80]
Conversion of glutamate into aspartate: $\frac{\Delta \text{Aspartate}}{\Delta \text{Glutamate}} \times 100$	78 (4) [62–89]	87 (8) [77–100]	88 (7) [82–97]	94 (3) [89–99]

Table 4. *Oxidation of [5-^{14}C]glutamate in brain mitochondrial systems*

The incubation mixture contained basic medium (see the Experimental section) and [5-^{14}C]glutamate (9·7mM; 40μc/m-mole), and also ADP (2mM) when added. The mitochondrial fraction contained 10·9mg. of protein per vessel. The incubation volume was 1·5ml. in the absence of ADP and 3·0ml. in the presence of ADP. Metabolic changes are given in μmoles/hr./vessel.

Vessel no. ...	1	2
Additions...	None	ADP
Metabolic changes:		
O_2	−5·50	−23·4
$^{14}CO_2$	+0·48	+ 1·46
Glutamate	−3·00	−14·9
Aspartate	+2·15	+11·0
α-Oxoglutarate	+0·92	+ 0·12
Rel. sp. activity of residual aspartate:	—	0·48

labelled equally in C-1 and C-4 due to randomization of ^{14}C. The $^{14}CO_2$ will be produced from the labelled oxaloacetate if further metabolized, either by decarboxylation to pyruvate or by entering the tricarboxylic acid cycle and yielding $^{14}CO_2$ at the steps of oxidation of isocitrate and α-oxoglutarate. The formation of $^{14}CO_2$ in the absence of ADP was 16% of the glutamate removed (vessel 1) and less than 10% in the presence of ADP (vessel 2). If the oxygen consumption calculated on the basis of glutamate removal through the transamination and dehydrogenase pathways is corrected for the further metabolism of oxaloacetate according to the results of Table 4, the oxygen uptake can be almost completely accounted for by the oxidation of glutamate even in the absence of ADP, e.g. the calculated value of oxygen uptake in vessel 1 of Table 2 is 5·3 and 5·6 depending on whether pyruvate was assumed to be formed from oxaloacetate or from endogenous sources.

In a few experiments the activity of C-4 of aspartate formed during the metabolism of [1-^{14}C]-glutamate was measured, but the counts were not significantly above the background, confirming the observations of Haslam & Krebs (1963*b*) that a γ-decarboxylation of glutamate, proposed by Cohen, Simon, Berry & Chain (1962), did not occur. Moreover, when [5-^{14}C]glutamate was the substrate the relative specific activity of residual aspartate was near to 0·5 (vessel 2 in Table 4), which is the theoretical value, when glutamate is not oxidized through γ-decarboxylation.

These results indicated that both in the resting and in the active state of mitochondrial respiration the major pathway of glutamate oxidation proceeds through transamination. This was confirmed by the effect of malonate on glutamate oxidation. Malonate inhibits succinate oxidation and thus should block the operation of the pathway of glutamate utilization through transamination with oxaloacetate. In the presence of malonate the conversion of glutamate into aspartate was negligible under the experimental conditions (Table 5). Oxygen and glutamate uptake in the presence of ADP or ADP plus 2,4-dinitrophenol was only a small fraction of that obtained in the uninhibited systems. The inhibition was the smallest in the absence of ADP, when oxygen uptake was about half of that obtained

Table 5. *Effect of malonate on glutamate oxidation in brain mitochondrial systems*

The incubation mixture contained basic medium (see the Experimental section) and L-glutamate (9·4 mM), and also ADP (2 mM), dinitrophenol (50 μM) and malonate (20 mM) when added. The incubation volume was 1·5 ml. in the absence of ADP and 3·0 ml. in the presence of ADP. Incubation was for 1 hr. in O_2 atmosphere. Changes in metabolites are given in μmoles/hr./vessel.

	Normal			Malonate		
Vessel no. ...	1	2	3	4	5	6
Additions...	None	ADP	ADP+ dinitrophenol	None	ADP	ADP+ dinitrophenol
Mitochondrial protein added (mg.)...	8·2	4·1	4·1	8·2	8·2	8·2
Metabolic changes:						
O_2	−4·81	−10·3	−19·5	−2·13	−3·62	−4·09
Glutamate	−2·25	−6·67	−11·1	−2·08	−3·54	−3·74
Aspartate	+1·75	+6·29	+9·21	0*	0*	0*
α-Oxoglutarate	+0·03	+0·16	+0·08	+0·01	+0·02	+0·02
$\Delta O_2/\Delta$ glutamate:	2·14	1·54	1·76	1·02	1·02	1·09
Rates in the presence of malonate as % of control:						
O_2				44	18	10
Glutamate				93	27	17

* Not measurable.

without malonate, and glutamate uptake was only slightly affected. The ratio of oxygen uptake to glutamate removal was about 1 in the presence of malonate, which is the theoretical value for the oxidation of glutamate to succinate through the glutamate-dehydrogenase route (reaction 1). It may be noted that in the presence of malonate the stimulation of glutamate oxidation by dinitrophenol is very small, whereas this uncoupler approximately doubles the uptake of glutamate in the absence of malonate. Since α-oxoglutarate accumulation was negligible in the presence of malonate the rate-limiting step of glutamate oxidation under these conditions in intact mitochondria is apparently the glutamate-dehydrogenase reaction.

Respiratory control with glutamate as substrate. It has been reported (Balázs, Biesold & Magyar, 1963) that with brain mitochondrial preparations the highest respiratory control ratio, measured by comparing the respiration rate in the presence of an excess of a phosphate-acceptor system with the rate in the absence of added ADP, can be obtained with glutamate as substrate. When pyruvate plus malate or α-oxoglutarate were the substrates the respiratory control ratio was 5–6 under these conditions, whereas it was 10–17 with glutamate. The high control ratio was mainly due to the low respiration rate with glutamate as substrate in the absence of added ADP. The rate was only half of that obtained with α-oxoglutarate (compare vessel 1 with vessel 7 in Table 6), whereas the oxygen uptakes in the stimulated state were comparable with the two substrates (Balázs *et al.* 1963).

Since a large fraction of the glutamate utilized yields aspartate (Table 3) it is possible that the rate of glutamate oxidation is controlled by the rate of oxaloacetate formation in the absence of ADP. This assumption was tested by supplementing the mitochondrial system with malate, which approximately doubled the rates of glutamate removal and of aspartate formation (vessel 2 in Table 6). In the presence of malate the oxidation of glutamate proceeded completely through the transamination pathway, whereas in the control (vessel 1) only about 70% was attributed to this route. Almost all the α-oxoglutarate formed was oxidized, since the amount accumulating, although increased significantly, was very low. The concentration of oxaloacetate was very low, although it was increased on the addition of malate. These experiments indicate that the low glutamate oxidation in the absence of ADP is related to a limitation in the supply of oxaloacetate.

Since in the resting state the mitochondrial nicotinamide nucleotides are mainly in the reduced form, it is possible that the oxidation of NAD^+-linked substrates was decreased by an unfavourable $NAD^+/NADH$ ratio. The effect of added NAD^+ was therefore tested. The mitochondria became apparently permeable to NAD^+ in the absence of added ADP, so it is probable that they were damaged under these conditions. In contrast with mitochondrial systems containing ADP, when added NAD^+ had no effect on the respiration rate, 0·1 mM-NAD^+ more than doubled the rate of glutamate oxidation in the unfortified system (vessel 3). The

Table 6. *Effect of malate and NAD+ on glutamate oxidation in the absence of ADP*

The incubation mixture contained basic medium (see the Experimental section) and [1-^{14}C]-glutamate (8·4 mM), and also α-oxoglutarate (10 mM), L-malate (7·3 mM) and NAD+ (0·1 mM) plus nicotinamide (25 mM) when added. The brain mitochondrial preparation contained 10·8 mg. of protein/vessel. Changes in metabolites are given as μmoles/50 min./vessel.

Vessel no.	1	2	3	4	5	6	7
Additions.....	Glutamate	Glutamate + malate	Glutamate + NAD+	Glutamate + malate + NAD+	Malate	Malate + NAD+	α-Oxoglutarate
Metabolic changes:							
O_2	−4·0	−8·6	−10·0	−14·4	−4·6	−11·6	−8·3
$^{14}CO_2$	+1·94	+3·71	+ 5·18	+ 4·73			
Glutamate	−2·1	−3·7	− 5·4	− 4·9			
Aspartate	+1·4	+3·6	+ 4·2	+ 4·1			
α-Oxoglutarate	+0·02	+0·23	+ 0·08	+ 1·38	+0·02	+ 0·10	−8·36
Malate	—	−5·56	—	−10·43	−4·59	− 8·82	+3·63
Oxaloacetate	+0·008	+0·019	+ 0·016	+ 0·005	+0·018	+ 0·003	0
Pyruvate	+0·008	+0·015	+ 0·027	+ 0·078	+0·009	+ 0·051	+0·04

Table 7. *Glutamate oxidation in liver mitochondrial systems*

Rat-liver mitochondrial preparations were incubated in the basic medium (see the Experimental section) with L-glutamate (8–10 mM) (in some experiments [1-^{14}C]glutamate, 10 μc/m-mole, was used), and also ADP (2 mM) and dinitrophenol (40 μM) when added. Incubation was for 40–60 min. at 37·5° in O_2 atmosphere. Metabolic changes are given in μmoles/hr./100 mg. of protein (± S.E.M.; no. of experiments in parentheses). The mean of the ratio of O_2 uptake to glutamate removal and the mean percentage conversion of glutamate into aspartate are given with the range in square brackets.

Additions...	None	ADP	ADP + dinitrophenol
Metabolic changes:			
O_2	−84·1 (2)	−139 ± 10·4 (6)	−495 ± 15·3 (7)
$^{14}CO_2$	+48·7 (2)	+ 87·1 ± 9·20 (4)	+327 ± 22·5 (5)
Glutamate	−61·9 (2)	− 96·0 ± 3·91 (9)	−357 ± 10·1 (9)
Aspartate	+41·0 (2)	+ 74·9 ± 4·09 (9)	+292 ± 11·7 (9)
α-Oxoglutarate	+ 1·55 (2)	+ 8·02 ± 1·41 (4)	+ 8·9 ± 1·88 (4)
$\Delta O_2/\Delta$glutamate:	1·35 (2)	1·46 (6) [1·28–1·62]	1·41 (7) [1·30–1·62]
Conversion of glutamate into aspartate: $\frac{\Delta \text{Aspartate}}{\Delta \text{Glutamate}} \times 100$	66 (2)	78 [70–88]	82 [70–94]

further addition of malate did not increase the uptake of glutamate in the presence of added NAD+ (vessel 4).

It is known that the oxidation of malate by mitochondrial preparations is very slow (cf. Chappell, 1964*a*), as is also shown in Table 6 (vessel 5). The equilibrium constant of the malate-dehydrogenase reaction is very strongly in favour of malate formation, so that the oxidation of malate will depend on the rate of removal of oxaloacetate. Another means of increasing the rate of malate oxidation is by increasing the concentration of NAD+. When NAD+ was added to a mitochondrial system containing malate the uptake of malate and oxygen was indeed doubled (vessel 6). Thus the low rates of glutamate and malate oxidation in the absence of added ADP are probably due to the same limiting factor, and only after the removal of this rate-limiting step by added NAD+ do the coupling reactions become the pace-makers as a result of a relative lack of phosphate acceptor. When these precautions are met the respiratory control ratio with glutamate as substrate is comparable with that obtained with α-oxoglutarate.

Oxidation of glutamate by liver mitochondrial preparations. Contradicting values have been reported from different Laboratories for the contribution of the transamination pathway to glutamate oxidation

Table 8. *Effect of malonate on glutamate oxidation in liver mitochondrial systems*

The incubation mixture contained basic medium (see the Experimental section) and [1-^{14}C]glutamate (10·4 mM), and also ADP (2 mM), dinitrophenol (40 μM) and malonate (20 mM) when added. The incubation volume was 1·5 ml. in vessels 1 and 4 and 3·0 ml. in the other vessels. Incubation was for 50 min. Metabolic changes are given in μmoles/50 min./vessel.

	Normal			Malonate		
Vessel no. ...	1	2	3	4	5	6
Additions...	None	ADP	ADP+ dinitrophenol	None	ADP	ADP+ dinitrophenol
Mitochondrial protein added (mg.)...	9·1	9·1	4·55	9·1	9·1	9·1
Metabolic changes:						
O_2	−5·99	−13·6	−21·3	−1·68	−11·3	−12·6
$^{14}CO_2$	+3·71	+ 8·36	+12·2	+1·88	+ 9·65	+11·6
Glutamate	−4·48	− 8·47	−13·7	−2·24	−11·7	−12·7
Aspartate	+2·48	+ 6·37	+10·9	+0·11	+ 0·32	+ 0·26
α-Oxoglutarate	+0·22	+ 0·57	+ 0·45	+0·37	+ 0·22	+ 0·25
Rates in the presence of malonate as % of control:						
O_2				28	83	30
Glutamate				50	138	46
$\Delta O_2/\Delta$glutamate:	1·34	1·61	1·55	0·75	0·97	0·99

by liver mitochondrial preparations (e.g. Krebs & Bellamy, 1960; Borst, 1962), but owing to the differences in the experimental conditions the results are not comparable. In the following experiments the effect of the state of mitochondrial respiration, which was one of the major differences in the conditions used by Krebs & Bellamy (1960) and Borst (1962), on the relative contribution of the different pathways to glutamate oxidation was studied (Table 7). The major pathway of glutamate oxidation was through transamination whether the mitochondria were incubated in the presence or absence of dinitrophenol, since about 80% of the glutamate uptake was accounted for as aspartate formed. As in brain mitochondria α-oxoglutarate accumulation was very small. Respiratory $^{14}CO_2$ formation was comparable with the removal of [1-^{14}C]glutamate (cf. Table 8), although in a few experiments significantly less $^{14}CO_2$ was formed relative to glutamate uptake. The addition of malonate blocked almost completely the transamination of glutamate, since aspartate formation was negligible (vessels 4–6 in Table 8). In the presence of malonate the ratio of oxygen uptake to glutamate consumption was near to 1. This value is to be expected when the oxidation of glutamate to succinate proceeds through the glutamate-dehydrogenase pathway (reaction 1). With mitochondrial preparations incubated with added ADP (vessel 5) the inhibition of respiration was small, whereas glutamate uptake was stimulated by 38%. Thus under these conditions the oxidation of glutamate proceeded exclusively through the glutamate-dehydrogenase pathway, which compensated almost completely for the oxygen uptake.

In the absence of ADP the oxidation of glutamate differed in some respects from that described above. The respiration rate was very low, and less glutamate was oxidized through the transamination pathway than in the presence of ADP (vessel 1). Malonate inhibited substantially both oxygen and glutamate uptake (vessel 4), whereas in the presence of ADP (vessel 5) this did not occur. Malonate had a similar, although less marked, effect on α-oxoglutarate oxidation in the absence of ADP (vessel 4 in Table 8), so that it is probable that the mitochondrial preparations were damaged under these conditions.

In the presence of dinitrophenol malonate inhibited oxygen uptake by 70% and the removal of glutamate by 54% (vessel 6 in Table 8). The rate-limiting reaction under these conditions must be that catalysed by the glutamate dehydrogenase, because α-oxoglutarate accumulation was very small. Further, when the oxidation of α-oxoglutarate was measured under comparable conditions (vessel 6 in Table 9), it was observed that the rate of α-oxoglutarate uptake was almost four times that of glutamate. When the oxidation of glutamate and α-oxoglutarate are compared in liver mitochondrial systems by calculating the ratios of oxygen uptake to substrate removal, the results indicate that a larger section of the tricarboxylic acid cycle is involved in glutamate oxidation (α-oxoglutarate to oxaloacetate) than in the oxidation of α-oxoglutarate (α-oxoglutarate to malate) (cf. Chappell, 1964*b*). Similar results were obtained with brain mitochondrial preparations.

'NADP-specificity' of glutamate dehydrogenase as

Table 9. *Oxidation of α-oxoglutarate in liver mitochondrial systems*

The incubation mixture contained basic medium (see the Experimental section) and α-oxoglutarate (20·1 μmoles to vessels 1 and 4, 30·1 μmoles to the other vessels), and also ADP (2 mM), dinitrophenol (10 μM) and malonate (20 mM) when added. The incubation volume was 2·0 ml. The same mitochondrial preparation as that in Table 6 was used. Incubation was for 50 min. Metabolic changes are given in μmoles/50 min./vessel.

	Normal			Malonate		
Vessel no. ...	1	2	3	4	5	6
Additions...	None	ADP	ADP+ dinitrophenol	None	ADP	ADP+ dinitrophenol
Mitochondrial protein added (mg.)...	9·1	9·1	4·55	9·1	9·1	4·55
Metabolic changes:						
O_2	−10·5	−14·0	−23·3	−5·5	−12·7	−12·0
α-Oxoglutarate	−11·1	−14·8	−25·4	−8·3	−24·5	−23·5
Rates in the presence of malonate as % of control:						
O_2				52	91	51
α-Oxoglutarate				75	165	92
$\Delta O_2/\Delta$ α-oxoglutarate:	0·95	0·95	0·92	0·66	0·52	0·51

Table 10. *Aspartate-aminotransferase and glutamate-dehydrogenase activities in liver and brain mitochondrial fractions*

The O_2 uptake was calculated from the mean values given in Tables 3 and 7 for the dinitrophenol-containing systems and is expressed as μg.atom/min./mg. of protein at 37·5°. The enzyme assays were as described in the Experimental section. The total activities were measured in mitochondrial preparations that had been diluted 20–40-fold in Triton X-100 (final concn. 0·1%) before the assay. The enzyme activities are given in μmoles/min./mg. of protein at 25°.

	Liver	Brain
O_2 uptake:	0·165	0·158
Aspartate-aminotransferase activity:		
(a) Glutamate + oxaloacetate → α-oxoglutarate + aspartate	0·891, 0·877, 1·429, 1·28, 1·0 (mean 1·10)	0·798, 1·085, 1·01 (mean 0·964)
(b) α-Oxoglutarate + aspartate → glutamate + oxaloacetate	3·16, 2·15, 2·10 (mean 2·65)	1·48, 1·53, 1·38, 0·91, 1·61 (mean 1·33)
Glutamate-dehydrogenase activity:		
(a) Glutamate + NAD^+ + H_2O → α-oxoglutarate + NH_3 + NADH + H^+	0·251, 0·229, 0·234, 0·22 (mean 0·233)	0·046, 0·030, 0·0295 (mean 0·035)
(b) α-Oxoglutarate + NH_3 + NADH + H^+ → glutamate + NAD^+ + H_2O	2·19, 2·86, 3·10 (mean 2·71)	0·33, 0·273, 0·252, 0·382 (mean 0·309)

a factor in the control of the glutamate-dehydrogenase pathway. The operating activity of glutamate dehydrogenase both in the active and the resting state was much higher in the presence than in the absence of malonate (Table 8). The total activity of this enzyme as measured in disrupted liver mitochondrial preparations is even higher than the respiration rate (Table 10) (cf. Jones & Gutfreund, 1961; Borst, 1962; Chappell, 1961*b*), but there is no reason to believe that malonate has 'unmasked' the glutamate-dehydrogenase activity. Thus these results suggest that the glutamate-dehydrogenase pathway is subject to some control mechanism. Borst (1962) suggested that the control may be explicable by the assumption of Klingenberg & Slenczka (1959) as to the NADP specificity of glutamate dehydrogenase in mitochondria (cf. the Discussion section). In the next experiments this assumption was tested (Table 11). The 'free' activity of glutamate dehydrogenase was assayed in dinitrophenol-stimulated systems in the presence of malonate by measuring oxygen and glutamate uptake, and the effect of added nicotinamide nucleotides on the enzymic activity was determined. The permeability of the mitochondrial membrane for nicotinamide nucleotides was increased by pretreatments that produced only slight or no 'unmasking', like a single freezing and thawing or hypo-osmotic conditions (0·1 M-sucrose; Bendall & de Duve, 1960). Whichever nicotinamide nucleotide is the cofactor of the glutamate dehydrogenase

Table 11. *Effect of mild structural damage of liver mitochondria on the glutamate-dehydrogenase activity in the presence and absence of NAD+ and NADP+*

The incubation mixture contained basic medium (see the Experimental section), L-glutamate (9·5mM), ADP (2mM), dinitrophenol (40μM) and malonate (20mM). A portion of the mitochondrial preparation was frozen and thawed once. Another portion was diluted to 0·1M-sucrose concentration and kept for 10min. in an ice–water bath before incubation started. Untreated mitochondrial fraction was used in the control (vessel 1). When nicotinamide nucleotides (2mM) were added nicotinamide (25mM) was also included in the medium. The osmolarity of all media was adjusted with KCl to 0·3. The mitochondrial protein added was 10·7mg. The incubation volume was 2ml. except for mitochondrial preparations pretreated in 0·1M-sucrose (3ml.). Metabolic changes are given in μmoles/40min./vessel.

Vessel no.	1	2	3	4	5	6	7
Pretreatment...	None	Freezing and thawing			Pretreatment in 0·1M-sucrose		
Additions	None	None	NAD+	NADP+	None	NAD+	NADP+
Metabolic changes:							
O_2	−11·3	−10·5	−13·6	−7·25	−10·3	−13·6	−7·80
Glutamate	− 9·58	− 8·60	−11·9	−6·02	−10·6	−13·3	−5·88
Aspartate	+ 0·40	+ 0·36	+ 0·14	+0·23	+ 0·10	0·0	+0·35
α-Oxoglutarate	+ 0·32	+ 0·31	+ 0·49	+0·16	+ 0·72	+ 0·93	+0·56

the rate of glutamate removal should be stimulated or unaffected by the addition of the cofactor, depending on whether or not the enzyme is saturated with the coenzyme. The results in Table 11 show that after both treatments the rate of glutamate uptake, which under the present conditions is by the glutamate-dehydrogenase pathway, was only slightly affected, unless nicotinamide nucleotides were added (compare vessel 1 with vessels 2 and 5). On the addition of NAD+ the rate of glutamate uptake was increased by 38% (vessel 3) and by 26% (vessel 6). On the addition of NADP+ the rate of glutamate removal was substantially decreased (vessels 4 and 7), a finding that is at variance with the alleged role of NADP+ in glutamate oxidation.

DISCUSSION

The observations reported in the present paper agree with the findings of Krebs & Bellamy (1960) in showing that transamination is the main pathway of glutamate oxidation in brain mitochondrial preparations. However, in contrast with their results, this was found to be the major route of glutamate oxidation also in liver mitochondrial preparations; this confirms the observations of other investigators (Jones & Gutfreund, 1961; Borst, 1962; Papa, Saccone, Palmieri, Francavilla & Quagliariello, 1963; Chappell, 1964*b*; Hird & Marginson, 1964; de Haan *et al.* 1964). The oxidation of glutamate by pathways other than transamination was estimated from the difference between glutamate removal and aspartate formation and also by blocking the transamination reaction by malonate. It was shown that the activity of the pathways other than transamination is much lower in brain than in liver mitochondrial preparations. The mean rates accounted for by these pathways in the active state in the presence and absence of malonate were in brain mitochondria 45 and 33 and in liver mitochondria 161 and 70μmoles/hr./100mg. of protein. Dinitrophenol increased the rate of oxidation of glutamate both in brain and in liver mitochondria without changing the relative proportions oxidized by transamination and by other routes (Tables 3 and 7). The ratio of these two pathways remained approximately constant during incubation for 1hr. at 37·5° (Fig. 1) and it was not affected by changing the glutamate concentration within the range 3·5–10mM. The glutamate that was not oxidized through the transamination pathways was oxidized in liver mitochondria by the glutamate dehydrogenase; this was also indicated by measurements of the formation of ammonia during the oxidation of glutamate (Papa *et al.* 1963; Hird & Marginson, 1964). In brain mitochondrial preparations the activity of glutamate dehydrogenase was much lower than in liver mitochondria (Table 10).

In brain preparations glutamate can also be metabolized by the glutamate decarboxylase. Quantitative amino acid analysis by paper chromatography was carried out in a few experiments, but only trace amounts of γ-aminobutyric acid were found at the end of the incubation. This would not exclude the possibility that this compound was formed and metabolized further by transamination with α-oxoglutarate (Bessman, Rossen & Layne, 1953). However, the contribution of this pathway must be small, since in previous experiments it was found that the respiration rate with or without

added α-oxoglutarate was only slightly increased by high concentrations of γ-aminobutyric acid (Balázs *et al.* 1963); also, the activity of the glutamate decarboxylase in these Triton-treated mitochondrial preparations was low (19·5 μmoles/hr./100 mg. of protein; compare with rates given in Table 10).

In liver mitochondrial preparations in the presence of ADP only a fraction of the total glutamate uptake (28 μmoles/hr./100 mg. of protein; Table 8) could be accounted for by the glutamate-dehydrogenase pathway, whereas when transamination was blocked with malonate the oxidation of glutamate by this route was increased about sixfold (154 μmoles/hr./100 mg. of protein). Thus the available (i.e. not latent) activity of glutamate dehydrogenase was much higher than the operating activity under these conditions.

It is suggested that the control of glutamate-dehydrogenase activity is attributable to a competition for NAD^+ between the enzymes oxidizing glutamate and α-oxoglutarate. The oxidation of glutamate yields α-oxoglutarate, which is further metabolized in the tricarboxylic acid cycle, as indicated by the low concentration (0·1 mM) of this metabolite during incubation. The aspartate-aminotransferase activity of the mitochondria is in great excess compared with the rate of respiration or with the activity of glutamate dehydrogenase (Table 10). Therefore oxaloacetate is continuously removed by the transamination reaction. Thus after the reactions are started α-oxoglutarate is formed independently of the dehydrogenation of glutamate. The main question is therefore how the glutamate-dehydrogenase activity is influenced by the presence of α-oxoglutarate. A direct inhibition of the glutamate-dehydrogenase reaction by α-oxoglutarate does not seem to be a major effect, since the final concentrations of α-oxoglutarate were of the same order of magnitude in the presence and absence of malonate; nevertheless, the oxidation of glutamate by the dehydrogenase was increased several-fold in the presence of malonate (vessels 2 and 5 in Table 8). The results indicate that competition takes place between the enzymes oxidizing the two substrates, resulting in preferential oxidation of α-oxoglutarate (Tables 3 and 7). This would agree with the general considerations of Krebs (1959). In view of the very complex nature of the two enzyme systems (α-oxoglutarate-dehydrogenase complex and glutamate dehydrogenase) and of our limited knowledge of their behaviour in the mitochondria, the detailed mechanism of the competition has yet to be elucidated.

The preferential oxidation of α-oxoglutarate is probably related to the observation that the apparent K_m for NAD^+ of the purified α-oxoglutarate-dehydrogenase complex is much lower ($4·5 \times 10^{-6}$ M; Sanadi, 1963) than that of the glutamate dehydrogenase (9×10^{-5} M; Frieden, 1959). It was also found that at high substrate concentrations the rate of α-oxoglutarate oxidation by 'intact' liver mitochondria was about four times as high as that of the oxidation of glutamate by the glutamate dehydrogenase in the presence of malonate and dinitrophenol (Tables 8 and 9). The relative contribution of the two pathways of glutamate oxidation will depend on the relative rates of substrate oxidation by the α-oxoglutarate dehydrogenase and glutamate dehydrogenase under any particular conditions. A further consideration is that some oxaloacetate will escape transamination by being further metabolized; this is indicated by the formation of $^{14}CO_2$ from [5-^{14}C]glutamate under these conditions (Table 4).

Klingenberg & Slenczka (1959) and Klingenberg & Pette (1962) gave circumstantial evidence as to the NADP-specificity of mitochondrial glutamate dehydrogenase. It was suggested by Borst (1962) that the control of the pathways of glutamate oxidation can be explained by the NADP-specificity of this enzyme, assuming a 'compartmentation' of nicotinamide nucleotides in the mitochondria. The cofactor specificity of glutamate dehydrogenase was tested under conditions when glutamate removal was due exclusively to the dehydrogenase pathway. The rate-limiting enzyme was glutamate dehydrogenase, and the permeability of the mitochondria to nicotinamide nucleotides was increased by mild pretreatment that hardly affected the latency of the enzymes (Table 11). Although the mitochondria were not 'intact' the rate of oxidation of glutamate without added cofactors (vessels 2 and 8) were comparable with that obtained with the untreated preparation (vessel 1). Thus probably no 'regrouping' of the nicotinamide nucleotides occurred. In these preparations the rate of the dehydrogenation of glutamate was increased by NAD^+ (about 30%) and the rate in the presence of $NADP^+$ was about half of that observed with NAD^+. Thus the properties of glutamate dehydrogenase under conditions when the latency was hardly affected were similar to those observed with the enzymes completely 'unmasked' (Gutfreund *et al.* 1961). These results make it unlikely that the control of the two pathways of glutamate oxidation would result from an NADP-specificity of mitochondrial glutamate dehydrogenase.

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Compartmentation of Glutamic Acid Metabolism in Developing Cerebral Cortex*

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As a consequence of studies on the exchange of ^{14}C-glutamic acid between circulating blood and brain cortex in the mouse and rat, data were obtained which indicated that the resultant ^{14}C-glutamine in the blood probably came from pools of tissue glutamic acid which were not in rapid equilibrium with the total content of the tissue glutamic acid (1). This was investigated further in brain by the administration of tracer quantities of ^{14}C-labeled glutamic acid or aspartic acid into the cisterna magna of rats and monkeys (2). These experiments left little doubt that more than one pool of glutamic acid was present in this tissue, and that one of these pools provided the glutamic acid required for the formation of glutamine. In studies of ammonia metabolism in which ^{15}N-ammonium acetate was administered into the systemic circulation of the cat, evidence was again elicietd which was entirely consistent with the contention that only a fraction of the total glutamic acid in brain cortex is available for rapid conversion to glutamine (3). Subsequent data obtained from studies on CO_2 fixation by brain tissue *in vivo* continued the support for this interpretation (4, 5).

An analysis of the changes in the levels of glutamic acid and glutamine during the process of maturation of the neocortex of the kitten suggested the presence at birth of this active glutamate compartment (6). Preliminary experiments with 1-day-old animals were negative for data which could be interpreted as clearly indicative of more than one pool of tissue glutamic acid (7). Since compartmentation in the neocortex appeared to be a phenomenon accompanying maturation, an investigation was undertaken to follow its development from the neonatal to the adult state.

EXPERIMENTAL PROCEDURE

Animals ranging in age from 1 week through adulthood were studied. Pentobarbital anesthesia (35 mg per Kg), respirometer-controlled respiration via a tracheal cannula, and local anesthesia (Xylocaine) were used during the experiments (8). In each experiment 2 litter mates were employed, one animal supplying two time points for a total of four per experiment. In most of the experiments 1-, 3-, and 5-, and 10-minute periods were taken and in others the time periods were 3, 5, 10, and 15 minutes. After bilateral exposure of the cortex, the dura and adhering arachnoid membrane were removed and rectangular pieces of Whatman No. 1 filter paper (0.8 × 1.0 cm in size) were placed on the surface of each hemisphere. The neutralized solution of L-glutamic-^{14}C acid, 20 μl, uniformly labeled (1.8 μc; 120 to 205 μc per μmole), was then applied to each piece of filter paper. This procedure was followed in all experiments but the one utilizing 16-week-old animals. In this case, Whatman No. 3 filter paper was used and 0.05 ml (0.45 μc) of glutamic acid solution was applied. The timing of the application of the substrate was so spaced as to allow 30 seconds between the removal of the two tissue samples. At the end of each experimental period, the piece of filter paper was removed, the sample of cortex (1 to 2 mm thick and 100 to 200 mg in weight) was excised and placed in ice-cold 0.9% NaCl solution. The tissue was then quickly blotted on filter paper and frozen in liquid nitrogen.

In several experiments of 10-minutes duration with adult cats, the tissue was frozen in Dry Ice to facilitate cutting and was sectioned horizontally with a scalpel blade into three or four slices of tissue each approximately 0.5 to 1.0 mm thick. These were then frozen in liquid N_2. Samples of tissue from varying depths of cortex were obtained in this manner. This procedure was followed after application of ^{14}C-glutamic acid (see above) as well as after application of 20 μl of L-aspartic-^{14}C acid, uniformly labeled (1.8 μc; 164 μc per mole).

The tissue samples were weighed and extracted with 5% trichloroacetic acid (2 ml/100 mg or less of tissue) in the frozen state. Glutamic acid, glutamine, aspartic acid, γ-aminobutyric acid, and glutathione were isolated on small columns (7 mm, inner diameter) of Ag 1 (anion exchange) and Ag 50 (cation exchange) resins[1] as described previously (2, 3). The GSH (with *p*-chloromercuriphenyl sulfonate omitted) was eluted from the Ag 1 column in the 9- to 12-ml fraction of 0.1 N HCl. The ninhydrin method of Moore and Stein (9) was used for amino acid determination, except for glutamine, which was measured by the hydroxamic acid procedure of Berl and Waelsch (10).

In preparation for counting, the glutamine was converted to glutamic acid by hydrolysis for 1 hour at 100° in 2 N HCl; the solution was taken to dryness at 60° under reduced pressure, desalted on Ag 50 columns (11), and reisolated from Ag 1 columns (2). The glutamic acid of the GSH was liberated by hydrolysis for 1 hour at 100° in 2 N HCl and reisolated from Ag 1 columns (2). The γ-aminobutyric acid was desalted on columns of Ag 50 (11) but gave a high ninhydrin blank probably due to retention of ammonia. It was therefore further treated as follows. It

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[1] Ag 1 and Ag 50 are purified Dowex 1 and 50 obtained from Bio-Rad Laboratories, Richmond, California.

was taken to dryness in a flash evaporator and the contents of the flask dissolved in 2.2 ml of 0.02 N NaOH. Columns of Ag 1, 3 × 0.7 cm, (partially converted to the acetate form (2)) were prepared and washed with water until the washings were no longer acid. Aliquots of solution, 2 ml, were passed through the columns followed by 10 ml of water and 3.2 ml of 0.1 N acetic acid. The γ-aminobutyric acid is eluted in the acetic acid and redetermined by the ninhydrin procedure.

The radioactivity in the amino acids was counted at infinite thinness on 1¼-inch planchets in a low background gas flow Geiger counter (Nuclear-Chicago Corporation). The neutralized trichloroacetic acid extracts, in 10 μl volumes, were also counted as above. Control experiments indicated that approximately 0.5% of the counts per minute in glutamic acid were carried over into the aspartic acid, and this was corrected for in the calculations of the radioactivity in the aspartic acid.

TABLE I

Labeling of glutamic acid, glutamine, and aspartic acid following application of ^{14}C-glutamic acid and ^{14}C-aspartic acid to surface of brain of adult cat

The brain cortex was cut into three or four horizontal sections each approximately 0.5 to 1.0 mm thick. The amount of substrate applied was 1.8 μc in 0.020 ml (205 μc per μmole) in Experiment 1 and 1.8 μc in 0.020 ml (164 μc per μmole in Experiments 2 and 3. The experimental time period was 10 minutes.

Substrate applied	Glutamic acid		Glutamine		Aspartic acid		Ratio of specific activities of glutamine to glutamic acid
	Amount	Specific activity	Amount	Specific activity	Amount	Specific activity	
	μmoles/g	*c.p.m./μmole*	*μmoles/g*	*c.p.m./μmole*	*μmoles/g*	*c.p.m./μmole*	
1. ^{14}C-Glutamic acid							
Section a	11.0	23,000	6.2	45,900	3.4	2,000	2.00
Section b	12.2	5,760	6.0	21,500	3.7	970	3.73
Section c	9.9	580	6.3	1,410	2.7	140	2.43
2. ^{14}C-Aspartic acid							
Section a	11.2	37,500	5.1	103,200	2.6	230,000	2.75
Section b	11.5	4,750	5.4	25,800	2.9	25,700	5.43
Section c	10.5	1,330	4.8	2,010	2.7	5,300	1.53
3. ^{14}C-Aspartic acid							
Section a	10.9	40,100	5.4	68,300	3.0	158,200	1.70
Section b	12.4	8,800	5.8	26,600	2.7	57,600	3.02
Section c	11.7	11,200	5.7	28,300	2.6	63,700	2.53
Section d	11.3	4,100	5.5	11,100	2.5	21,200	2.71

TABLE II

*Percentage distribution of ^{14}C in glutamic acid, glutamine, and aspartic acid**

	Section a		Section b		Section c		Section d	
	Total counts†	Per cent of total	Total counts	Per cent of total	Total counts	Per cent of total	Total counts	Per cent of total
Experiment 1								
Trichloroacetic acid extract	719,500	(100)	253,400	(100)	18,130	(100)		
Glutamic acid		35.2		27.7		31.4		
Glutamine		39.6		50.9		49.1		
Aspartic acid		0.9		1.4		2.1		
Total		75.7		80.0		82.6		
Experiment 2								
Trichloroacetic acid extract	1,690,000	(100)	268,800	(100)	47,100	(100)		
Glutamic acid		24.9		20.3		29.7		
Glutamine		31.1		51.8		20.8		
Aspartic acid		35.4		27.7		30.4		
Total		91.4		99.8		80.9		
Experiment 3								
Trichloroacetic acid extract	2,123,800	(100)	623,000	(100)	607,000	(100)	205,800	(100)
Glutamic acid		20.6		17.5		18.8		22.5
Glutamine		17.4		21.8		23.1		29.7
Aspartic acid		22.3		25.0		23.8		25.8
Total		60.3		67.3		65.7		78.0

* See Table I for experimental conditions.

† Total counts per minute per g of tissue, wet weight.

RESULTS

To determine whether the topically applied ^{14}C-glutamic acid was metabolized only at the surface of the brain or whether it could penetrate deeply into the cortex, the tissue experimental site was divided into three or four sections and analyzed for amino acid levels and for distribution of radioactivity. The results of such an experiment are given in Table I (Experiment 1).

It is evident that the applied glutamic acid did penetrate into

TABLE III

Percentage distribution of total counts per minute in glutamic acid and glutamine

	1 min		3 min		5 min		10 min		15 min	
	Total counts*	Per cent of total	Total counts	Per cent of total	Total counts	Per cent of total	Total counts	Per cent of total	Total counts	Per cent of total
1 week										
Trichloroacetic acid extract			515.0	100	552.2	100	956.1	100	369.5	100
Glutamic acid				66.7		63.3		63.3		61.0
Glutamine				16.0		15.3		17.4		23.7
2 weeks										
Trichloroacetic acid extract	185.1	100	809.1	100	838.1	100	576.8	100		
Glutamic acid		77.4		80.8		69.5		67.7		
Glutamine		11.1		12.7		16.8		24.4		
3 weeks										
Trichloroacetic acid extract	52.8	100	171.0	100	335.9	100	465.5	100		
Glutamic acid		86.6		72.6		54.2		49.3		
Glutamine		5.9		12.7		23.0		31.0		
3½ weeks										
Trichloroacetic acid extract			137.5	100	356.6	100	399.7	100	1156.9	100
Glutamic acid				75.5		70.8		45.5		43.0
Glutamine				18.0		15.1		42.7		44.4
4 weeks										
Trichloroacetic acid extract	92.8	100	78.2	100	103.6	100	169.3	100		
Glutamic acid		61.2		52.9		50.8		48.4		
Glutamine		35.3		44.8		47.1		46.9		
4 weeks										
Trichloroacetic acid extract			72.5	100	114.2	100	748.0	100	302.5	100
Glutamic acid				59.5		56.5		35.7		35.2
Glutamine				28.5		35.0		53.4		56.5
5 weeks										
Trichloroacetic acid extract	124.9	100	105.5	100	296.8	100	514.0	100		
Glutamic acid		59.6		52.9		50.4		46.4		
Glutamine		27.4		41.6		44.0		50.6		
6 weeks										
Trichloroacetic acid extract	65.7	100	274.0	100	302.9	100	186.5	100		
Glutamic acid		66.0		43.5		40.2		35.7		
Glutamine		27.7		46.6		62.7		61.7		
6 weeks										
Trichloroacetic acid extract			244.1	100	289.7	100	686.6	100	722.5	100
Glutamic acid				46.0		43.3		38.0		34.3
Glutamine				40.8		49.1		54.2		60.0
8 weeks										
Trichloroacetic acid extract					714.3	100	522.5	100		
Glutamic acid						31.7		27.4		
Glutamine						52.7		60.7		
14 weeks										
Trichloroacetic acid extract	81.4	100	287.3	100	577.7	100	571.7	100		
Glutamic acid		51.5		45.3		44.9		40.5		
Glutamine		31.7		36.4		64.4		50.0		
16 weeks										
Trichloroacetic acid extract	7.7	100	13.8	100	15.8	100	10.8	100		
Glutamic acid		64.9		52.9		45.6		39.8		
Glutamine		31.2		39.9		51.3		59.3		
Adult										
Trichloroacetic acid extract	153.2	100	339.4	100	514.7	100	1021.6	100		
Glutamic acid		46.3		36.0		27.3		27.3		
Glutamine		34.7		48.0		54.7		50.4		

* Total counts per minute per g of tissue, wet weight $\times 10^{-3}$.

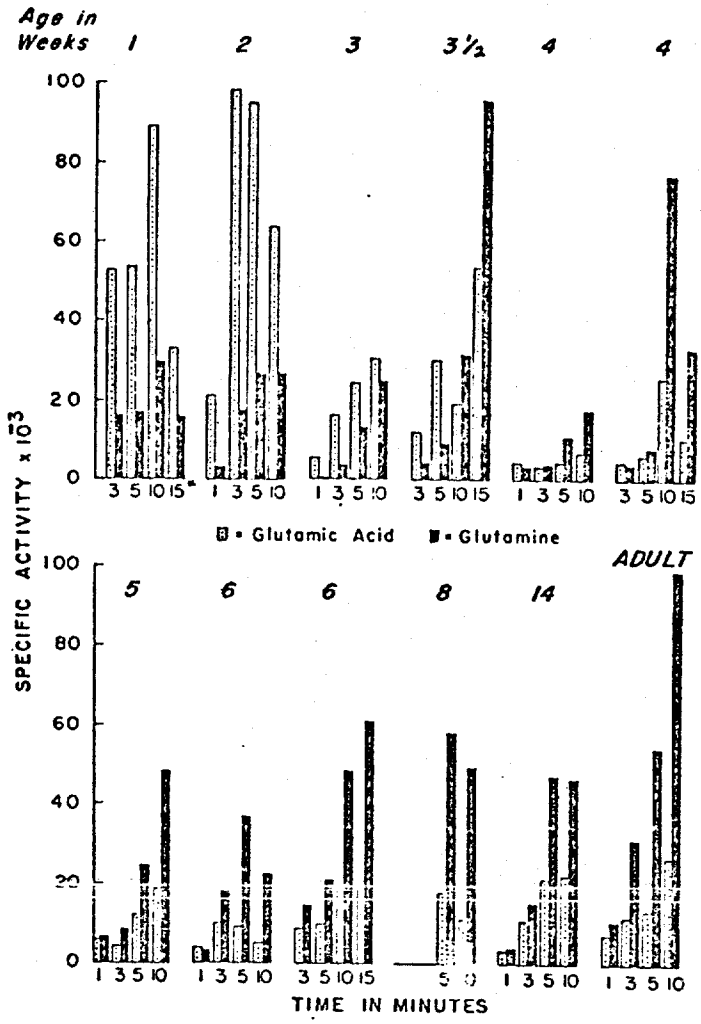

FIG. 1. Specific activities (counts per minute per μmole) of glutamic acid and glutamine in cerebral cortex following application of L-glutamic-^{14}C acid, uniformly labeled (20 μl, 1.8 μc: 120 to 205 μc per μmole), to the surface of the brains of kittens of different ages.

the deep layers of the cortex. In all sections of the cortex the specific activity (counts per minute per μmole) of the glutamine was considerably greater than that of the glutamic acid. The ratio of specific activities of glutamine to glutamic acid was highest in the section approximately 0.5 to 1.0 mm beneath the surface of the brain (Section b), achieving a value of 3.73; this was true even though the highest concentration of radioactivity was in the outermost section of the cortex.

To determine further whether or not one was indeed dealing with an intracellular process occurring throughout the layers of the cortex another metabolite which could be converted to glutamic acid was used; uniformly labeled ^{14}C-aspartic acid was applied to the surface of the brain (Table I, Experiments 2 and 3).

The results obtained were similar to those observed with ^{14}C-glutamic acid, namely, radioactivity was present in all sections of the cortex, the ratios of the specific activities of glutamine to that of glutamic acid were significantly greater than 1 in all sections, and the highest ratio was present in the section of cortex beneath the topmost one (5.43 and 3.02 for Experiments 2 and 3, respectively).

In all three experiments the concentration of amino acids did not vary sufficiently from section to section to affect significantly the ratios of the specific activities.

The percentage distribution of the radioactivity in the glutamic acid, glutamine, and aspartic acid of the trichloroacetic acid tissue extract is given in Table II. Whether ^{14}C-glutamic acid or ^{14}C-aspartic acid was applied to the surface of the brain, glutamine contained more total radioactivity than glutamic acid did in almost every section of cortex; this was particularly evident in Section b of Experiments 1 and 2. The three amino acids accounted for approximately 76 to 83%, 81 to 100%, and 60 to 78% of the total radioactivity of the trichloroacetic acid extracts in Experiments 1, 2, and 3, respectively.

The developmental data are presented in Tables III to V and in Figs. 1 to 5. In general, in each experiment the total number of counts per minute per g of tissue (Table III) was higher in those tissue sites at which the substrate was applied for a longer period of time. The specific activities of the amino acids (Fig. 1) appeared to reflect the total counts per minute present in the tissue and were also higher when the latter was higher.

The total number of counts per minute per g of tissue, wet weight, in the trichloroacetic acid extract showed considerable variation from animal to animal, as well as from point to point of time (Table III). These values are no doubt dependent on many external factors as well as on the rate of removal by the circulation and by metabolic processes such as oxidation to CO_2. The effect of dilution of the applied tracer can be seen in the experiment with the 16-week-old animals (Table III). Here 0.45 μc of L-glutamic acid in 0.05 ml of solution was applied on Whatman No. 3 filter paper. Although one-fourth of the ^{14}C-glutamic acid applied in the other experiments was used in these animals the solution was 10 times as dilute and the total number of counts per minute in the tissue samples was approximately one-tenth of that found in the other experiments.

It is evident from Fig. 1 that in younger kittens the specific activity of the glutamine remains lower than that of its precursor, glutamic acid; this relationship reverses in the 4th week of life when the specific activity of the glutamine is able to achieve a value considerably above that of the total tissue glutamic acid.

Since the total number of counts per minute present in the tissue varied from animal to animal and since the specific activities of the amino acids followed the trend of the total number of counts per minute, the specific activities obtained in the different experimental animals (Fig. 1) were adjusted to a common arbitrary base of 100,000 total c.p.m. per g of tissue. Where the total counts per minute per g of tissue (Table III) were greater than 100,000 the specific activities were proportionately decreased, and where the total counts per minute per g of tissue were less than 100,000 the specific activities were proportionately increased. The data thus obtained is plotted in Fig. 2. A steady state of the glutamic acid-glutamine system is approached in approximately 10 to 15 minutes in animals under 4 weeks of age and in approximately 5 to 8 minutes in animals 4 weeks of age and older; the rate of conversion of glutamic acid to glutamine appears to increase until about 4 weeks of age. The specific activity of total glutamic acid at the steady state level decreases until about 4 to 6 weeks of age while the specific activity of the glutamine rises and the latter becomes greater than the former in the 4th postnatal week. This is portrayed more sharply in Fig. 3 where the adjusted specific activities at the 10- to 15-minute points are plotted against the age of the animals. The plot of the ratios of the specific activities of glutamine to glutamic

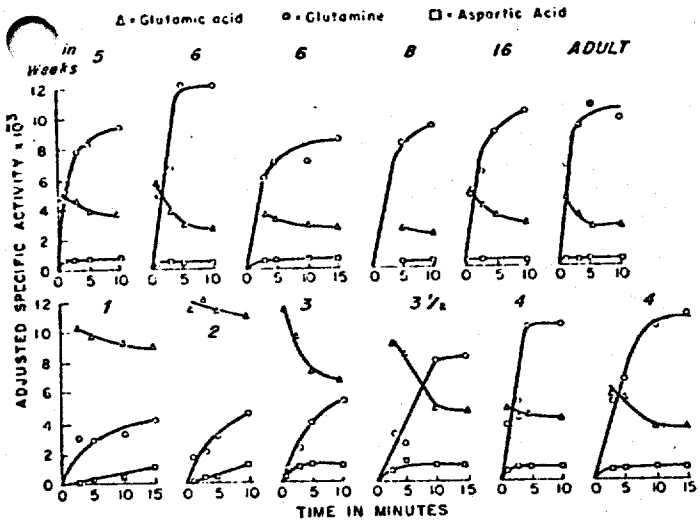

FIG. 2. Data from Fig. 1 and aspartic acid specific activities ljusted to an arbitrary base of 100,000 total c.p.m. per g of tissue (wet weight) for each time point.

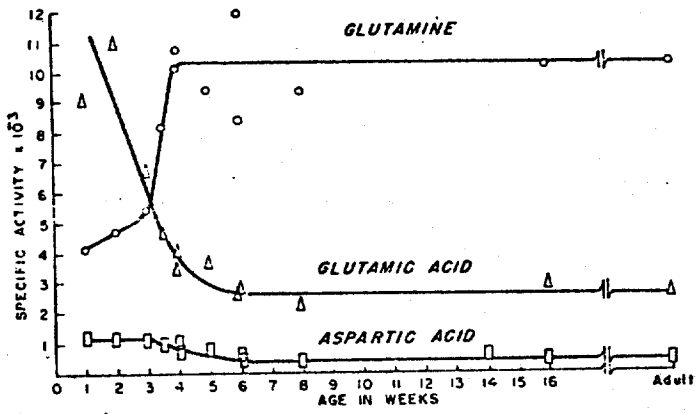

FIG. 3. Composite of the 10-to 15-minute time points from Fig. 2

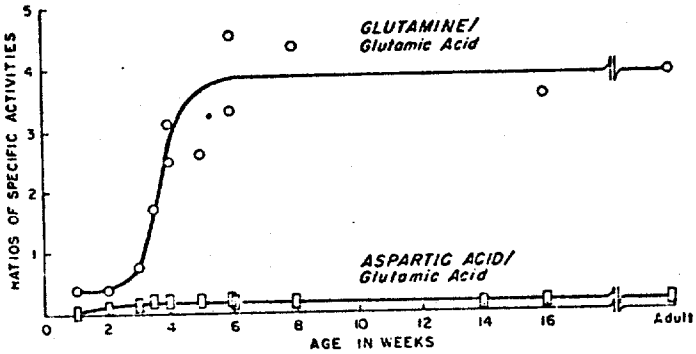

FIG. 4. Ratios of the specific activities of glutamine and aspartic acid to that of glutamic acid at the 10- to 15-minute time points following application of L-glutamic-^{14}C acid to the surface of the cerebral cortex of kittens.

acid (Fig. 4) shows that during the first 2 weeks after birth they remain at about 0.4, rise to about 0.8 at 3 weeks of age, sharply increase to about 2.75 during the 4th week, and approach ratios of 4 in animals 6 weeks of age.

The percentage distribution of the radioactivity is described in Tables III and IV. The glutamic acid and glutamine accounted for 80 to 98% of the total counts per minute at all ages (Table III); thus radioactivity remains essentially in the glutamine and glutamic acid during the experimental time periods. The amount of radioactivity present in the glutamine at the steady state point rapidly increases from approximately 24% at 1 to 2

TABLE IV

Percentage distribution of total counts per minute in aspartic acid, γ-aminobutyric acid, and GSH

	1 min	3 min	5 min	10 min	15 min
	% total counts				
1 week					
Aspartic acid		0.67	1.0	1.4	2.7
γ-Aminobutyric acid			0.11	0.14	0.11
GSH		1.7	1.8	1.5	1.9
2 weeks					
Aspartic acid	0.65	1.1	1.4	2.8	
γ-Aminobutyric acid	0.08	0.04	0.14	0.11	
GSH	0.78	0.59	0.54	0.81	
3 weeks					
Aspartic acid	1.0	2.3	2.6	2.4	
γ-Aminobutyric acid					
GSH	0.73	0.66	0.96	1.1	
3½ weeks					
Aspartic acid		1.8	3.4	2.7	2.6
γ-Aminobutyric acid		0.19	0.36	0.24	0.51
GSH		0.58	0.77	0.86	0.69
4 weeks					
Aspartic acid	2.2	2.7	2.3	1.9	
γ-Aminobutyric acid	0.11	0.17	0.44	0.50	
GSH	1.2	1.3	1.5	1.5	
4 weeks					
Aspartic acid		1.7	2.1	2.3	2.1
γ-Aminobutyric acid		0.29	0.19	0.39	0.54
GSH		1.5	1.5	1.8	1.8
5 weeks					
Aspartic acid	2.4	2.4	2.3	2.2	
γ-Aminobutyric acid	0.20	0.33	0.42	0.75	
GSH	0.91	1.3	0.96	0.93	
6 weeks					
Aspartic acid	1.1	1.4	1.3	1.3	
γ-Aminobutyric acid	0.11	0.09	0.61	0.56	
GSH	1.6	1.6	1.9	2.6	
6 weeks					
Aspartic acid		1.8	1.7	1.6	1.6
γ-Aminobutyric acid		0.09	0.09	0.24	0.42
GSH		1.0	0.97	1.1	1.1
8 weeks					
Aspartic acid			1.1	1.2	
γ-Aminobutyric acid			0.39	0.33	
GSH			1.3	1.3	
14 weeks					
Aspartic acid	2.0	2.4	2.5	1.7	
γ-Aminobutyric acid	0.39	0.40	0.65	0.90	
GSH	0.78	0.96	0.72	0.74	
16 weeks					
Aspartic acid	1.2	1.3	1.4	1.4	
γ-Aminobutyric acid					
GSH	0.86	0.72	1.2	1.5	
Adult					
Aspartic acid	1.4	1.4	1.4	1.1	
γ-Aminobutyric acid	0.05	0.18	0.86	0.75	
GSH	0.46	0.53	0.40	0.46	

TABLE V

Specific activities of glutamic acid, γ-aminobutyric acid, and GSH 10 to 15 minutes after application of L-glutamic-^{14}C acid to cerebral cortex (kitten)

Age	Specific activity			Relative specific activity (glutamic acid = 1)	
	Glutamic acid	γ-Aminobutyric acid	GSH	γ-Aminobutyric acid	GSH
wks	*c.p.m./μmole*				
1	89,000	800	8,820	0.009	0.096
2	63,680	330	2,700	0.005	0.042
3	30,960		3,330		0.107
3½	19,450	470	1,790	0.024	0.092
4	6,770	510	1,200	0.075	0.177
4*	10,270	700	2,740	0.068	0.267
5	18,500	2,190	2,060	0.113	0.118
6	4,970	690	1,140	0.139	0.228
6*	18,620	1,670	3,620	0.090	0.194
8	11,280	970	3,160	0.086	0.280
14	18,980	3,370	1,930	0.178	0.102
16	310	50	75	0.165	0.239
Adult	26,600	5,560	2,200	0.209	0.083

* These are 15-minute points; all others are 10-minute points.

weeks of age to approximately 30% at 3 weeks of age, 50% in the 4- to 5-week animals, and reaches a maximum of about 60% at 6 weeks of age. The sum of the counts per minute in the aspartic acid, γ-aminobutyric acid, and GSH add up to approximately 3 to 4% of the total radioactivity in the trichloroacetic acid extract (Table IV). The percentile counts in the aspartic acid (Table IV), as well as its specific activities (Figs. 2 and 3), decreases with maturation. The rate at which the aspartic acid reaches a steady state, however, is increased (Fig. 2). The radioactivity in the γ-aminobutyric acid increases from about 0.1% of the total counts per minute in the younger animals to about 0.9% in the older animals. Its specific activity relative to that of glutamic acid shows a 10- to 20-fold increase, while the glutamic acid in the GSH shows a 2- to 3-fold increase with development (Table V).

DISCUSSION

Compartmentation of metabolic events has received increasing attention in recent years. In the area of amino acid metabolism heterogeneous functional pools have been postulated for microorganisms (12–14), plants (15, 16), and animal tissue (17, 18).

In several earlier studies on brain metabolism evidence was obtained suggesting that glutamic acid in brain is present in more than one metabolic pool or compartment. This conclusion was first arrived at by intracisternal administration of ^{14}C-glutamic and ^{14}C-aspartic acids *in vivo* (2) and received unequivocal support in systemic studies on ^{15}N-ammonia metabolism (3) and $^{14}CO_2$ fixation by the brain (4, 5). The similarity of the data reported here to that previously described (2–5) suggests that the topical approach does indeed give data on a physiological process present *in vivo*. There is no assurance, however, that the different approaches study the same metabolic compartments to the same extent.

In the interpretation of the results obtained by topical application it was of interest to determine whether the substrates are retained at the surface of the brain or can indeed penetrate into the tissue. If the applied glutamic acid penetrated very poorly or was perhaps rapidly removed by the circulatory system from all but the superficial cell layers, when the tissue is removed and washed the heavily labeled superficial glutamic acid could be carried away while the glutamine might be retained more strongly by the tissue because of binding or transport properties. Thus the results would be distorted.

It is also possible that the data may reflect a surface phenomenon (19). Glutamine may be synthesized more rapidly in the surface layers of the cortex than in the underlying layers and the closer proximity of the active glutamine-synthesizing cells to precursor of higher specific activity could result in the compartmentation observed.

The data presented in Table I show that the above mentioned possibilities do not occur. The applied glutamic and aspartic acids do penetrate deeply into the cortex and are metabolized there. Glutamine is formed at all levels in the cortex and its specific activity is always greater than that of the total tissue glutamic acid. The highest ratio of specific activities of glutamine to glutamic acid does not occur at the surface of the brain, where the specific activity of glutamic acid is greatest, but approximately 0.5 to 1.0 mm below the surface of the brain. This is in line with the contention that the metabolic events are intracellular for it is in this location that the greatest concentration of cortical cells occur. There can be little doubt that the conversion of aspartic acid to glutamic acid in the Krebs cycle and then further to glutamine is an intracellular process. It therefore appears that evidence for compartmentation of glutamic acid metabolism can be obtained at all depths of cortex and that it is greatest beneath the surface of the brain in an area most dense in neuronal cells.

It is clear from the data that similar evidence for compartmentation of glutamic acid metabolism, obtained from the mature rat, monkey, and adult cat, is not obtainable in kittens under 3 weeks of age but develops rather rapidly between the 3rd and 6th week of life. It is possible that in the younger animals most of or the total tissue glutamic acid is in rapid equilibrium and therefore there are no operational pools. Such pools do appear with maturation but at present we are not able to correlate these biochemically defined pools with anatomical changes.

With maturation there appears to be a contraction of the pool of glutamic acid available for glutamine formation and an expansion of the pool or pools of glutamic acid not immediately available for glutamine synthesis. Thus there is an approximately 10-fold increase in the ratios of specific activities of glutamine to glutamic acid occurring as a result of growth and maturation of the cerebral cortex (Fig. 4) while during the same period the glutamic acid levels approximately double and the glutamine levels remain essentially unchanged (Fig. 5). One is led to the consideration, therefore, that the size of the pool of glutamic acid available for glutamine formation may decrease by a factor of 5 during maturation. Also consistent with this concept is the larger percentage of radioactivity remaining in the glutamic acid in the younger animals (Table III) for in these kittens the applied ^{14}C-glutamic acid enters and is diluted by the larger pool of cold tissue glutamic acid available for the formation of glutamine.

The data in Fig. 5 are similar to the data previously reported for glutamic acid and glutamine in the developing neocortex of the kitten (6) with the exception that the 4-week-old animals in the present study have an average glutamic acid level somewhat higher than in the earlier report. In addition, the data show that

the glutamic acid level may continue to increase beyond 6 weeks and return to mature levels sometime after the 4th month.

The results presented in this paper parallel to some extent those obtained in previous experiments in which ^{14}C-glutamic acid or aspartic acid was administered into the cisterna magna of rats (2). In these earlier experiments the specific activity of the glutamine rapidly rose above that of the glutamic acid to ratios of about 5:1. As in the present experiments, during this period the sum of the radioactivities in glutamic acid and glutamine remained constant, there being merely a redistribution of the counts from the glutamic acid to the glutamine. The ratios of specific activities of γ-aminobutyric acid to glutamic acid were about the same as in the present results but the glutamic acid in the GSH contained relatively more of the radioactivity in the rat as compared to the kitten. The differences no doubt reflect species differences as well as experimental variations.

It is of interest to note the rather small amount of radioactivity in the γ-aminobutyric acid of kittens shortly after birth and the rapid increase following the 3rd postnatal week (Table V). It is not possible at present to calculate a valid turnover rate because the specific activity of the glutamic acid pool from which it arises is not known. However, the almost negligible amount of labeling present in this metabolite in the younger animals indicates that its metabolism may be of minor importance to these animals and become of greater value only later in development, unless another source of γ-aminobutyric acid other than glutamic acid or another pool of glutamic acid which mixes very slowly with the topically applied glutamate is utilized for its formation. The glutamic acid moiety of GSH also shows an increase in turnover rate with maturation, but it is less striking than that of γ-aminobutyric acid.

Although the development of glutamine synthetase activity in the maturing kitten remains to be determined, it has been reported (20) that in the pig and monkey this enzyme increases rapidly during the last phase of fetal development with high activity present at birth and continues to increase for several more weeks postnatally in the monkey. In the chick central nervous system and particularly the retina, the enzyme shows rapid increase during the latter part of the incubation period and continues to increase for some time after hatching (21). Work is now in progress to determine how the level of glutamine synthetase changes with development and to evaluate whether such changes contribute significantly to the development of the compartmentation picture drawn from the experiments described in this paper.

The site of glutamine formation is still not entirely clear. The synthetase enzyme has been reported to be present mainly in the microsomes (22). In a previous paper, it has been suggested that the administered glutamic acid is amidated in the endoplasmic reticulum (3). In another recent report (23) the membranous part of the endoplasmic reticulum is described as the most likely site for localization of the synthetase enzyme. However, we have no knowledge at all as to how much of the glutamine synthetase activity is contributed by the glial cells and how much by the neuronal cells.

In the kitten the morphophysiological development of the neuronal cells appears to be complete by the end of the 3rd postnatal week (24-28). Therefore, the development of the compartmentation of the glutamic acid-glutamine system is completed after the anatomical and physiological development of the neurons. Little is known about the early development of

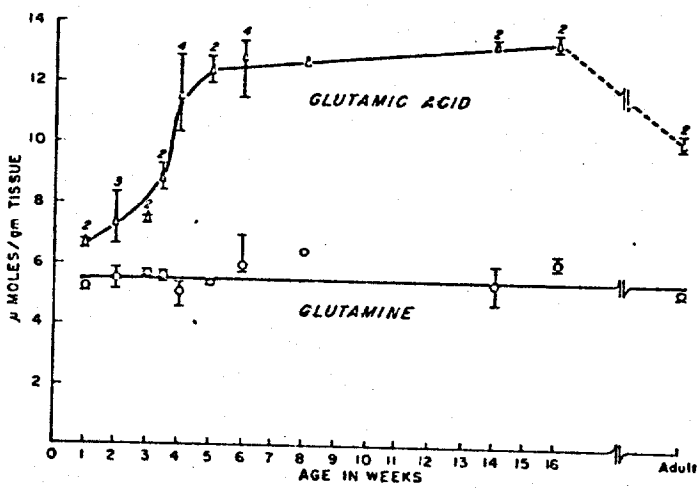

FIG. 5. Concentration of glutamic acid and glutamine (micromoles per g of tissue, wet weight) in cerebral cortex of kittens at different ages. The *bars* indicate the range of values, the *symbol* the average value, and the *figure* the number of animals for each point of the curves.

the glia in the cerebral cortex but it does continue to develop in the cat until about 6 months of age (29). Thus, one must give consideration to the possibility that glial metabolic activity does contribute to the total picture of glutamic acid compartmentation.

In general two categories of compartmentation can be envisioned, cellular and intracellular. Since one deals with a mixed population of cells, the compartments may simply consist of two or more cell types, one of which contains only a low level of glutamate but converts the amino acid rapidly to glutamine, resulting in a compound of high specific activity. The other cell type (or parts of cells such as dendrites or axons) could contain substantial quantities of glutamate and relatively less glutamine with little capacity to make it and would thus dilute the isotopic glutamic acid to a greater extent. Compartmentation of this kind would reflect changes in cell type or cellular extensions with maturation.

In the corpus callosum, white matter consisting essentially of myelinated axons and glial cells, both glutamic acid and glutamine levels are proportionately lower than in the cortex in the rat (10) and in the cat.[2] There is no evidence of disproportionate distribution of glutamic acid and glutamine within the elements of cerebral tissue. It is also unlikely that the applied amino acid could penetrate myelinated fibers and therefore compartmentation very probably reflects metabolic events associated with the cell body and possibly the dendrites.

The cerebral cortex consists in the main of two general types of cells, neural and glial. In the mature cortex the greatest evidence for compartmentation is attained from the layers of cortex most dense in neuronal cells. During ontogenesis compartmentation continues to increase during active proliferation of glial cells beyond the period of neuronal development. Thus, both types of cells appear to contribute, if to varying degrees; this suggests that cellular compartmentation can only in small part explain the total picture. It would seem, therefore, that intracellular compartmentation, the relative metabolic contribution of subcellular structures and their associated enzymes may be of greater importance.

[2] Unpublished observations.

The similar findings obtained with isotopic glutamic and aspartic acids (2) and in the studies of $^{14}CO_2$ fixation (4, 5) and $^{15}NH_3$ metabolism (3) also support intracellular compartmentation as the functional mechanism.

SUMMARY

The metabolism *in vivo* of ^{14}C-L-glutamic acid, topically applied to the cerebral cortex of kittens and adult cats, was studied.

The ratios of specific activities of glutamine to that of glutamic acid rose to values significantly greater than 1 after the 3rd postnatal week.

The data suggest that the compartmentation of glutamic acid metabolism in the cerebral cortex follows a developmental pattern.

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2491 NUTRITION

MONOSODIUM GLUTAMATE METABOLISM IN THE NEONATAL PRIMATE.

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Oral or intraperitoneal administration of large quantities of monosodium glutamate (MSG) to the infant mouse results in a massive hypothalamic lesion. The occurrence of such MSG-induced lesions in species other than the rodent is highly controversial. The original report of an extensive MSG-induced lesion in the neonatal monkey has not been confirmed by other groups. The failure to note the lesion has been ascribed to an inadequate elevation of plasma glutamate levels. We have studied the effect of oral MSG loads (1 to 4 gm/kg body wt.) upon plasma amino acid levels in the neonatal monkey, and have isolated and identified the major glutamate metabolites (glucose and lactate) present in the blood. Glutamate load results in a rapid and extreme elevation of plasma glutamate levels, with peak elevations roughly proportional to the load administered (range 200-400 μ moles/dl, normal 6-10). Plasma aspartate levels were also elevated, but all other amino acid levels were unchanged. Neonatal monkeys studied had higher fasting plasma glutamate levels than did adult animals. Two of the youngest animals studied exhibited a decreased ability to metabolize glutamate, resulting in abnormal tolerance curves. No animal studied, including those with abnormal tolerance, showed signs of the massive hypothalamic lesion. Supported in part by a grant-in-aid from the Gerber Products Co.

yawning elicited by startling varied from 1 to 20 s). In such a scheme, a yawn could have some regulatory function in response to a sudden mobilization of neural resources.

During and between bouts of CSD which induced yawning rats frequently exhibited penile erections. Typically they arched their backs, trembled, reared up on their hind legs, and exhibited a remarkable erection, often with waving of the penis. This was followed by licking and grooming of the genital area. The relationship in these experiments between yawning and sexual excitement is not clear, but it is interesting in the light of recent reports of yawning and sexual arousal induced concurrently by injection of adrenocorticotropin into the brain of rats, rabbits and cats[5-7], and by the release of erections and yawning in monkeys in response to their mirror images[8].

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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 aetiology 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.

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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Handedness not a Function of Birth Order

It has been suggested[1] on the basis of a survey of 648 university students (95 left handed = 14.7%) that handedness is a function of birth order and that left handedness may be caused by neurological insults associated with prenatal or birth trauma. I here report a similar survey of 974 university students (141 left handed = 14.5%).

Handedness was classified as in the original investigation[1] by asking each subject "Which hand do you use for writing?". As expected, the population of left handed students included a high proportion of twins ($P<0.01$) and a nonsignificantly smaller proportion of women than did the population of right handed students.

Table 1 Handedness and Birth Order and Parental Handedness

Birth order	R	L	Parental handedness	R	L
1, 4+	445	62	R, R	722	117
2, 3	388	79	RL, LR or LL	98	23
	$\chi^2=4.31$			$\chi^2=2.18$	
	$P=0.05$			$P=0.15$	

Table 1 shows the relationship between handedness and the two categories of birth order—high risk (first and fourth or later birth) and low risk (second or third births). For the whole group there are significantly more ($P=0.05$) left handed persons in the low risk category, the exact opposite of the previous findings[1]. If the results of these and previous investigations are combined, there are almost equal numbers in both risk categories for both right and left handed series. These results strongly suggest that the apparent effect of birth order[1] on handedness is simply a sampling error.

It is more usual to attribute handedness to inheritance[2] although opinion is divided about the nature and strength of the effect[3]. In this study, 960 of the students were able to answer the questions "What hand does your mother write with?" and "What hand does your father write with?". Sometimes it was reported that one or the other parent wrote with the right hand now but changed in grade school. Such parents were counted as "left". Table 1 shows the distribution of parental handedness of the right and left handed students. None of the 140 left handed students had two left handed parents although three right handed students did. Although the proportion of left handed students with one left handed parent (16.5%) was slightly larger than the proportion of right handed with one or two left handed parents (12%), the

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Monosodium Glutamate: Necrosis of Hypothalamic Neurons in Infant Rats and Mice Following either Oral or Subcutaneous Administration. R. M. Burde*, B. Schainker*, and J. Kayes*, *St. Louis, Mo.*

Conflicting reports have appeared recently concerning the effects of monodosium glutamate (MSG) on the developing hypothalamus. Olney described necrosis of hypothalamic neurons in infant mice or rats following administration of either oral or subcutaneous MSG (1–4 mg/gm). Adamo and Ratner detected no cytopathology in infant rats treated subcutaneously (4 mg/gm). Arees and Mayer observed hypothalamic lesions in MSG-treated mice (2–4 mg/gm) but interpreted the pathology as "microglial" necrosis without neuronal involvement.

In the present study infant rats and mice were treated orally or subcutaneously with MGS (1–4 mg/gm) or with equimolar doses of sodium chloride. Five hours following a single treatment each animal was sacrificed by perfusion fixation and brains were processed for combined light and electron microscopic examination.

Two observers, performing independent blind analysis of coded microscopic slides, separated sodium chloride controls (no cytopathology) from MSG-treated animals (conspicuous hypothalamic lesions) with 100% accuracy. Structures undergoing acute degeneration in the hypothalamus (arcuate nucleus) of MSG-treated animals were interpreted by electron microscopy as neuronal cell bodies and dendrites based on their post-synaptic involvement at axosomatic and axodendritic synapses. "Microglial" necrosis was not detected, but phagocytosis of degenerating neurons by cells, possibly of glial origin, was already apparent five hours after treatment. (NEY EY-00016 and EY-00015.)

CONFIDENTIAL

3490/70/302

THE EXAMINATION OF NATURAL MONO-SODIUM GLUTAMATE FOR THE PRESENCE OF CHEMICAL CARCINOGENS

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A sample of natural mono-sodium glutamate was received from Dr. T. Yoshida of the Ajinomoto Co. Inc., Tokyo, Japan for examination for the possible presence of chemical carcinogens.

All analyses were carried out as detailed in our previous report number 3451/70/263, "The examination of synthetic mono-sodium glutamate for the presence of chemical carcinogens". These analyses failed to indicate the presence of any compounds in the classes under investigation, above the previously stated detection limits, which were:

Polycyclic aromatic hydrocarbons	0.1μg = 0.001ppm
Phenolics	5.0μg = 0.0005ppm
Nitrosamines	0.5μg = 0.005ppm

E9

The Effect of Monosodium Glutamate on Food Flavor

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Author's abstract

Studies have been made to determine the role of monosodium glutamate in food flavoring. While some observers assert a meat-like taste for monosodium glutamate, we have found it to have only a sweet saline taste accompanied by some astringency. It appears to have a notable and usually favorable effect on food flavor by blending and rounding out the notes without contributing any noticeable odor or taste. This improvement in flavor appeal was most notable in the case of meats, seafoods, stews, soups and chowders. Many vegetable dishes developed added attraction by addition of glutamate. It was noted that glutamate suppressed undesirable flavor factors in some cases. Glutamate also aided in combating over-cooked flavor often noted in steam table food. Fruits, fruit juices, sweet baked goods, some dairy products and cooked cereals were not considered to be benefited.

Attempts to determine the relative effect of pH on glutamate effect in a single food were unsuccessful as changing pH produced flavor changes greater than those produced by glutamate. Glutamate appears to be effective in the pH range investigated, 3.5 to 7.2, the limits being represented by certain tomato products and hominy.

Monosodium 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 in fat-free foods than it has when fat is present.

The flavor of food is composed of many different 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 means of studying the effect of added materials without regard to individual preferences. A seasoning accentuates desirable notes and suppresses objectionable ones in food without itself being evident. For this reason, monosodium glutamate may be classified as a seasoning agent; its unique action being more of a salt than a condiment type. Because of its importance and the fact that it can effect the balance of other seasoning and flavoring, it should be added first.

Although monosodium glutamate has been available for many years, the literature on its fundamental role in food flavoring is extremely limited. No published work supplies a general theory to explain its action or to define its place in the field of salt, spices and seasoning. It has frequently been referred to as a material having a meat-like flavor but this viewpoint has also been contradicted.

Our work on monosodium glutamate was initiated over a year ago with the purpose of answering some of the questions which have arisen in connection with its use through the development of a background of information on its mode of action and general effect on food flavor.

Results of these studies will be published soon in *Food Research* and *Food Industries*, and the two papers being presented today are drawn in part from articles already submitted for publication. The following is a list of the principal topics studied:

1. General orientative study of monosodium glutamate in sixty different cooked foods.
2. Taste and flavor evaluation of highly purified MSG, commercial MSG, isolated impurities, D- and L-glutamic acids.
3. Taste evaluation of MSG at food levels in the range of 0.1-.0.3%.
4. Taste and flavor evaluation of common meats.
5. Effect of MSG on fundamental tastes (sweet, salty, sour and bitter).
6. Effect of MSG on aromatic components of flavor.
7. General relationship of glutamate effect and pH.
8. Influence of thickening agents, fats and related materials on glutamate activity.

General Observations

When tasted in Nuchar-treated water at food levels of 0.1-0.3%, commercial monosodium glutamate had a sweet saline taste accompanied by some astringency. It stimulated all surfaces of the tongue and oral cavity, producing a slight sensation of "furriness" on the tongue. A mild but lasting aftertaste resulted. Solutions were almost odorless and therefore at these concentrations MSG would be expected to contribute only sweet salty taste to food flavor.

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.)

Under the same conditions glutamate had an effect on food aroma, without contributing any noticeable odor itself. (0.1-0.3%)

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, seafoods, stews, soups, and chowders. These are applications with which you are most familiar.

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 suggested application of monosodium glutamate in protecting the flavor of foods held on steam tables.

For example, in canned corn, canned lima beans, and dehydrated and reconstituted vegetables maintained for more than an hour over a hot water bath, monosodium glutamate aided in combating steam-table flavor.

Fruits, fruit juices, sweet baked goods, some dairy products and cooked cereals were not considered to be benefited by use of MSG.

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 note in boiled rice.
5. Bitter tastes in a few freshly opened canned vegetables.
6. A fishy note sometimes present in lima beans.

The Effect of pH

Glutamate has been investigated in foods in the pH range 3.5 to 7.2. A taste titration curve indicated that glutamate taste was notable throughout this range, and was accentuated at the lower values of pH. The foods in which the effect was most desirable happened to lie in the range pH 5.5 to 6.5. Attempts to determine the influence of pH on glutamate effect in a single food were, however, unsuccessful. Changing the pH of chicken broth by as little as ± 0.5 units produced flavor changes greater than those produced by glutamate. These unfavorable changes greater than those produced by glutamate. These unfavorable changes were not corrected by adding glutamate. However, our general observation was that glutamate was effective in the pH range of 3.5 to 7.2, the limits being represented by certain tomato products and hominy.

We are intrigued by the apparent intensification of glutamate taste in the more acid solutions and feel that more work should be done on the relationship of glutamate taste to glutamate effect at various pH values.

Effect of Demulcents and Fats

Observations made on aqueous solutions and dispersions of flavoring agents do not correlate exactly with those found in food. Foods generally have higher viscosity and usually contain fats and oils. These factors markedly affect the balance of a flavoring system. Consequently we have begun a series of studies on the fundamental taste factors in thickened non-food media. Using one per cent solutions of carboxy methyl cellulose we have observed a pronounced demulcent action, by

which sweet, salty, sour and bitter tastes and the effect of low concentrations of glutamate were suppressed. At higher concentrations taste seemed to be more pronounced and longer lasting. At higher concentrations of glutamate, bitterness and sourness were suppressed. Well seasoned non-meat gravies were prepared with cereal thickening agents with no added fat, and glutamate was found to improve their flavor. Addition of fat suppressed all flavor, requiring development of a new seasoning and flavoring at a higher level. Glutamate effect in this system was less pronounced than in the case of the non-fat gravy.

Effect of MSG on Sweet, Salty, Sour and Bitter Tastes

In examining fundamental tastes we have found that glutamate accentuates sweetness in a food when sweetness is low or near optimal values. When sweetening was optimal, glutamate had no great influence. The same applied to saltiness. When farina or mashed potatoes, for example, were salted with 75% of the optimal amount of salt, 0.1-0.2% MSG could supply the remainder. Glutamate alone could not supply adequate salt value to these foods.

Tests with plain water indicated that glutamate appeared to be more salty than salt at 0.1-0.2% levels (subthreshold levels of salt) but at threshold levels glutamate added some saltiness to the salt solutions. Above threshold, the saltiness of salt solutions was not greatly increased by adding MSG.

Sourness accentuated glutamate taste. Glutamate seemed to suppress sour taste and odor in certain vegetables but not by affecting pH.

Glutamate appeared to suppress bitterness in saccharin solutions and in certain vegetables, but this action was not general.

The Philosophy of Seasoning

As a prerequisite to classifying monosodium glutamate among common seasoning and flavoring agents, we have attempted to find objective data on how salt, pepper and spices react upon food flavor. A review by Dunn (17) on the flavor effects of salt and original work by Fabian (21) and Blum supply some understanding as to the complex possibilities with salt, sugar and acids. Similar background on spices and condiments is lacking. Some speculation as to the general philosophy of seasoning is possible from our work with monosodium glutamate and it is presented here, tentatively, with the idea of suggesting approaches to the problem.

In attempting to make objective measurements on flavor effects one is handicapped by the usual limitations of subjective impressions, preferences and prejudices, especially in dealing with food. Our approach has been to conduct an open panel discussion to determine the principal notes or points of odor and flavor on the unseasoned food. A

composite of these points is called the odor or flavor profile. Subsequent observations may then be made on the effect of an added ingredient upon particular aspects of the profile, without reliance upon preference.

Spices and condiments were said to have been used originally to suppress objectionable unsavory flavors in spoiled meats. Why then are they necessary in modern cooking? Our observations indicate that unseasoned food generally has a sharp open profile with many notes individually evident. Such flavor lacks blending and balance. Cooking makes for notable improvement in character but still leaves rawness and sourness, particularly in vegetables. Salting is the first step in correction of rawness and other objectionable flavors may be controlled by sugar, acid, pepper and flavoring agents.

Seasoning is the art of making fit; it has much to do with palatability and appeal. Seasoning can be accomplished without the additives being noticeable. Bizarre or outstanding notes may then be added for special effects but this is a step beyond minimal seasoning requirements. Seasoning increases total volume of flavor and although it may suppress some outstanding notes of natural flavor it does not destroy identity. Some parts of the natural flavor profile may be accentuated and others suppressed; a good seasoning accentuates the desirable notes and suppresses objectionable ones.

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 effect 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.

References: (17) (21).

Discussion

Dr. Fellers: In regard to canned products, have you determined whether processing, such as you would use with process meat, corned beef hash, clam chowder, etc., affects the monosodium glutamate flavor? Does it change the blend of flavor or destroy the fullness?

Dr. Cairncross: In our work we have not dealt with the food technology aspects of canning procedures, etc. Our work has been done on fresh vegetables and freshly prepared dishes.

Mr. Galvin: With reference to canned soups, the glutamate taste is not very much affected by ordinary heat processes. You will find, I think, that other flavors of foods—flavors of carrots, peas and corn—will change a greater amount than will the glutamate.

Mr. Stateler: In vegetable soups where potatoes are an ingredient—too

often in these soups when canned, there is a sort of staleness in the flavor of the potatoes. If glutamate is present or is added to the soup, will that staleness be dispersed?

Dr. Cairncross: We have observed that glutamate in freshly opened canned goods frequently suppresses an undesirable note that was apparent on canning. In the case of canned bean sprouts and in the case of a number of other vegetables, there is often a correction of an overcooked or undesirable flavor.

Mr. Sjostrom: We did some work on canned potatoes and noticed a decided cut-down on earthiness. Earthiness is associated with the flavor of the peel which develops in canned potatoes. It is also tied in with overcooked flavor. Glutamate when added to these potatoes did improve palatability.

Dr. Cairncross: We also had limited experience with dehydrated potatoes.

Mr. Sjostrom: Several years ago when we were getting into some of the dehydrated work, we attempted to use dried potatoes in dehydrated soups. One fault at that time was that potatoes were not stable over long enough periods. The glutamate, however, did add something to the freshly dehydrated potatoes to make a better flavor.

Mr. Bauer: Our type of investigation has not been along this line.

Dr. Melnick: One of the major problems facing the Institute is canned meat flavor. I wonder if glutamate is capable of masking that taste.

Dr. Cairncross: We have done very little work in that connection. We have taken it for granted that glutamate fits in with meat preparations in general, and we agree that it supports the flavor of any meat dish or animal protein preparation, but we have used vegetables primarily because we get a greater range of complex flavors.

Dr. Buchanan: In your paper you discussed very briefly glutamate taste versus glutamate effect; I wonder if you have done enough with the advantages of glutamate in prepared foods to comment further, and particularly, on what you meant by glutamate taste versus glutamate effect as far as concentrations of glutamate in food and preparation go. Can you tell us what you mean by glutamate effect?

Dr. Cairncross: We think of food flavor as a sort of pin cushion model, an open profile with many characteristic points easily discernible, such as sour, sweet, bitter and astringent notes. We know the spectrum of glutamate very well. If we superimpose that upon a food flavor we can predict what it will add to the spectrum of the whole flavor. When we add glutamate to food we do not observe a simple additive flavor effect but we have observed major changes in the flavor profile and in

the overall volume of flavor. In Farina for example, we observed the blending of natural flavor and suppression of sourness and rawness. By glutamate effect we mean that overall effect on flavor which occurs and which is not predictable from addition of the known spectrum of the glutamate taste. We believe that that effect includes the sensations produced in the mouth—possibly salivary stimulation and other factors which we have not as yet analyzed. We mentioned that the aroma of the food is generally affected by glutamate, and often with the highly purified glutamate added to the cooked food there is a marked difference in odor appeal.

QUESTION FROM THE FLOOR: Would you say a few words about the comparison of the D and L forms?

Dr. Cairncross: We have done very little in comparing the two forms of glutamate except as Dr. Crocker mentioned the glutamate taste is characteristic only of the naturally occurring form and the glutamate effect is characteristic of the same form. We have not done any extensive food tests with the two isomers except to confirm the fact that the taste was predominantly common to the L-plus glutamic acid.

Dr. Anson: The unnatural form is not devoid of taste, and the taste is not completely different from that of the natural form. It is considerably lower in taste, but not enough to be negligible.

QUESTION FROM THE FLOOR: Is there much difference whether glutamate is added during cooking or just before serving?

Dr. Cairncross: Experiments with processing would give us a better idea on that. We often prefer to cook glutamate with the food in order to get it well blended with the food. I would say it would be more desirable to cook it into the food. I cannot predict whether there would be any great economy or practical gain thereby.

Dr. Shannon: Regarding the canned taste that was mentioned with canned foods, maybe some of you have tasted Oscar Mayer's product, wieners with barbecue sauce in a separate sack. The reason behind that is to mask the canned flavor, and we have masked that fairly well.

QUESTION FROM THE FLOOR: I wonder if you have noticed anything in green vegetables. Have you tried preservation of color?

Dr. Cairncross: There may be small difference in preservation of natural color, but in our experience the effect is not to be compared with that obtained with bicarbonate or some other device.

What Glutamate Does in Food

By S. E. CAIRNCROSS and L. B. SJOSTROM
Arthur D. Little, Inc., Cambridge, Mass.

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What Glutamate Does in Food

M. S. G., utilized in pure form, has its own value as a flavor adjunct, distinct from the place occupied by protein hydrolysate

By S. E. CAIRNCROSS and L. B. SJOSTROM
Arthur D. Little, Inc., Cambridge, Mass.

WHEN used in food, monosodium glutamate augments and improves most flavors even when added so sparingly that its own taste is not noticeable. It blends and rounds out flavoring, often suppressing undesirable notes.

Like many of the things added to foods to improve flavor and palatability, glutamate came originally from the Orient. While its use in Chinese food and in canned and dehydrated soups is fairly well known, it has a much broader application in the preparation of cooked foods generally, but its possibilities are not so well known.

Because of opinions expressed in the early literature, the idea exists that monosodium glutamate has a meat-like flavor capable of producing meat flavor in non-meat dishes. It is true that vegetable protein hydrolysates which contain glutamate do have a peptone, broth-like flavor which lends itself to the preparation of meaty-tasting but meatless gravies and dishes. In the early commercial forms, monosodium glutamate was a crude, colored product of high salt content and possessing a considerable amount of protein hydrolysate flavor.

Because of lack of experience with the pure chemical, its true effect was not dissociated from that of the accompanying hydrolysate flavor. The taste and flavor characteristics of the pure substance were then known only to the few who had examined the freshly purified chemical.

Chemically Pure

With the increasing commercial use of glutamate in America and the application of American technology to its production, the product now is becoming standardized as a material of high chemical purity. It is available on the market as a pure white crystalline powder, free from discoloration, and practically free from odor and hydrolysate flavor.

In surveying the field of application of glutamate in its newly-available form in foods, the question naturally arises as to the relative importance of the pure chemical and of the highly flavored hydrolysate components which formerly accompanied it.

Not much information was available on pure glutamate. Accordingly, the role of pure glutamate in both meat and non-meat dishes was examined with the purpose of determining its action in altering and improving food flavor. Also, the relationship of glutamate to meat flavor was investigated.

This work showed that monosodium glutamate in its present highly purified form is low in odor and contributes primarily a sweet, salty taste to food which is of lower intensity than many of the flavors commonly present in the finished food.

Effect Compared

By contrast, the hydrolysate products, derived from protein containing up to 20 percent of glutamic acid, exert their effect in part by the activity of the glutamate but in larger part by that of strongly-flavored chemical derivatives of the amino acids.

The natural amino acids, other than glutamic, have in general very little flavor, but when heated together or prepared in crude condition, the mixtures develop characteristic odors and tastes, which are best described as "hydrolysate" flavor. The hydrolysate type of product is useful in supplying its own characteristic peptone-like flavor to foods and sauces. Glutamate, on the other hand, modifies existing flavor without adding anything new. In modifying sauces with either glutamate or hydrolysate it is important to judge the flavor effect in two ways. One is the effect on the sauce, the other is the effect when the sauce is added to food. Glutamate appears to be quite compatible with a much wider class of foods than are the hydrolysates and has been found to improve the palatability and appeal of most cooked dishes.

What It Does

The following are some general observations on the effect of glutamate in food:

Monosodium glutamate added in small amounts has a pronounced effect on the flavor of practically all foods to which it is added, without itself being noticeable. (Exceptions: It is very noticeable in certain fruits and dairy products.)

Under the same conditions glutamate has an effect on food aroma, without contributing any noticeable odor itself. (0.1-0.3 percent.)

The principal effect on food flavor is a balancing, blending and rounding out of total flavor.

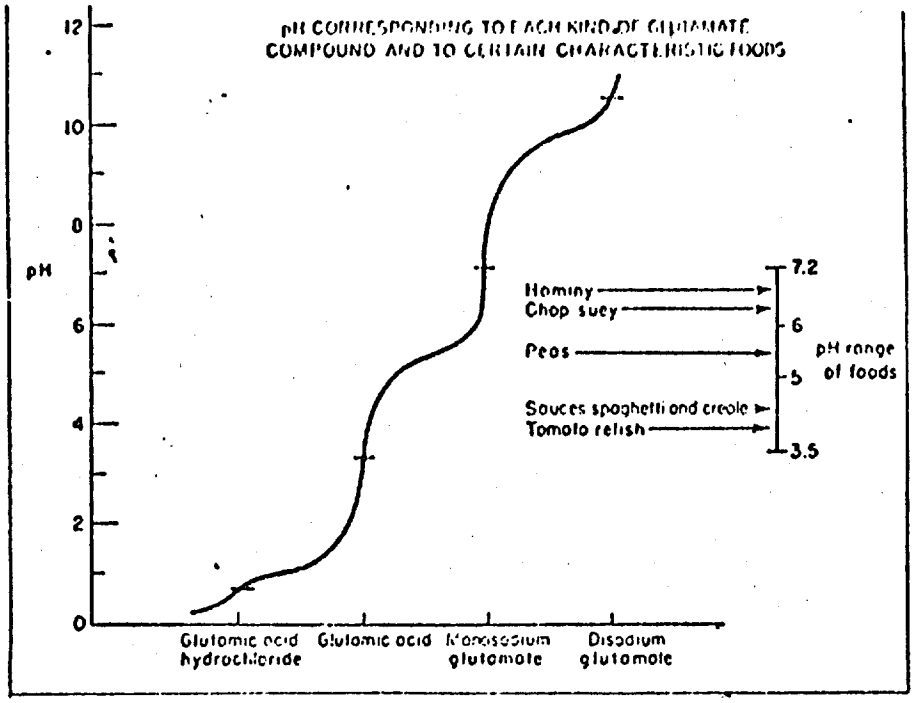
Flavor appeal is frequently improved, most notably in the case of meats, seafoods, stews, soups and chowders.

Cooked vegetables are markedly improved in flavor by glutamate addition but the following are 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 such cases as carrots and cauliflower the natural flavor characteristics are intensified. This suggests application for reinforcing the flavor of foods held on steam tables. For canned corn, canned lima beans, and dehydrated and reconstituted vegetables maintained for more than an hour over a hot water bath, monosodium glutamate aids in combatting steam-table flavor.

Fruits, fruit juices, sweet baked goods, some dairy products and cooked cereals, in a limited series of experiments, were not found to be benefited by use of MSG.

It has been found that glutamate suppresses 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.



MONOSODIUM GLUTAMATE covers pH range of many foods. Effect is accentuated at lower pH values, is most desirable in foods in the pH range of 5.5 to 6.5.

4. A volatile characteristic note in boiled rice.
5. Bitter tastes in a few freshly opened canned vegetables.
6. A fishy note sometimes present in lima beans.

General Physiological Effect

When a small quantity of monosodium glutamate is placed on the tongue, an immediate and long lasting effect is noted. The taste is pleasant, mild, slightly sweet and saline, imparting a general sense of satisfaction. Salivary secretion is increased, and this effect is noted for approximately half an hour. The dry salt produces a slight sensation of furriness on the tip of the tongue, and mild stimulation in the throat and the back part of the mouth. Although the above effects produced by the dry salt are not prominent when properly added to foods, they do supply some clue to the mode of action of glutamate in modifying food flavors. Aqueous solutions gave similar taste effects but were practically odorless. It has been postulated that increased salivary secretion favors release of flavor from food and provides a more intimate contact between the food and the taste buds. The long lasting stimulation of the mouth by glutamate probably serves to out-last and counteract some of the unfavorable after-tastes. The feeling of mouth satisfaction experienced with glutamate may be a factor in augmenting food flavor appreciation.

Glutamate has been investigated in foods in the pH range 3.5 to 7.0. A taste titration curve indicated that glutamate taste was notable throughout this range, and is accentuated at the lower values of pH. The foods in which the effect was most desirable happened to lie in the range pH 5.5 to 6.5. Attempts to determine the relative effect of pH on glutamate effect in a single food were, however, unsuccessful. Changing pH of chicken broth by as little as ± 0.5 units produced flavor changes greater than those produced by glutamate.

The flavor changes were not corrected by adding glutamate. Our general observation was that glutamate was effective in the pH range of 4.0-7.0, the limits being represented by certain tomato products and hominy.

Effect of Demulcents and Fats

Observations made on aqueous solutions and dispersions of flavoring agents do not correlate exactly with those found in food. Foods generally have higher viscosity and usually contain fats and oils. These factors markedly affect the balance of a flavoring system. Consequently, we have begun a series of studies on the fundamental taste factors in thickened non-food media. Using one percent solutions of carboxy methyl cellulose we have observed a pronounced demulcent effect; sweet, salty, sour and bitter tastes were suppressed and the effect of low concentrations of glutamate was suppressed. At higher concentrations taste seemed to be more pronounced and longer lasting. At bitterness and sourness were suppressed. Well seasoned non-meat gravies were prepared with cereal thickening agents and no fat, and glutamate was found to improve their flavor. Addition of shortening suppressed all flavor, requiring development of a new seasoning and flavoring at a higher level. Glutamate effect on this system was less pronounced than on non-fat gravy.

Effect of MSG on Other Tastes

In examining fundamental tastes we have found that glutamate accentuates sweetness in a food when sweetness is near its optimal value. At higher values it has no great effect. The same rule applies to saltiness. When farina and mashed potatoes were salted with 75 percent of the optimal amount of salt, 0.1-0.2 percent MSG could supply the remainder. Glutamate alone could not adequately supply salt value to these foods.

Tests with aqueous solutions indicated that glutamate appears to be more salty than salt at the 0.1-0.2 percent level (subthreshold level of salt) and at threshold levels glutamate adds some saltiness to salt solutions. Above threshold, saltiness of salt solutions is not greatly increased by adding MSG.

Sourness accentuates glutamate taste. Glutamate seems to suppress sour taste and odor in certain vegetables but not by affecting pH. The relationship of pH and glutamate effect needs further investigation.

Glutamate appears to suppress bitterness in saccharin solutions and in certain vegetables, but the effect is not general.

The Philosophy of Seasoning

Full understanding of the effect of glutamate and related materials requires development of a general philosophy of seasoning. The best flavor in food is one which appeals simultaneously to all of our flavor-detecting senses, i.e., odor, taste and feeling. An aroma is needed to make known the presence of the food, taste to entertain the tongue, and finally, a pleasant combination of texture and tang to make us realize that the food is at its best in all ways. If any of these sensory elements is missing, the food is flat and uninteresting. It is the function of additives to supply the missing element.

Seasonings supply taste but no odor. They are of value whenever taste is deficient and aroma is adequate. A case in point is roast beef,

which without salt is seriously flat. Nearly all meat products are low in true taste, and so are most vegetable and cereal products, and these benefit greatly from added seasoning. Milk, however, has already so much taste that the addition of salt or other seasoning upsets the balance and the effect is often bad.

At the moment of prime condition, when rushed from garden to kitchen to table, peas or sweet corn have excitingly fine flavor and require the minimum of any addition: little more than salt and butter. "Garden-fresh" is the standard for most vegetables, and is the aim of all cooks in preparing the articles available to them for use. Fortunately, the average quality of fresh, frozen or canned items is better and better, but still it is so far short of the garden-fresh ideal that help is much appreciated from seasonings, condiments and flavorings.

A typical seasoning of great desirability is salt. Sugar is another, of especial value in beverages, in fruits and importantly, in vegetables that have lost some of the garden-fresh sweetness. Monosodium glutamate is another seasoning which has no odor but which builds up taste. It is "sweet-salty" in general effect. It, like sugar or salt, is more or less specific or selective in the foods which it improves, but in general it is nearer to salt than to sugar in character. It builds up a very desirable flavor in cooked meat and vegetable products. Most interestingly, it also provides feeling stimulus, and is unique among seasonings in this respect. Its over-all influence is that of a seasoning for the moment, which gradually stimulates mouth-feeling nerves to create a lasting feeling of satisfaction.

Where aroma is deficient, odor-stimulating herbs and spices, called condiments, often can supply the lack. Many of these produce immediate bite or tingle as well, such as pepper, cloves, mustard and other spices, but some, such as marjoram or sage, are used mostly for aroma.

When food is very flat, such as cooked, dehydrated vegetables, steam table dishes, or some fish, nearly every type of seasoning must be added. The first and most important step is the building of a pleasant taste background by addition of salt, glutamate, sugar and simple seasoning. If this step is properly accomplished, the initial flavor will be well blended and well rounded out. The second step consists of the addition of aromatic spices and condiments and herbs in low intensity to build complexity and character.

Finally, bizarre and highly specific flavor effects may be produced by the addition of higher concentrations of aromatic spices and condiments, including such food components as onions, tomatoes and cheese. Thus, food seasoning and flavoring may be accomplished in three distinct steps and in all of these monosodium glutamate mediates to assist in amplifying and blending the total flavor.

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Crystals of Pure Monosodium Glutamate

α-アミノ酸の味と構造との關係に就て (第二報)[1]

アミノ酸の立體化學的研究 (II)

(昭和十四年四月廿四日受領)

金子武夫

一般に R-CH(NH$_2$)COOH なる α-アミノ酸に於て *l* 系 (天然系) に屬するものは常に無味乃至は苦味を呈するに反し, *d* 系 (非天然系) に屬するものは甘味を呈するものである.

從來チロジン及びフェニルアミノ酢酸は *d* 系, *l* 系の間に味の差がなく且つ無味なりとせられてゐたが前報に於てチロジンが何等例外でないことを明かにした. 今回はフェニルアミノ酢酸に於ても亦この現象に例外のないことを明かにした.

然るにアラニン及び α-アミノ酪酸は *d* 系, *l* 系共に甘く例外である.

そこで R$_1$(R$_2$)C(NH$_2$)COOH なる α-アミノ酸に於て

R$_1$ と R$_2$ が同一の置換基である場合: R$_1$, R$_2$ が CH$_3$, C$_2$H$_5$ 及び C$_3$H$_7$ (新アミノ酸)

R$_1$ と R$_2$ が異つた置換基である場合: (R$_1$ が CH$_3$, R$_2$ が C$_2$H$_5$), (R$_1$ が CH$_3$, R$_2$ が C$_3$H$_7$), (R$_1$ が C$_2$H$_7$, R$_2$ が C$_6$H$_5$CH$_2$) (新アミノ酸)

なる各種のアミノ酸を合成して, R$_1$R$_2$ の大きさとその味との關係を檢べた結果, α-アミノ酸が甘味を發現する爲には次の條件の必要なことを明かにした.

R$_1$(R$_2$)C(NH$_2$)COOH なるアミノ酸に於て R$_1$ = H の場合が天然系 (*l* 系) アミノ酸とすれば, 甘味を呈する爲には R$_1$ は任意の大いさで差支ないが R$_2$ は C$_3$H$_7$ よりかなる原子團であることを必要とする.

上記味よりの結果

(−)-Phenylaminoessigsäure 及び (−)-Isovalin は *d* 系

(+)-Phenylaminoessigsäure 及び (+)-Isovalin は *l* 系

に屬することを知つた.

前報に於ては一般に R-CH(NH$_2$)-COOH なる α-アミノ酸の光學的異性體の味を比較するに, 天然系卽ち *l* 系に屬するものが苦味乃至は無味又は不快味を有するに反し非天然系卽ち *d* 系のアミノ酸が例外なく甘味を有してゐる事實を表示し, 從來チロジンは *d* 系, *l* 系共に無味なりとせられてゐたが, それは誤りでこの場合も非天然系卽ち *d* 系に屬するチロジン並びに其誘導體は總て甘味を有し, 之に反し天然系卽ち *l* 系に屬するものは總て苦味或は不快味を有すること全く脂肪屬アミノ酸に於ける關係と同一であつて例外ではないことを明かにした.

其後文獻に徵するに E. Fischer, O. Weichhold[2] 兩氏は Phenylaminoessigsäure の場合には *d* 系に屬するものも, *l* 系のものも殆ど無味であつて他のアミノ酸の光學的異性體に見られる樣な味の差が無いことを報告してゐる. もしこれが事實であるとすれば唯一の例外となるわけであるが, 恐らくこれはその溶解度の小たるために起きた誤りであらうと思はれるので Fischer 等の實驗を追試した. 卽ち, *dl*-Phenylaminoessigsäure を Formyl 誘導體として後 Cinchonin 及び Chinin を用ひて光學

1) 第一報: (アミノ酸の立體化學的研究 I) 本誌, 59(1938), 433. 2) *Ber.*, 41(1908), 1293; *Ber.*, 35 (1902), 2663.

的分割を行ひその味を檢したるに，左旋光性(−)-Phenylaminoessigsäure の濃水溶液は明かに微甘味あるに反し右旋光性(＋)-Phenylaminoessigsäure は無味にして後に弱き不快味を殘すことを知り，この場合も脂肪族アミノ酸に於ける關係と同一であつて何等例外ではないことが明かになつた．

α-アミノ酸の光學的異性體のうち甘味を有するものは非天然系卽ち *d* 系に屬することから考へて，甘味ある左旋光性(−)-Phenylaminoessigsäure は *d* 系卽ち *d*(−)-Phenylaminoessigsäure であり，右旋光性のものは *l* 系卽ち *l*(＋)-Phenylaminoessigsäure であると思はれる．E. Fischer[3] が右旋光性(＋)-Phenylaminoessigsäure に亞硝酸を作用せしめて *d* 系に屬する左旋光性マンデル酸を得たる結果から見ると右旋光性(＋)-Phenylaminoessigsäure は *d* 系卽ち *d*(＋)-Phenylaminoessigsäure であると考へられるが，この場合には Walden 轉移の起ることが考へられる[4] から右旋光性(＋)-Phenylaminoessigsäure は *l* 系卽ち *l*(＋)-Phenylaminoessigsäure であつて上記味よりの結果と一致する．又 F. Ehrlich[5] がラセミ體を酵母により選擇的に醱酵せしめて(−)-Phenylaminoessigsäure を得てゐる結果と矛盾しない．

偖て前報に於て既に述べた如く天然系卽ち *l* 系のアミノ酸は一般には甘味を有しない筈であるが，これには例外があつて α-Alanin, α-Aminobuttersäure[6] の如く R が比較的小さい場合は天然系と雖も甘味(非天然系のものよりは當然弱いが)を呈するに至るのである．卽ち $R_1(R_2)C(NH_2)COOH$ なる α-アミノ酸に於て R_1, R_2 の大きさと甘味との間には或る關係のあることが考へられる．

そこで先づ簡單なる場合として光學的異性體の存在しないもの卽ち R_1, R_2 が同一である三，四の α-アミノ酸を合成してその味を檢べた．

(1)　R_1, R_2 が共に水素の場合：　これは Glykokoll であつて無論甘味を有する．

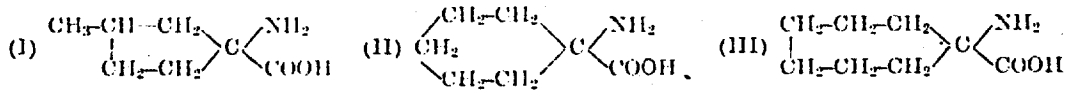
(2)　R_1, R_2 が共に CH_3 の場合：　これは α-Aminoisobuttersäure[7] であつて常法によりアセトンより合成したるに *dl*-Alanin と Glykokoll との中間位の甘味[8] を有してゐる．

(3)　R_1, R_2 が共に C_2H_5 の場合：　これは α-Aminodiäthylessigsäure[9] であつて Diäthylketon より常法によつて合成したるに(2)の場合よりは弱いが甘味[9] を有してゐる．

(4)　R_1, R_2 が共に C_3H_7 の場合：　これは α-Aminodipropylessigsäure であつて未だ文獻に記載がない．常法により Dipropylketon のアルコール溶液に青酸加里と鹽化アンモンの濃溶液を作用せしめて合成した．水より光輝ある柱狀晶．毛細管中に速かに熱するも熔融することなく昇華する．甘味は全然なく，却つて弱い苦味を呈する．

卽ち R_1, R_2 が C_3H_7 以上になると甘味は遂に無くなるに至る．この結果から見ると R_1 と R_2 が同一でなく互に異つてゐる場合，卽ち光學的異性體の存在する場合でも R_1, R_2 が共に大きくなれば *l* 系は勿論のこと，*d* 系のものに於ても甘味は消失するに至ることが考へられる．

N. Zelinsky, G. Stadnikow[10] 等は Methyl-(1)-amino-(3)-cyclopentan-carbonsäure-(3) (I), Amino-(1)-cyclohexan-carbonsäure-(1) (II) 及び Amino-(1)-cycloheptan-carbonsäure-(1) (III).



3) *Ber.*, 41(1908), 1294.　4) K. Freudenberg: "Stereochemie," 888, Leipzig und Wien (1933).
5) *Biochem. Z.*, 8(1908), 445.　6) Klein: "Handbuch der Pflanzenanalyse," IV Bd. 1ste Hälfte spezielle Analyse III 41.　7) *N. Zelinsky, G. Stadnikow: Ber.*, 39(1906), 1726; W. Gulewitsch; *Ber.*, 33 (1900), 1900.　8) F. Urech: *Ann.*, 164(1872), 271; F. Tiemann, L. Friedländer: *Ber.*, 14(1881), 1972.
9) K. W. Rosenmund: *Ber.*, 42(1910), 4473; W. Gulewitsch, T. Wasmus: *Ber.*, 39(1906), 1191.　10) *Ber.*, 39(1906), 1727, 1728, 1730.

が甘味あることを記載してゐる．同氏の記載に從ひ Cyclohexanon より (II) を作りたるに多少甘味を有しては居るが非常に弱く後に苦味を殘し寧ろ苦甘味である．(I) と (II) が甘味あることは R_1, R_2 が C_3H_7 より小である場合に相當するから當然と考へられるが，(III) が甘味を呈することは α-Aminodipropylessigsäure が苦味を有してゐることから見ると矛盾してゐるが白人は味に對する感覺が鈍い樣であるからこれは少しく疑問である．(尤もこの場合は R_1 と R_2 とが環を形成してゐるから一般の場合と同一に取扱ふことは不當であるかも知れない．)

先に R_1 と R_2 が異つてゐてしかも大きい場合には *d* 系，*l* 系共に苦味を呈するに至るであらう事を述べたがその一例として *dl*-α-Amino-*n*-propyl-benzylessigsäure を合成してその光學的分割を試みた．

先づ Benzylmagnesiumchlorid と *n*-Butyronitril とより *n*-Propylbenzylketon[11] を作つた．沸點 109～110°/3 mm, Semicarbazon の融點 85～87° で餘り明確ではなく *cis*, *trans* の混合物であらうと思はれる．この Semicarbazon をトルオールを用ひて分別結晶を行ひ，トルオール易溶部より融點 84～85°，難溶部より融點 123～125° のものを得た．兩者を等量混じたものの融點は 87～89° である．常法に從ひここに得たケトンと青酸加里及び鹽化アンモンの濃水溶液との混液にアルコールを加へて均一となし，加壓瓶中，100° に 10 時間反應せしめた後鹽酸で加水分解して目的の *dl*-α-Amino-*n*-propylbenzylessigsäure を得た．このものは既に苦味を呈する．ラセミ體が苦味を有してゐることから考へると *d* 系に屬するものも當然無味乃至は苦味あることが想像されるが，これを Formyl 誘導體となし，Brucin を用いて光學的に分割したるに豫想通り *d* 系，*l* 系共に苦味を呈することを知つた．兩異性體の苦味の差が明かにあれば苦味の少ない方は *d* 系に屬することを云ひ得るが，この場合苦味の差が非常に少ないから右旋光性及び左旋光性の兩異性體が夫々 *d* 系，*l* 系の何れに屬するかは不明である．

$R_1 = R_2$ の簡單なる場合にはそのアミノ酸が甘味を失ふ限界が C_3H_7 基である事が實驗的に判つたが，それならば R_1 と R_2 が同じアルキル基でなく，しかも C_3H_7 基より小さい場合には先に述べた Alanin 等の場合の樣に天然系卽ち *l* 系のものと雖も甘味を呈する．卽ち甘味の差はあるけれども *d* 系 *l* 系共に甘い筈である．その一例として α-Aminomethyläthylessigsäure (Isovalin) に就て實驗した．E. Fischer, R. Grüvenitz[12] は既にこのアミノ酸を光學的に分割して (+)-α-Aminomethyläthylessigsäure のみを得て居るが，味に就ては記載がない．著者は同氏の方法に從つて Methyläthylketon より *dl*-α-Aminomethyläthylessigsäure (Glykokoll より甘い[13]) を作りこれを光學的に分割したるに，右旋光性及び左旋光性イソヴァリン共に甘味を有してゐて，左旋光性イソヴァリンの方が右旋光性イソヴァリンよりは甘味の強い事を知つた．卽ち左旋光性イソヴァリンは非天然系卽ち *d* 系に屬し從つて右旋光性イソヴァリンは天然系卽ち *l* 系に屬するものであらう．且て F. Ehrlich[14] は *dl*-Isovalin を酵母によつて選擇的に天然系アミノ酸のみを分解せしめ，分解されずに殘つた方から左旋光性イソヴァリンを得てゐる．卽ち(−)-Isovalin は非天然系卽ち *d* 系に屬することになり上記の結果と一致する．

更に Methylpropylketon より常法によつて *dl*-α-Aminomethylpropylessigsäure[15] を作つた．無論甘味はあるが，この場合 *l* 系のものは極めて弱い苦味あるか乃至は無味に近いことが豫想される．卽ちこのアミノ酸が *l* 系に於て甘味を消失する限界であらう．α-Aminodipropylessigsäure が苦味を呈することから考へると *d* 系アミノ酸が甘味を消失する限界は α-Aminoäthylpropylessigsäure であらう．

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11) Blaise: *Compt. rend.*, 133(1902) 1218. 沸點 238～241°, Semicarbazon 融點 84°.　12) *Ann.*, 406 (1914), 1.　13) M. D. Slimmer: *Ber.*, 35(1902), 406.　14) *Biochem. Z.*, 8(1908), 459. $[\alpha]_D^{18} = -9.10°$ (H_2O).　15) 黑野: 本誌, 45(1924), 239.

以上の結果を表示すれば次の如くになる.

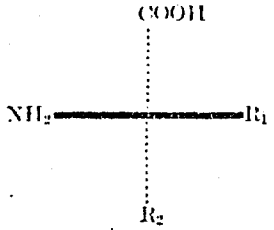
第　1　表

アミノ酸		d 系 味	d 系 旋光性	l 系 味	l 系 旋光性	dl-及び不活性化合物 味
Glykokoll	$CH_2(NH_2)COOH$					甘
Alanin	$CH_3CH(NH_2)COOH$	強甘	(−)	弱甘	(+)	甘 (d-, l-の中間)
α-Aminoisobuttersäure	$(CH_3)_2C(NH_2)COOH$					甘
Isovalin	$CH_3(CH_3CH_2)C(NH_2)COOH$	強甘	(−)	弱甘	(+)	甘
α-Aminodiäthylessigsäure	$(CH_3CH_2)_2C(NH_2)COOH$					弱甘
α-Aminomethylpropylessigsäure	$CH_3(CH_3CH_2CH_2)C(NH_2)COOH$	甘*		無味*		甘
α-Aminodipropylessigsäure	$(CH_3CH_2CH_2)_2C(NH_2)COOH$					弱苦
α-Aminopropylbenzylessigsäure	$(CH_3CH_2CH_2)(C_6H_5CH_2)C(NH_2)COOH$	弱苦		弱苦		弱苦
Phenylaminoessigsäure	$C_6H_5CH(NH_2)COOH$	微甘	(−)	無味 (不快味)	(+)	無味

* 印を附せるものは dl-アミノ酸の味より推想せられるもの.

以上の結果から一般に α-アミノ酸が甘味を呈するためには次の條件が必要であることが明かになつた. W. Kuhn[16] によれば α-アミノ酸の Absolute Konfiguration は略次の様に決定された. 即ち下圖に於て R₁ が H で置換されたものが天然系 α-アミノ酸である.

16) *Naturwissenschaften*, 26 (1938), 345.



水平に太い線で連つた置換基は紙面の前方に，縦の點線で連つた置換基は紙面の後方にある．

上圖の立體構造を有するアミノ酸が甘味を呈する爲に必要な條件としては，アミノ酸が水に可溶性なる限り R_1 は如何なるものでも差支へないが R_2 は H, CH_3, 或は C_2H_5 でなければならぬ．R_2 が C_3H_7 以上の大きな原子團では甘味を呈しない．

斯の如く α-アミノ酸の味は外に表はれる旋光性の正なると負なるとに關せずその立體的配位及び R_1, R_2 の大きさに支配せられるものであつて，R_1, R_2 の大きさを考慮に入れる時は未知 α-アミノ酸の味を檢することによつてその α-アミノ酸の不齊炭素原子の相對的配位を相當確實に豫知することが出來る．

オキシプロリン及びプロリンに就てはその立體構造稍不確實なる故論外としたがこれに就ては目下研究中である．

實驗の部

α-Aminoisobuttersäure　アセトンより Strecker 法によつて合成した．[7] 相當な甘味を呈する．

l(＋)-Isovalin 及び *d*(－)-Isovalin　1) *l*(＋)-Isovalin　全く E. Fischer[12] の記載に從ひメチールエチールケトンより *dl*-Isovalin ($C_5H_{11}O_2N \cdot H_2O$) を合成し，これを Formyl 誘導體として後ブルチンを用ひて光學的分割を行ひ，結晶し易いブルチン鹽より得た．光輝ある針狀晶．熔融することなく昇華する．甘味は *d*(－)-Isovalin より弱い．

$[\alpha]_D^{21°} = +9.1°$[17]　試料 0.0698 g を水 1.2 cc に溶かす．1 dm 管，$\alpha = +0.53°$

元素分析：　試料（風乾物）3.707 mg，N_2 0.315 cc (16.7°, 767.1 mm)；　N 10.11 %

$C_5H_{11}O_2N \cdot H_2$ としての計算値　N 10.36 %

2) *d*(－)-Isovalin　Formyl-*dl*-isovalin 22 g，ブルチン ($C_{23}H_{26}O_4N_2 \cdot 2H_2O$) 65.2 g 及び 85 % アルコール 647 cc より成る溫溶液を一夜冷所に放置して結晶性のブルチン鹽を析出せしめ，これを濾別して得た濾液を減壓にて溶媒を溜去せしめ，殘渣を水 180 cc に溶かし，1 N 苛性加里 72 cc を加へ，析出せるブルチンを濾別，洗滌．濾液をクロロホルムで三回，エーテルで二回抽出してブルチン及びクロロホルムを可及的に除去した後 5 N 鹽酸 12 cc を加へ，減壓で濃縮して *d*-Formylisovalin を析出せしめる．收量 7.6 g．これを約 10 % 臭化水素酸 70 cc と 75 分間湯浴上に熱した後減壓で濃縮，乾固せしめる．殘渣を水 100 cc に溶かし密陀僧 23 g と 20 分間煮沸後濾過，洗滌．濾濾液に硫化水素を通じて鉛を除去した後湯浴上に蒸發乾固すれば 3 g のアミノ酸を殘す．水とアルコールより再結晶して光輝ある針狀晶 2.3 g を得た．熔融することなく昇華する（封管中）．*l*(＋)-Isovalin より甘味强．

$[\alpha]_D^{21°} = -9.2°$[10]　試料 0.0772 g を水 1.2 cc に溶す．1 dm 管．$\alpha = -0.59°$

元素分析：　試料（風乾物）3.455 mg，N_2 0.298 cc (17.5°, 764 mm)；　N 10.19 %

$C_5H_{11}O_2N \cdot H_2O$ としての計算値　N 10.36 %

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17) E. Fischer, R. Grävenitz: *Ann.*, 406 (1914), 6.　$[\alpha]_D^{19°} = +11.0°$ (H_2O).

α-Aminodiäthylessigsäure　常法により Diäthylketon より合成した.[9] 弱い甘味を呈する.

***dl*-α-Aminomethylpropylessigsäure**　黒野氏[15] の記載に従ひ常法により Methylpropylketon より合成した. 水より一分子結晶水を含む無色柱狀晶熔融することなく昇華する（封管中）. 稍甘味を有する.

元素分析:　試料（風乾物）3.785 mg,　N_2 0.298 cc (16.6°, 766.7 mm);　N 9.36 %
〃　〃　0.1671 g,　H_2O 0.0204 g (減量 100°, 2.5 時間);　H_2O 12.21 %
$C_6H_{13}O_2N \cdot H_2O$ としての計算値　N 9.40 %,　H_2O 12.09 %

Amino-(1)-cyclohexan-carbonsäure-(1)　Zelinsky, Stadnikow[10] 氏の方法に従ひ Cyclohexanon より合成した. 微弱な甘味あるも苦味を殘す.

α-Aminodipropylessigsäure　青酸加里 4 g 及び鹽化アンモン 3.5 g を夫々水 12 cc に溶かしたものに正酪酸のカルシウム鹽を乾溜して得た Butyron（沸點 141～145°）7 g とメタノール 30 cc を加へ, 加壓瓶中 60～70° に 8 時間加熱後氷冷しつつ等容の濃鹽酸を加へ, 更に乾燥鹽酸瓦斯を飽和せしめ一夜放置. 等容の水を加へ二時間煮沸した後湯浴上に蒸發乾固する. 殘渣を濕無水アルコールにて抽出. 抽出液より減壓にてアルコールを溜去, 殘渣を再び少量のエーテルを含むアルコール (4:1) にて抽出し可及的に無機物を除き, 溶媒を溜去して得た殘渣を水に溶かし過剰の密陀僧と煮沸して後濾過, 水洗. 濾液に硫化水素を通じて鉛を除き, 活性炭で脱色後湯浴上に濃縮すれば柱狀結晶析出する. 熱水に溶かした後濃縮して再結晶せしめる. 收量 4 g. 光輝ある柱狀晶, 急速に熱するも熔融することなく昇華する（封管中）. 冷水に比較的難溶, アルコールに難溶, メタノールに稍易溶, 氷醋酸に易溶, エーテル, アセトン, ブタノールに不溶. 甘味は全然なく弱い苦味を呈する.

元素分析:　試料 3.133 mg,　CO_2 6.956 mg,　H_2O 2.954 mg;　C 60.55 %,　H 10.55 %
〃　3.059 mg,　CO_2 6.814 mg,　H_2O 2.912 mg;　C 60.75 %,　H 10.65 %
〃　3.451 mg,　N_2 0.261 cc (13.3°, 774.6 mm);　N 9.18 %
〃　3.356 mg,　N_2 0.253 cc (13.3°, 774.6 mm);　N 9.12 %
$C_8H_{17}O_2N$ としての計算値　C 60.34 %,　H 10.77 %,　N 8.81 %

***dl*-α-Amino-*n*-propylbenzylessigäure の合成** 1) ***n*-Propylbenzylketon**　先づマグネシウム 17.8 g, 無水エーテル 150 cc 及び Benzylchlorid 90.5 g の 100 cc 無水エーテル溶液とより常法によつて Benzylmagnesiumchlorid を作り, これに Butyronitril 42.5 g を無水エーテル 100 cc に溶かしたものを良く攪拌しつつ, 常に緩かな沸騰を持續せしめる程度に滴下する. ニトリルを加へ終つた後 2 時間煮沸し, 冷後内容物を約 320 g の氷水中に注加して分解せしめ, 更に 8 N 硫酸 280 cc を加へて湯浴上に 2 時間加熱後エーテルにて抽出. 抽出液を乾燥後エーテルを溜去して得た粗ケトンを減壓蒸溜して沸點 100～110°/3 mm のもの 54 g を得た.（收量約 50 %）.

2) **Semicarbazon**　セミカルバヂツド鹽酸鹽 2.2 g と醋酸加里 2.0 g を夫々水 3 cc に溶かしたものを混じ, これにケトン 3.2 g と適量のアルコールを加へて均一となし湯浴上に約 9 時間加熱後アルコールを溜去すれば油狀物を殘す. これをエーテルにて抽出. 抽出液を無水芒硝で乾燥後エーテルを溜去して冷所に放置すれば細針狀晶析出する. 結晶を石油エーテルでよく洗滌後アルコールと水より再結晶. 融點 85～87°.

元素分析:　試料 2.988 mg,　N_2 0.485 cc (12.5°, 767 mm);　N 19.21 %
$C_{12}H_{25}ON_3$ としての計算値　N 19.18 %

上記 Semicarbazon をトルオールを用ひて分割結晶を行ひ, トルオール易溶部より融點 84～85° のものを, 難溶部より融點 123～125° のものを分離し得た. 兩物質等量混合物の融點は 87～89° である.

3) *dl*-α-Amino-*n*-propylbenzylessigsäure　青酸加里 4.5 g を水 10 cc に, 鹽化アンモン 4 g を水 15 cc に溶かしたものに *n*-Propylbenzylketon 10 g 及び アルコール 50 cc を加へて加壓瓶中, 100° に 10 時間熱する. 以下 α-Aminodipropylessigsäure の場合と全く同様に處理して得た濃厚アミノ酸水溶液を湯浴上に蒸發乾固する. 殘渣を熱アルコールに溶かして放冷すれば細柱狀晶析出する. アルコールより再結晶して無色柱狀の *dl*-α-Amino-*n*-propylbenzylessigsäure 2.3 g を得. 分解點 282° (同時に一部昇華). 水に易溶. 弱い苦味を呈する.

元素分析:　試料 3.151 mg,　CO_2 8.063 mg,　H_2O 2.253 mg;　C 69.78 %,　H 8.00 %
〃　3.672 mg,　CO_2 9.401 mg,　H_2O 2.628 mg;　C 69.82 %,　H 8.01 %
〃　2.550 mg,　N_2 0.137 cc (9.5°, 773.5 mm);　N 6.46 %
〃　6.473 mg,　N_2 0.350 cc (9.5°, 773 mm);　N 6.50 %
$C_{12}H_{17}O_2N$ としての計算値　C 69.52 %,　H 8.27 %,　N 6.76 %

***dl*-α-Amino-*n*-propylbenzylessigsäure の光學的分割**　1). *dl*-Formyl-α-amino-*n*-propylbenzylessigsäure　*dl*-α-Amino-*n*-propylbenzylessigsäure 6 g を蟻酸 („Kahlbum") 12 g と 3 時間煮沸した後減壓にて蟻酸を溜去. 殘渣に就き同樣の操作を更に 2 回繰返す. 最後に蟻酸を減壓下に完全に溜去して得た結晶塊を冷水とよく混和して濾過. 冷水にてよく洗滌. 水に難溶結晶 3.8 g を得 (全濾液を湯浴上に蒸發乾固すれば 2.7 g の未反應アミノ酸を回收し得). これを熱無水アルコールに溶かし白濁を生じない程度に水を加へて放置すれば Formyl 誘導體の結晶析出する. 濾過. 水洗. 收量 3.1 g. 更にアルコールと水より再結晶して分析. 無色板狀晶. 分解點 209.5～210° (發泡). 水に極めて難溶. 熱無水アルコールに易溶.

元素分析:　試料 4.253 mg,　N_2 0.214 cc (17.1°, 767.5 mm);　N 5.98 %
$C_{13}H_{17}O_3N$ としての計算値　N 5.96 %

2). (+)-Formyl-α-amino-*n*-propylbenzylessigsäure　*dl*-Formyl-α-amino-*n*-propylbenzylessigsäure 6.65 g とブルチン ($C_{23}H_{26}O_4N_2 \cdot 2H_2O$) 12 g とを熱水 650 cc に溶かし一夜放置すればブルチン鹽の美しき板狀晶析出する. 濾過. 冷水洗. 收量 13 g. 濾液は (−)-Formyl 誘導體の分離に用ふ. 13 g の結晶を約 170 cc メタノールより三回再結晶して 8 g の板狀晶を得. これを細粉して水とよく混じ. 1 N 苛性曹達 15 cc を加へ析出せるブルチンを濾別. 水洗. 濾液をクロロフオルムと二回. 次にエーテルと一回振盪後. 2 N 鹽酸 7.5 cc を加へ放置すれば (+)-Formyl 誘導體析出する. 濾過. 水洗. 收量 2.8 g, 熱無水アルコールに溶かし水を加へて再結晶すれば柱狀晶 2.4 g を得. 分解點 210.5～211° (發泡). 微苦味を呈する.

$[\alpha]_D^{17°} = +1.2°$　試料 0.0332 g を 0.5 N 苛性曹達 1 cc に溶かす. 1 dm 管. $\alpha = +0.04°$
元素分析:　試料 3.711 mg,　N_2 0.186 cc (16.8°, 766.8 mm);　N 5.96 %
$C_{13}H_{17}O_3N$ としての計算値　N 5.96 %

全メタノール再結晶母液よりメタノールを溜去して得たブルチン鹽を上記と同樣に處理して. 分解點 210.5～211°, $[\alpha]_D^{17°} = +1.1°$ のもの 1.5 g を得.

3). (−)-Formyl-α-amino-*n*-propylbenzylessigsäure　最初に (+)-Formyl 誘導體のブルチン鹽を濾別した濾液に 1 N 苛性曹達 1 cc を加へてブルチンを除き. (+)-Formyl 誘導體の場合と全く同樣に處理して 1.5 g の (−)-Formyl 誘導體を得. 微苦味ある無色柱狀晶. 分解點 210.5～211° (發泡).

$[\alpha]_D^{17°} = -1.4°$　試料 0.0563 g を 0.5 N 苛性曹達 1 cc に溶かす. 1 dm 管. $\alpha = -0.08°$
元素分析:　試料 4.211 mg,　N_2 0.208 cc (17°, 767.3 mm);　N 5.87 %
$C_{13}H_{17}O_3N$ としての計算値　N 5.96 %

4). (+)-α-Amino-n-propylbenzylessigsäure　(+)-Formyl-α-Amino-n-propylbenzylessigsäure 2.2 g を 3N 鹽酸 50 cc と 4 時間煮沸後減壓にて水を溜去して可及的に鹽酸及び蟻酸を除く. 殘渣を水 30 cc に溶かし 4～5 g の醋酸鉛と共に約 20 分間煮沸後鹽化鉛及び過剰の酸化鉛を濾別. 水洗, 濾液に硫化水素を通じて鉛を除き, 活性炭で脱色後湯浴上に蒸發乾固して得たアミノ酸を二回無水アルコールより再結晶して 0.4 g の美しき無色針狀晶を得. 分解點 283° (收縮發泡しつつ一部昇華). 水より菱形板狀晶. 弱い苦味を呈する.

$[\alpha]_D^{22°} = +1.2°$　試料 0.0546 g を水 1.2 cc 及び濃鹽酸 0.03 cc に溶かす. 1 dm 管, $\alpha = +0.05°$

元素分析:　試料 3.077 mg,　N_2 0.171 cc (17°, 770 mm);　N 6.63 %

$C_{12}H_{17}O_2N$ としての計算値　N 6.76 %

5). (−)-α-Amino-n-propylbenzylessigsäure　(−)-Formyl-α-amino-n-propylbenzylessigsäure 1.3 g より上記と全く同樣に處理して得. 無水アルコールより美しき無色針狀晶, 水より菱形板狀晶. 分解點 283° (收縮發泡しつつ一部昇華). 弱い苦味を呈する.

$[\alpha]_D^{22°} = -1.9°$　試料 0.0459 g を水 1.2 cc 及び濃鹽酸 0.03 cc に溶かす. 1 dm 管, $\alpha = -0.07°$

元素分析:　試料 3.931 mg,　N_2 0.222 cc (16.5°, 769.5 mm);　N 6.74 %

$C_{12}H_{17}O_2N$ としての計算値　N 6.76 %

dl-Phenylaminoessigsäure N. Zelinsky, G. Stadnikow[18] の方法に從ひ Benzaldehyd より合成した. 光輝ある鱗片狀晶. 殆んど無味. 多少甘味を呈する.

dl-Phenylaminoessigsäure の光學的分割　全く E. Fischer, O. Weichhold[19] の記載に從ひ *dl*-Phenylaminoessigsäure 30 g を Formyl 化後, 先づシンコニン鹽を作り, 結晶性シンコニン鹽から *d*-(−)-Phenylaminoessigsäure 2 g を得た. 水より光輝ある細長い板狀又は柱狀晶. 分解點 305～308° (封管中速熱). 水溶液は微弱な甘味を呈する.

$[\alpha]_D^{22°} = -165.1°$[20]　試料 0.0651 g を 7 % 鹽酸 1.2 cc に溶かす. 1 dm 管. $\alpha = -8.71°$

元素分析:　試料 3.923 mg,　N_2 0.299 cc (16.8°, 769.5 mm);　N 9.09 %

$C_8H_9O_2N$ としての計算値　N 9.27 %

結晶性シンコニン鹽を濾別した濾液よりアルカリで分解してシンコニンを除去後ヒニン鹽を作り, これを分解して *l*(+)-Phenylaminoessigsäure 1.5 g を得. 水より光輝ある細長い板狀又は柱狀晶. 分解點 305～308° (封管中, 速熱). 水溶液は無味多少不快味を殘す.

$[\alpha]_D^{22°} = +161.4°$[20]　試料 0.0534 g を 7 % 鹽酸 1.2 cc に溶かす. 1 dm 管, $\alpha = +7.18°$

元素分析:　試料 3.234 mg,　N_2 0.247 cc (16.5°, 769.6 mm);　N 9.12 %

$C_8H_9O_2N$ としての計算値　N 9.27 %

終りに臨み移轉繁忙なる御指導と御鞭撻を賜つた赤堀先生に厚く感謝の意を表する. 本實驗の一部は日下御出征中の望月幸一郎氏の御援助を得た. 玆に厚く感謝の意を表する.

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18) *Ber.*, 39 (1906), 1722.　19) *Ber.*, 41 (1908) 1286.　20) E. Fischer, O. Weichhold: *Ber.*, 41 (1908), 1292, 1293. $[\alpha]_D^{22°} = -165.43°$ (10 % 鹽酸), $[\alpha]_D^{22°} = +158.09°$ (2.5 % 鹽酸).

EFFECTS OF FEEDING INDIVIDUAL AMINO ACIDS UPON THE DISTRIBUTION OF OTHER AMINO ACIDS BETWEEN CELLS AND EXTRACELLULAR FLUID

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Amino nitrogen is present in much higher concentration in tissues than in plasma (1–3). Nevertheless injected amino acids are taken up by the cells so that the tissue concentrations are increased more than the plasma concentration (1). Glutamine is present at higher concentrations in the tissues than in the plasma of dogs (3). In contrast the sum of the concentrations of valine and leucine is not appreciably higher in the tissues than in the plasma of guinea pigs (4).

Although the tissue amino acids appear to be free by customary analytical methods, they may be present in some unstable combination which does not pass through the cell wall. Thus, administered creatine enters cells against apparent concentration gradients to form phosphocreatine (5, 6). Such unstable compounds of the amino acids might be identical with the high energy intermediates postulated in peptide bond formation (7), for example N-phosphoryl compounds. We have been interested in the possibility that the magnitude of the distribution ratios of amino acids between cells and extracellular fluids might determine whether growth or wasting occurs.

In the present communication evidence is reported that glycine is distributed according to a characteristic ratio between the cells (liver and muscle) and the extracellular fluid. Amino acids which produced high concentrations in the organism when fed produced a diminution of the distribution ratio (cells to extracellular fluid) for glycine. Similarly when high glycine levels were produced by feeding glycine, the distribution ratios for the non-glycine amino acids were reduced. Of the amino acids tested, only glutamic acid produced *increases* rather than decreases in the distribution ratios of the three categories of amino acids determined. We interpret these findings to mean that each of the amino acids, when at high concentration, competes with other amino acids for the means employed by cells for the concentration of amino acids, and that L-glutamic acid in some way contributes to the intracellular concentration of other amino acids.

EXPERIMENTAL

Guinea pigs of both sexes (300 to 550 gm., Rockland Farms) were maintained upon the Rockland guinea pig diet with greens in addition two or three times a week. Amino acids and sodium benzoate were administered by mouth to animals after a 12 hour fast, in either a neutral solution or a suspension by a dropper pipette, and washed down with water. Feedings were practically quantitative. Dosages were usually 10 to 20 millimoles of amino acid per kilo of body weight. With a view to having more or less steady absorption from the gut for nearly 3 hours, two-thirds of the total amount of amino acid was given at the beginning of the experiment, one-sixth an hour later, and one-sixth 2 hours later. The animals were sacrificed at 3 hours. Omitting the later two portions and sacrificing the animals at 2 hours did not modify greatly the effects observed with glycine.

Other experimental details have been described (8), including the determination of glycine (9) and of glutamine α-amino nitrogen and non-glutamine α-amino nitrogen, both at pH 4.7 with ninhydrin (10). The α-amino nitrogen was thus divided into three categories, that due to glutamine, that due to glycine, and that due to neither, which for convenience will be called the "residual" α-amino acid nitrogen. Glycine was recovered as well (101 ± 3 per cent) from the picric acid-lead acetate filtrates at various levels of enrichment of tissues, as from standard solutions. Corrections (small, but appreciable) were made routinely for the formaldehyde obtained in the absence of ninhydrin. The specificity of the glycine method (9, 11) is supported by the observation that the deduction of the values for glycine nitrogen from the values for the total α-amino nitrogen eliminated most of the variability of the latter values. To enhance the precision of the glycine determination, aliquots usually were selected which gave optical transmittances between 45 and 60 per cent.

The concentration of each category of nitrogen in mg. per gm. of cell water was calculated from the proportions of extracellular and intracellular water, determined according to Lowry and Hastings (12, 8). The water distribution was determined in the tissues of the control animals and of those receiving sodium benzoate or glycine, but average normal values were assumed for other animals when it became apparent that variations in water distribution were not of significant magnitude. The differences reported have been checked for statistical significance (13).

Peptides in Tissues—We have attempted to measure peptides in tissues by analyzing filtrates prepared with picric acid, followed by neutral lead acetate (10). The picric acid filtrates contained conjugated α-amino nitrogen not passing through cellophane equivalent to about 2 mg. per 100 gm. of tissue, indicating about 99.9 per cent removal of protein. The lead

acetate treatment, however, left only 0.02 mg. per cent of non-dialyzable conjugated α-amino nitrogen, so that dialysis of these filtrates was unnecessary.

The following results for conjugated α-amino nitrogen were obtained: five guinea pigs, liver 0.2 mg. per cent ($\sigma = 0.2$), muscle 3.2 mg. per cent ($\sigma = 1.1$), kidney 1.0 mg. per cent ($\sigma = 0.8$); mouse carcass 1.9 mg. per cent, mouse carcass with skin 7.0 mg. per cent.

The lead acetate precipitation, designed to remove the abundant glutathione, undoubtedly removed other peptides. From a fibrin hydrolysate, for example, containing about two-thirds of the amino acids in conjugated form, 42 per cent of the conjugated amino acid nitrogen was removed by lead acetate under conditions simulating those used in preparing tissue filtrates. One may conclude that tissues, especially muscle, contain appreciable concentrations of non-protein amino acid conjugates other than glutathione. Anfinsen and Beloff[1] have concurrently made similar observations upon the livers of rats.

Effect of Lead Acetate Treatment upon Free Amino Acids—The lead acetate filtrates were not ideal for the measurement of free amino acids but seemed preferable to glutathione-rich filtrates. About one-tenth of the α-amino acid nitrogen was lost from plasma by the treatment, and 14 per cent from a fibrin hydrolysate. The amino acids removed most extensively by lead acetate when they were added to plasma and tissue filtrates were glutamic acid (60 to 80 per cent), aspartic acid (50 to 70 per cent), and histidine (about 50 per cent), as indicated by the loss of α-amino nitrogen. Glycine, alanine, serine, phenylalanine, and proline were not lost to a measurable extent; methionine, tyrosine, and leucine suffered losses of 10 per cent or less. For many of the plasma samples, α-amino nitrogen was determined both before and after lead acetate treatment; the only plasmas showing severe losses were those obtained after histidine feeding.

RESULTS AND DISCUSSION

The glycine nitrogen of the plasma of normal guinea pigs varied for unknown reasons from 0.4 to 2.6 mg. per cent; the coefficients of variation (σ/mean) were 0.60, 0.50, and 0.52 respectively for glycine in plasma, liver cells, and muscle cells. Glycine contributed most of the variability of the amino acid concentrations of these tissues (Table I). The variations in glycine, however, *occurred together in the three tissues*, so that coefficients of correlation among them were above 0.9, or, expressed in another way, the coefficients of variation of the distribution ratios were 0.17 and 0.25.

[1] Personal communication.

The liver cell tended to contain 33 times as much glycine as the extracellular fluid; the muscle cell, 8.3 times as much. The residual amino acids showed distribution ratios of 15.7 (σ = 3.0) and 5.3 (σ = 1.1) for liver and muscle respectively. The residual amino acids were not sufficiently variable to establish that their changing values in the liver, muscle, and plasma were positively correlated. The surprisingly large proportion of the free amino acids represented by glycine (31 to 63 per cent of the non-

TABLE I

Effects of Ingestion of Benzoate (300 Mg. per Kilo) and of Glycine, Glutamate, and Aspartate (Each 20 mM per Kilo) upon Distribution of Amino Acids between Extracellular Fluid and Cells of Liver and Muscle

The mean values are given, followed by the standard deviations.

	Glutamine	Glycine		"Residual"	
	α-Amino N in cell water	α-Amino N in cell water	(Cellular)/(Extracellular)	α-Amino N in cell water	(Cellular)/(Extracellular)
	mg. per cent	*mg. per cent*		*mg. per cent*	
Controls (13)*					
Liver	5.5 ± 1.4	32 ± 16	33 ± 5	33 ± 6	15.7 ± 3.0
Muscle	5.5 ± 1.8	8.6 ± 4.5	8.3 ± 2.0	13.2 ± 2.9	5.3 ± 1.1
Benzoate (3)†					
Liver		9.0 ± 4.2	19 ± 2.4	51 ± 12	25 ± 7
Muscle		7.6 ± 3.0	16.5 ± 1.6	27 ± 14	13.5 ± 8
Glycine (4)					
Liver		129 ± 13	5.5 ± 0.7	22 ± 9	7.0 ± 2.6
Muscle		41 ± 8	1.7 ± 0.2	9.0 ± 1.8	3.0 ± 0.4
Glutamate (5)					
Liver	10.3 ± 2.1	33 ± 6.2	40 ± 3.8	49 ± 4.4	21 ± 3.7
Muscle	8.5 ± 2.4	10.0 ± 2.2	12.2 ± 2.0	21.5 ± 5.5	7.9 ± 1.1
Aspartate (3)					
Liver		37 ± 3	31 ± 2	44 ± 1.4	21 ± 3
Muscle		10.6 ± 1.7	8.8 ± 0.9	17 ± 1.8	7.9 ± 1.5

* The figures in parentheses represent the number of animals.

† Animals sacrificed 70, 80, and 120 minutes after feeding sodium benzoate.

glutamine α-amino nitrogen in liver, 19 to 56 per cent in muscle) may be related to the relatively large contribution of glycine to urinary amino acid conjugates in man (11).

Sodium Benzoate—A dose of sodium benzoate (300 mg. per kilo of body weight) which should stimulate a nearly maximum rate of synthesis of hippuric acid reduced the free glycine of liver and secondarily that of plasma, so that the distribution ratios, liver cell to plasma, and muscle cell to plasma, moved downward and upward respectively (Table I). This illustrated what happens when the withdrawal of an amino acid is

stimulated in one of the two tissues, and is to be contrasted with the effects of L-proline or DL-serine, for example, which caused both liver and muscle cells to lose glycine (see below). The results indicate a predominance of the liver over the kidneys (14) in hippuric acid synthesis in the guinea pig, the distribution ratio for glycine between kidney and plasma being little changed. Accumulation of hippuric acid in the liver (measured as conjugated amino acid nitrogen extractable by ethyl acetate) was small, in one case 1.7 mg. per cent of nitrogen.

L-Amino Acids—Of the amino acids administered (excepting DL forms) L-proline, L-histidine, glycine, and L-methionine caused large rises in plasma amino acids. These were also the amino acids giving the largest rises in

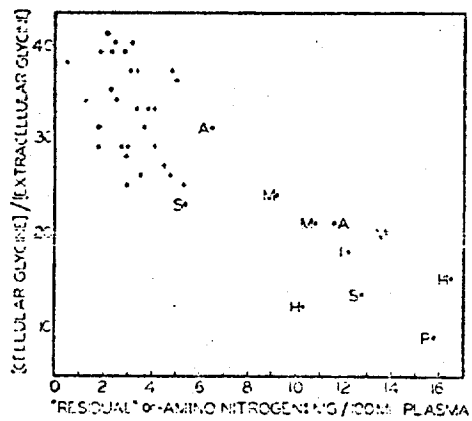

FIG. 1. Relation of the distribution ratio for glycine between liver cells and extracellular fluid, to the plasma residual α-amino acid nitrogen after the feeding of individual amino acids. *A* = DL-alanine; *H* = L-histidine hydrochloride; *M* = L-methionine; *P* = L-proline; *S* = DL-serine; *T* = DL-threonine; *V* = DL-valine. +, experiments with other amino acids; ○, fasting controls.

the amino acids of liver and muscle cells. As the glycine concentration of the plasma was increased after glycine feeding, the distribution ratios for glycine were decreased (although the absolute increase in the glycine concentration was always greater in the cells than in the plasma). Similarly, at high concentrations of residual α-amino nitrogen, the distribution ratio of this category was reduced. This would be expected if the capacity of cells for concentrating each of the amino acids is limited. But whenever large elevations of the plasma glycine were produced, the distribution ratios of the other (residual) amino acids, between the cells (liver and muscle) and the plasma, were also depressed (Table I), with an accompanying rise in plasma concentration. Similarly, whenever the plasma residual amino acids were elevated, the distribution ratio of *glycine* between liver

cells and extracellular fluid was decreased approximately in proportion (Fig. 1). The ingestion of L-proline caused the plasma glycine nitrogen to rise to the extraordinary level of 7.7 mg. per cent with the distribution ratios falling below one-third of normal. An effect upon the muscle glycine was not apparent under the conditions of the experiments, except in the case of proline.

All the other L-amino acids examined gave undetectable to moderate increases in the plasma α-amino acid nitrogen, smaller effects upon the α-amino acid nitrogen of liver cells, and no significant effect upon the distribution of glycine. The majority of the amino acids tested caused increases of the α-amino nitrogen of muscle cells of the guinea pig (*cf.* (2)). Several of the differences in plasma response to ingested amino acids recorded here have been noted before in other species (15–23).

DL-*Amino Acids*—In cases where comparisons were made (alanine, serine, leucine, and phenylalanine), the DL-amino acids elevated the plasma amino acid concentration more than the corresponding L acids. The intense effect upon glycine distribution produced by high accumulations of an amino acid was also obtained with DL-amino acids (threonine, serine, valine, and alanine), muscle as well as the liver being affected (Table II; Fig. 1).

The amino acids appear to show competitive inhibition among themselves for the means by which the cells concentrate amino acids, indicating that these means do not operate independently for each amino acid. An identical mechanism could scarcely concentrate each of the large number of amino acids and yet maintain characteristic distributions for each in the face of the variations of the amino acid mixtures presented. Thus, a high plasma glycine level reduced the distribution ratio of other amino acids much less than that of glycine itself. The slope indicated roughly by the points of Fig. 1 suggests that an increase of about 9 mg. per cent of α-amino nitrogen due to a single amino acid in the plasma (*i.e.*, about 9 times the glycine concentration) halves the ability of the liver cell to hold glycine. Protein synthesis obviously requires the presence in some form and at some limiting concentration of every amino acid (essential or non-essential) which must be built into the molecule. As a corollary, it may be inferred that, when a strongly asymmetric accumulation of amino acids is produced by feeding or injection, the ability of the cells to retain other amino acids will be handicapped and one may expect an inhibitory effect upon growth or nitrogen retention. Thus, the difficulty in producing optimum growth of rats with diets containing gelatin supplemented with amino acids, or with diets containing 5 to 6 per cent of glycine, L-proline, or DL-phenylalanine (24, 25), may well be an effect of the excessive accumulation of one or more amino acids. The amino acids producing high blood concentrations upon feeding are the ones which cause extra energy production ("heat of specific dynamic action") and in some cases extra urea excretion (16, 26).

TABLE II

Effects of Ingestion of Several Other Amino Acids upon Distribution of Amino Acids between Extracellular Fluid and Cells of Liver and Muscle

Because of the great variability in glycine concentrations the distribution ratio (glycine nitrogen per 100 gm. of cell water to 1.05 × mg. of glycine nitrogen per 100 ml. of plasma) appears more useful. For the residual α-amino acids the cellular concentration is the more useful value because of its relative constancy. Mean values followed by standard deviations are given where two or more animals are grouped.

Amino acid fed	Dosage, mg per kilo	Glycine			Residual α-amino N		
		Plasma	Liver	Muscle	Plasma	Liver	Muscle
		α-Amino N	Distribution ratio	Distribution ratio	α-Amino N	Concentration in cell water	Concentration in cell water
		mg. per cent			*mg. per cent*	*mg. per cent*	*mg. per cent*
Fasting (13 animals)		1.02	33	8.6	2.46	38	13.2
		±0.61	±5	±2.1	±0.53	±6	±2.9
L-Alanine	10	0.92	31	8.6	3.86	46	24
" *	10	0.73	26	7.1	4.76	34	22
DL-Alanine*	10	1.22	31	6.2	6.5	46	21
" *	20	1.52	21	6.4	11.6	41	31
L-Serine	12.5	1.95	28	6.5	2.92	60	20
DL-Serine*	12.5	0.82	37	8.0	3.19	62	19
"	12.5	2.4	23	5.9	5.39	64	22
DL-Serine*	20	3.1	13.5	3.4	12.8	95	21
DL-Threonine*	15	1.89	18	8.0	12.2	93	23
DL-Valine*	15	1.0	20	6.6	13.8	49	28
L-Leucine (3 animals)	10	1.24	33	9.0	4.4	34	15.2
		±0.46	±6	±1.7	±0.9	±2	±4.7
DL-Leucine*	10	0.51	39	11	6.54	35	21
L-Proline*	15	7.7	9	2.2	15.8	72	30
L-Methionine (2 animals)	10	0.85	22	9.8	10.0	83	23
		±0.05	±2	±1.0	±0.8	±2	±1
L-Phenylalanine*	10	0.81	33	8.6	3.81	42	17.0
DL-Phenylalanine*	10	0.99	36	9.7	5.00	43	17.9
L-Tyrosine	10	0.95	29	10.3	4.1	38	19.2
L-Tryptophan	10	0.77	37	11	4.8	38	18.5
L-Ornithine	6.4	0.76	37	10.9	3.12	48	20.0
L-Lysine	10	1.96	27	9.2	4.20	43	16
L-Arginine (2 animals)	10	0.94	33	10.1	2.6	48	17
		±0.15	±1	±0.3	±0.8	±7	±4
L-Histidine (2 animals)	10	1.07	13.7	9.7	13.4	76	24
		±0.12	±1.5	±0.5	±3.1	±11	±1

* Basal ration supplemented with pyridoxine, 5 mg. per kilo.

The elevation of the alanine concentration of plasma when glycine is ingested by man, and of the glycine concentration when DL-alanine is fed (13), is an additional illustration of the response of cells to high concen-

trations of an amino acid. Decreases in the plasma concentration of several amino acids in dogs after DL-methionine feeding were noted by Hier and Bergeim (21). Humans fed glycine showed increased amino acid conjugates in plasma ((11); *cf.* (17)). The conjugated amino acids of an ultrafiltrate of the plasma of a dog were doubled after a dose of L-glutamic acid which elevated the portal α-amino nitrogen to 10.7 mg. per cent.[2] High plasma concentrations of an amino acid may provoke loss of peptides as well as of free amino acids from cells.

The observations recorded here may also assist in explaining the urinary losses of amino acids in mice fed peroxide-treated proteins (27), and the production of vomiting during the infusion of DL-amino acids and of L-glutamic and L-aspartic acids (28–30).

L-*Glutamic Acid*—The failure of L-glutamic acid absorption to increase noticeably the amino nitrogen of peripheral blood was noted by Seth and Luck (16). Increases occurred in portal blood, however. In our experiments, none of the three fractions of the amino acids of cardiac plasma was increased during glutamic acid absorption by guinea pigs; in fact, the residual amino acids were decreased (means, fasting, 2.46 mg. per cent; after glutamic acid, 2.01 mg. per cent).

In a partially gastrectomized dog receiving by mouth 20 mM of L-glutamic acid (as sodium glutamate) per kilo of body weight, we observed, in collaboration with Dr. Charles Dent,[2] a net decrease in the glycine, glutamine, and residual α-amino nitrogen in both the portal and jugular plasma, following preliminary increases, especially of the residual α-amino nitrogen in the portal plasma.

The explanation, we believe, lies in an increased uptake of various amino acids by tissues when glutamate is fed. Each of the three categories of amino acids determined was increased in liver and muscle cells of guinea pigs, and each of the distribution ratios was elevated while glutamate was being absorbed. Glycine does not appear to undergo transamination with the glutamate-α-ketoglutarate system (31, 32).

After feeding L-aspartic acid, as with L-glutamic acid, the plasma concentrations were not elevated, whereas the residual amino acids in liver and muscle were increased. The distribution of glycine was unaffected, however.

It is concluded that glutamate (or α-ketoglutarate) is somehow involved in the system by which the cells concentrate the amino acids presented to them in the extracellular fluid. Whether the observed effects are related to the conversion of glutamate to α-ketoglutarate (transamination) or to the disposition of the α-ketoglutarate with resultant formation of high

[2] Unpublished results with Dr. Charles Dent.

energy phosphate compounds, or to other reactions, remains to be determined.

SUMMARY

1. Glycine represented a large and highly variable portion of the amino acids of skeletal muscle and liver of guinea pigs. The glycine of the muscle cells, liver cells, and plasma were covariant, so that fairly constant distribution ratios were maintained. The glutamine levels of these tissues were also highly variable but with little covariance among them. The concentrations of the non-glutamine, non-glycine ("residual") amino acids were moderately constant.

2. Those amino acids which upon feeding caused high plasma concentrations (L-proline, L-histidine, glycine, L-methionine, DL-threonine, DL-serine, DL-valine and DL-alanine) caused the largest increases in the amino acids of liver and muscle cells.

3. When high glycine levels were produced by feeding glycine, not only was the distribution of glycine affected but the distribution ratios of the residual amino acids (cellular to extracellular) were also reduced.

4. Those amino acids which produced high concentrations in the organism caused a reduction not only of the distribution ratios for the category to which they belong but of the glycine distribution ratios as well. Those amino acids which gave small rises in the plasma concentration had little if any effect upon glycine distribution.

5. Glutamic acid alone of the amino acids studied produced an *increase* in the distribution ratios of each of the three categories of amino acids determined. The plasma amino acids were *diminished* during glutamate absorption.

6. These observations are interpreted to mean that the various L- and D-amino acids compete with each other, although only to a restricted extent, for the means by which cells concentrate the amino acids presented to them by the extracellular fluid, and that L-glutamic acid contributes in some way to this concentrating mechanism.

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CLXXXII. TRANSAMINATION IN PIGEON BREAST MUSCLE

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D. M. NEEDHAM [1930] first observed that in pigeon breast muscle added glutamic acid disappears from the tissue while the concentration of amino groups, as measured by the Van Slyke method, remained unchanged. She concluded that the amino group of glutamic acid is transferred to "some reactive carbohydrate residue" to form a new amino-acid. Braunstein & Kritzmann [1937; 1938] succeeded in elucidating this reaction in detail. They showed that glutamic acid reacts as follows:

(1) Glutamic acid + α-ketonic acid ⇌ α-ketoglutaric acid + amino-acid. The "reactive carbohydrate residue" of Needham is thus an α-ketonic acid. The reaction is reversible and proceeds with great rapidity under suitable conditions. It is a general reaction, occurring in almost every animal tissue, and has been termed "transamination" [Schaeffer & Le Breton, 1938; Cohen, 1939; Braunstein, 1939]. The widespread occurrence and the rapid rate of transamination suggest that the reaction plays an important part in tissue metabolism, although its significance is as yet by no means clear.

The detailed study of transamination has been hampered by the lack of suitable analytical methods. The procedure used by Braunstein & Kritzmann [1937] does not appear to be very satisfactory for the authors themselves [1938] question their analytical data in several instances. Since the determination of glutamic acid synthesis in the presence of α-ketoglutaric and amino-acids is one of the ways of measuring transamination, a more reliable method for glutamic acid determination was recently worked out by the author [1939].

The preliminary experiments with this method showed that although most tissues are capable of bringing about transamination, the different tissues show great differences in detail. In muscle, for example, only three amino-acids, *l*(+) glutamic, *l*(−)aspartic and *l*(+)alanine react rapidly; two others, *dl*-α-amino-butyric and *l*(+)valine react slightly; while the other amino-acids do not react appreciably. In kidney, on the other hand, many more amino-acids react.

This paper is confined to a study of transamination in pigeon breast muscle. Experiments on the rates of transamination in this tissue and the reactivity of different amino-acids are reported.

Procedure and methods

Principle of the procedure. For the study of transamination it is desirable to separate this reaction from other reactions in which amino- and α-keto-acids take part. Many of the other reactions require molecular O_2 and they can therefore be eliminated by working under anaerobic conditions. Since it is simpler we have preferred this method to the use of inhibitors. Comparative experiments showed that the results obtained under anaerobic conditions are not essentially different from those obtained aerobically in the presence of bromo-acetate [Braunstein & Kritzmann, 1937], an inhibitor which prevents certain

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side reactions. Neither method however eliminates the side reactions completely, for glutamic acid slowly disappears anaerobically, as well as aerobically in the presence of iodoacetate, without the addition of ketonic acids. The mechanism of this reaction is not clear; it may be due to transamination to ketonic acids provided by the muscle. The rate of the reaction however is relatively slow so that it generally does not interfere with experiments on transamination of added substrates.

Transamination was studied by the determination of glutamic acid formed on the addition of α-ketoglutaric acid and amino-acids. In all experiments a blank was carried out with α-ketoglutaric acid alone to determine the glutamic acid formation from preformed NH_2-donators (NH_3 or amino-acids). The values of the "blanks" in most experiments were considerable, showing that a relatively high concentration of NH_2-donators is present in pigeon breast muscle.

An increased glutamic acid formation from added α-ketoglutaric acid and amino acids may be considered as conclusive proof of transamination. Euler *et al.* [1939] studied the disappearance of oxaloacetic acid in the presence of amino-acids and they concluded that transamination occurs when the added amino-acids increased the rate of disappearance of oxaloacetic acid. This type of experimentation may suggest that transamination takes place, but cannot provide conclusive proof. The demonstration of aspartic acid formation in this reaction would in our view be the only conclusive evidence of transamination.

Experimental procedure. Pigeon breast muscle was chilled immediately after the death of the animal and finely divided in a Latapie mincer. The minced muscle was suspended in 0·10 *M* phosphate buffer, *p*H 7·4. Unless otherwise stated the muscle suspension used in the different experiments consisted of 1 part muscle plus 7 parts phosphate buffer. The suspension (3 ml.) was pipetted into the main compartment of conical Warburg manometer flasks provided with a side arm, which contained the substrate, and a centre well which contained a stick of yellow phosphorus. The substrates were used as neutral 0·2 *M* solutions (0·4 *M* for *dl*-amino-acids) and usually added in amounts to give a final concentration of 0·017 *M*. Substrates which were soluble with difficulty were weighed directly into the side arm of the cup.

Anaerobic conditions were maintained by filling the vessels with N_2 and by the yellow phosphorus in the centre well. The vessels were shaken at 40° and unless otherwise stated the substrate was added from the side arm after 5 min. shaking. At the end of the experimental period the flasks were detached and 1 ml. 10 % H_2SO_4 added to stop the reaction. The solutions were washed into graduated cylinders with 6–7 ml. distilled water, 1 ml. 10 % Na_2WO_4 was added and the solutions made up to a volume of 15–20 ml. The solutions were filtered and an aliquot of the clear filtrate employed for the glutamic acid determination.

Analytical procedure. Glutamic acid was determined by the method recently described by the author [1939]. Interfering succinic acid, and malonic acid when added, were removed by ether extraction before the glutamic acid determination. The aliquot of the clear filtrate was acidified with 2 ml. 10 % H_2SO_4 and extracted with ethyl ether for 2 hr. The ether was removed from the aqueous phase and the latter brought to a *p*H of approximately 5 by the addition of alkali in the presence of a drop of bromocresol purple. The glutamic acid in the aqueous phase was then determined.

The results are expressed as μl. glutamic acid (147 mg. glutamic acid being equivalent to 22,400 μl.). This procedure was used since the glutamic acid was determined as μl. O_2 (1 μl. O_2 is equivalent to 2 μl. glutamic acid).

Results

Glutamic acid formation from amino-acids and α-ketoglutaric acid

The question was first investigated as to which amino-acids react with α-ketoglutaric acid to form glutamic acid. In these experiments a relatively long incubation period of 40 min. was chosen to allow for the formation of measurable amounts of glutamic acid from slowly reacting amino-acids. The results of these experiments are listed in Table I.

Table I. *Glutamic acid formation from α-ketoglutaric acid and different amino-acids*

Substrate conc. 0·017 *M* (for *dl*-amino-acids, 0·034 *M*). N_2. Yellow phosphorus. 40°.

Amino-acid added	Period of incubation min.	μl. Glutamic acid formed: Without amino-acid	μl. Glutamic acid formed: With amino-acid	Increase in glutamic acid due to added amino-acid μl.	% of added α-ketoglutaric acid converted into glutamic acid
l(−)Aspartic acid	40	161	815	654	49
l(+)Alanine	40	96	636	543	40
l(+)Valine	40	96	220	124	9
l(−)Phenylalanine	40	96	170	74	—
l(−)Tyrosine	40	142	204	62	
l(−)Cysteine	40	94	152	56	—
d(−)Valine	40	96	149	53	—
l(−)Histidine	40	96	146	50	—
d(+)Histidine	40	96	140	44	—
l(−)Leucine	40	142	180	38	—
d(−)Alanine	40	130	160	30	—
l(+)Citrulline	40	142	168	26	—
l(+)*iso*Leucine	40	142	166	24	—
l(−)Methionine	30	138	156	18	—
l(+)Arginine	40	96	99	3	—
Glycine	40	96	95	—	—
l(+)Ornithine	40	142	104	—	—
l(−)Proline	40	142	142	—	—
d(+)Phenylalanine	40	96	92	—	—
l(−)Tryptophan	40	142	124	—	—
dl-Serine	40	142	140	—	—
dl-Lysine	30	138	113	—	—
Homologous series					
dl-Alanine	40	130	564	434	32
dl-α-Aminobutyric acid	40	130	266	136	10
dl-α-Aminovaleric acid	40	130	145	15	—
dl-α-Aminohexoic acid	40	130	150	20	—

Of the 21 different amino-acids tested, *l*(−)aspartic acid and *l*(+)alanine are the only ones which show a large glutamic acid formation; *dl*-α-aminobutyric acid and *l*(+)valine show a slight activity. None of the remaining amino-acids is significantly active.

These results differ in various points from those reported by Braunstein & Kritzmann [1938] whose experiments were carried out aerobically in the presence of bromoacetate. In order to ascertain whether the differences in the results are due to differences in experimental conditions, transamination was also studied under conditions similar to those of Braunstein & Kritzmann. It is seen from Table II that the results obtained aerobically in the presence of iodoacetate are essentially the same as those obtained anaerobically. If anything these experi-

Table II. *Glutamic acid formation from α-ketoglutaric acid and different amino-acids under aerobic conditions in presence of iodoacetate*

Substrate conc. 0·016 *M*. Iodoacetate conc. (final), 0·002 *M*. (Iodoacetate plus muscle suspension incubated 15 min. before substrate added). 1 part minced muscle plus 5 parts phosphate buffer. Reaction time, 60 min. Air.

Amino-acid added	Glutamic acid formed µl.	Increase in glutamic acid due to added amino-acid µl.	% of added α-ketoglutaric converted into glutamic acid
—	92	—	—
l(+)Valine	98	6	—
d(−)Valine	33	—	—
d(+)Histidine	98	6	—
l(−)Histidine	55	—	—
l(+)Arginine	127	35	—
l(+)Alanine	450	358	27
	No iodoacetate		
l(+)Alanine	214	122	9

ments show that the formation of glutamic acid is greater under anaerobic conditions. The inhibitory effect of iodoacetate on the aerobic disappearance of glutamic acid formed from *l*(+)alanine and α-ketoglutaric acid is seen from Table II. This is in agreement with the findings of Braunstein & Kritzmann [1937] who first reported this effect with bromoacetate.

Braunstein & Kritzmann [1938] found 14 different amino-acids to react with α-ketoglutaric acid in pigeon breast muscle, but the authors themselves question their results for 3 amino-acids. Of these amino-acids only the findings for alanine are confirmed by our experiments. From our data previously discussed it follows that the discrepancy between our results and those of Braunstein & Kritzmann cannot be attributed to a difference in experimental procedure. The different results may however be explained by the omission of a suitable control by Braunstein & Kritzmann. As can be seen from Table I α-ketoglutaric acid when added to a muscle suspension forms considerable amounts of glutamic acid in the absence of any added amino-acid. The failure of Braunstein & Kritzmann to take this into account is the chief reason for the high values obtained for all the amino-acids. An additional factor is to be found in the method used by Braunstein & Kritzmann for the determination of glutamic acid. The method employed by these workers measures the NH_2-N which is insoluble in alcohol after the addition of $Ba(OH)_2$ and thus would include aspartic acid and possibly other amino-acids. The high values for certain of the amino-acids, e.g. cystine, reported by these workers is probably due to the insolubility of these amino-acids under the conditions used for the analysis.

On the other hand, of all the amino-acids studied we find *l*(−)aspartic to be the most active. Braunstein & Kritzmann have reported no data for this amino-acid, presumably because their method does not permit the differentiation between glutamic and aspartic acids.

None of the amino-acids of the *d*-series tested was found to be active (Table I).

Effect of ketonic acids on the anaerobic disappearance of glutamic acid

Under anaerobic conditions glutamic acid slowly disappears when added to pigeon breast muscle [Needham, 1930; Braunstein & Kritzmann, 1937; Cohen, 1939]. Braunstein & Kritzmann [1937] showed that the disappearance of glutamic acid is markedly accelerated if pyruvic acid is added, and that the disappearance of glutamic acid is accompanied by the formation of equivalent

amounts of alanine. These authors thus demonstrated the reversibility of transamination.

In order to determine whether pyruvic acid can be replaced by other ketonic acids, the effects of a series of α- and other keto-acids on the anaerobic disappearance of glutamic acid were studied. If the activities of the members of the homologous series of α-ketomonocarboxylic acids are compared (Table III)

Table III. *Disappearance of added glutamic acid in the presence of ketonic acids*

Substrate conc. 0·017 *M*. N_2. Yellow phosphorus. 40°. Incubation period, 40 min.

Ketonic acid added	Glutamic acid added μl.	Glutamic acid found μl.	Glutamic acid disappeared μl.	% Glutamic acid disappeared
—	1344	1240	104	7·5
Oxaloacetic	1344	654	690	51·5
Pyruvic	1344	688	656	49
α-Ketobutyric	1344	1090	254	19
α-Ketovaleric	1344	1180	164	12
α-Ketocaproic	1344	1190	154	11
Mesoxalic	1344	1105	239	18
Acetopyruvic	1344	1162	182	13·5
Acetoacetic	1344	1192	152	11
Laevulic	1344	1360	—	—

it is seen that pyruvic acid is the most active, α-ketobutyric acid is slightly active, while α-ketovaleric and α-ketohexoic acids are not appreciably active. These results are in keeping with the findings of the reverse reactions (Table I). Of the α-ketonic dicarboxylic acids, oxaloacetic acid shows an activity of the same order as pyruvic whilst mesoxalic shows a small activity. Acetoacetic, acetopyruvic and laevulic acids are not appreciably active. Laevulic acid appears to inhibit the small anaerobic disappearance of glutamic acid.

All the preceding observations on transamination in pigeon breast muscle can be explained on the assumption that three different enzyme systems are concerned with transamination in this tissue, one each for the three reactions:

Glutamic acid ⇌ α-ketoglutaric acid
Aspartic acid ⇌ oxaloacetic acid
Alanine ⇌ pyruvic acid

The slight activities of *l*(+)valine and *dl*-α-aminobutyric acid, on the one hand, and α-ketobutyric acid and mesoxalic acid on the other, may be explained by assuming slight affinities of *l*(+)valine, *dl*-α-aminobutyric and α-ketobutyric acids for the alanine ⇌ pyruvic acid system, and of mesoxalic for the aspartic ⇌ oxaloacetic acid system. It is well known that the affinities of other enzyme systems, for example, lactic dehydrogenase, are not strictly limited to one substrate. Homologous compounds may also react but the rate of reaction rapidly falls off as the carbon chain is lengthened. If this holds for the transaminating enzymes, then the failure of the higher homologues of alanine and pyruvic acid to react is easily understood. The fact that α-aminobutyric acid is physiologically a rare compound makes the explanation of its activity difficult on any other basis.

Glutamic acid formation from amino compounds other than α-amino-acids

A number of different amino compounds—β-, δ-, and ε-amino-acids, amines and aminopurines—were tested for glutamic acid formation in the presence of

α-ketoglutaric acid, but no significant increase was observed with any of these compounds (Table IV). These findings confirm those of Braunstein & Kritzmann [1938].

Table IV. *Glutamic acid formation from α-ketoglutaric acid and amino compounds other than α-amino acids*

Substrate conc. 0·017 *M*. N_2. Yellow phosphorus. 40°.

Amino-acid added	Period of incubation min.	Glutamic acid formed: Without amino-acid μl.	Glutamic acid formed: With amino-acid μl.
β-Alanine	30	85	114
δ-Aminovaleric	30	85	114
ε-Aminohexoic	30	85	92
dl-β-Aminobutyric	40	130	127
Adenine	40	138	95
Histamine	40	138	118
Putrescine	20	122	125
Spermine	40	122	116
Heptylamine	40	122	92

Rates of formation and disappearance of glutamic acid by transamination

The data in the previous sections are not accurate measurements of the rate of transamination, since in cases where the rate is very rapid, as with *l*(−)aspartic acid and *l*(+)alanine, the reaction comes to an equilibrium before the end of the incubation period. To determine the rate of transamination shorter periods of incubation were therefore chosen. The data from such experiments are shown in Figs. 1, 2 and 3.

From Fig. 1, it is seen that the initial rates are very rapid for *l*(−)aspartic acid and *l*(+)alanine. In the case of *dl*-α-aminobutyric acid and *l*(+)valine, the initial rates are also appreciable but the curves show an early plateau, for some unexplained reason. This is not due to a true equilibrium since the plateaux reached from both sides of the reaction lie at different levels (Fig. 2).

Under the experimental conditions employed the reaction:

(2) glutamic acid + pyruvic acid ⇌ ketoglutaric acid + alanine

has an equilibrium constant

$$\frac{[\text{glutamic}] \times [\text{pyruvic}]}{[\text{ketoglutaric}] \times [\text{alanine}]}$$

of approximately 1 (see Fig. 2). This is in agreement with the previous finding reported by Braunstein & Kritzmann [1937]. None of the remaining systems shown in Fig. 2 shows any evidence of reaching an equilibrium.

The reaction:

(3) glutamic acid + oxaloacetic acid ⇌ ketoglutaric acid + aspartic acid

reaches an equilibrium even faster than reaction (2) (Fig. 3). The equilibrium constant of this reaction is of an order similar to that of reaction (3). Owing to some side reactions in which oxaloacetic acid disappears, reaction (3) does not come to a standstill and this complicates the accurate determination of the equilibrium constant.

If the rates for reactions (2) and (3) are expressed as $Q_{\text{glutamic acid}}$,

$$\frac{\mu\text{l. glutamic acid formed}}{\text{mg. dry wt. of tissue} \times \text{hr.}},$$

values of 39 and 44 respectively are obtained. Q_{O_2} values for respiration of pigeon breast muscle under optimum conditions are of the same order.

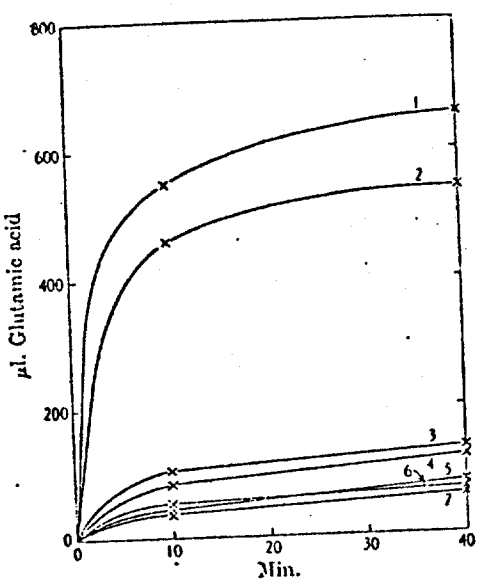

Fig. 1.

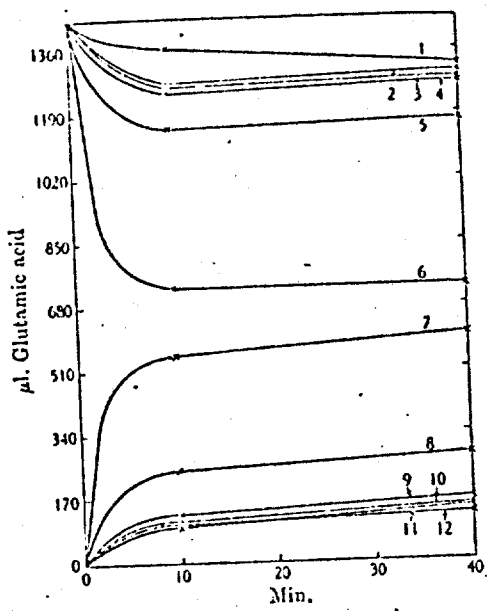

Fig. 2.

Fig. 1. Rate of glutamic acid formation from α-ketoglutaric acid and amino-acids. Substrate conc. 0·017 *M* (for *dl*-amino-acids, 0·034 *M*). N_2. Yellow phosphorus. 40°. (Corrected for blank.) Curves: 1, *l*(−)Aspartic acid + α-ketoglutaric acid; 2, *l*(+)alanine + α-ketoglutaric acid; 3, *dl*-α-aminobutyric acid + α-ketoglutaric acid; 4, *l*(+)valine + α-ketoglutaric acid; 5, *l*(+)phenylalanine + α-ketoglutaric acid; 6, *l*(−)cysteine + α-ketoglutaric acid; 7, *l*(−)tyrosine + α-ketoglutaric acid.

Fig. 2. Rate of glutamic acid formation in the presence of α-ketoglutaric acid plus amino-acids, and rate of glutamic acid disappearance in the presence of ketonic acids. Substrate conc. 0·017 *M* (*dl*-amino-acids, 0·034 *M*). N_2. Yellow phosphorus. 40°. Curves: 1, Glutamic acid; 2, glutamic acid + acetoacetic acid; 3, glutamic acid + α-ketohexoic acid; 4, glutamic acid + pyruvic acid; 5, glutamic acid + α-ketobutyric acid; 6, glutamic acid + α-ketovaleric acid; 7, α-ketoglutaric acid + *dl*-alanine; 8, α-ketoglutaric acid + *dl*-α-aminobutyric acid; 9, α-ketoglutaric acid + *dl*-α-aminovaleric acid; 10, α-ketoglutaric acid + *dl*-α-aminohexoic acid; 11, α-ketobutyric acid + *dl*-β-aminobutyric acid; 12, α-ketoglutaric acid.

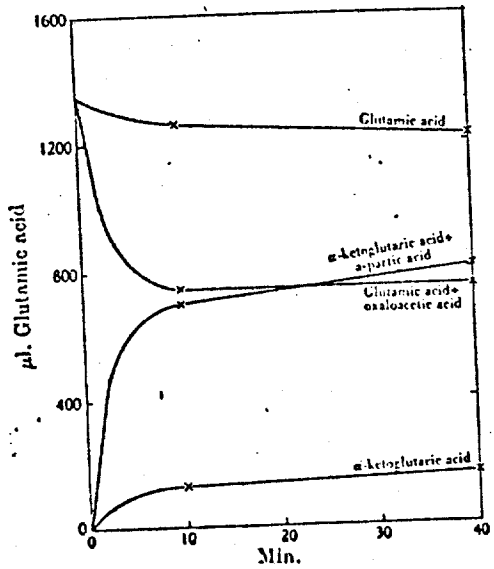

Fig. 3. Rate of glutamic acid formation in the presence of α-ketoglutaric acid plus aspartic acid, and rate of glutamic acid disappearance in the presence of oxaloacetic acid. Substrate conc. 0·017 *M*. N_2. Yellow phosphorus. 40°.

Effect of small amounts of aspartic acid on transamination

The lack of a suitable method for the determination of aspartic acid has so far prevented an investigation of the reaction:

(4) oxaloacetic acid + amino-acid ⇌ aspartic acid + ketonic acid

(except when the amino-acid is glutamic acid). However, reaction (4) can be studied in an indirect way by coupling it with reaction (3). Experimentally this means the addition of α-ketoglutaric acid to the system represented by reaction (4). The balance sheet of reactions (3) and (4) is:

(5) α-ketoglutaric acid + amino-acid ⇌ glutamic acid + α-ketonic acid.

Thus it should be possible to detect transamination according to reaction (4) by the addition of α-ketoglutaric acid, aspartic acid and another amino-acid to a muscle suspension. When these three substances are added, reaction (3) takes place and oxaloacetic acid is formed. Oxaloacetic acid would then be expected to react according to reaction (4), and aspartic acid would be regenerated. Since aspartic acid is expected to act in the manner of a catalyst in this reaction, small concentrations of aspartic acid should suffice to bring about an effect. With this in mind previously studied amino-acids were tested with and without the addition of aspartic acid. From the data in Table V it is seen that there is no significant

Table V. *Effect of small concentrations of aspartic acid on transamination from α-ketoglutaric acid and different amino-acids*

Final conc. of α-ketoglutaric acid and amino-acids, 0·016 *M*. Final conc. of aspartic acid, 0·0016 *M*. Substrate added after 5 min. incubation. N_2. Yellow phosphorus. 40°.

Amino-acid added	Period of incubation with substrate min.	μl. Glutamic acid formed: Without aspartic acid	μl. Glutamic acid formed: With aspartic acid	Increase in glutamic acid due to aspartic acid μl.
—	40	110	194	84
l(+)Alanine	40	520	560	40
l(+)Valine	40	164	218	54
l(−)Phenylalanine	40	140	210	70
l(+)Arginine	40	129	222	93
l(−)Leucine	40	138	241	103

increase in glutamic acid formation from the various amino-acids in the presence of aspartic acid, beyond that of aspartic acid itself. The conclusion may be drawn from these experiments that oxaloacetic acid reacts with no amino-acids other than glutamic acid and alanine. However direct proof for the reaction with alanine has not been produced.

The effect of inhibitors on transamination

The effects of different inhibitors on transamination are listed in Table VI. In these experiments the inhibitors were incubated with the muscle suspension for 15 min. before the substrate was added from the side arm.

Transamination is markedly inhibited by high concentrations of cyanide. As seen from the data the percentage inhibition is roughly proportional to the concentration of cyanide. The effect of high concentrations of cyanide suggests that the inhibition may be due to cyanohydrin formation [Green & Williamson, 1937]: however, the 30 % inhibition by 10^{-3} *M* cyanide indicates that some other basis for the inhibition may exist.

Table VI. *Effect of inhibitors on glutamic acid formation from α-ketoglutaric acid and l(+)alanine and l(−)aspartic acid*

Final substrate conc. 0·015 *M*. Inhibitor incubated with muscle suspension for 15 min. before substrates added. N_2. Yellow phosphorus. 40°.

Inhibitor	Final conc. *M*	Incubation period min.	Glutamic formed µl.	Inhibition %
	α-Ketoglutaric acid + *l*(+)alanine			
—	—	30	576	—
Cyanide	0·05	30	122	79
"	0·01	30	288	50
"	0·001	30	400	30
"	0·0001	30	505	12
Malonate	0·10	30	484	10
"	0·01	30	542	6
Pyrophosphate	0·01	30	534	7
NaF	0·02	30	490	15
Iodoacetate	0·002	30	550	5
Bromoacetate	0·002	30	526	9
As_2O_3	0·01	30	488	15
Octyl alcohol	Sat.	30	498	14
	α-Ketoglutaric acid + *l*(−)aspartic acid			
—	—	40	592	—
Malonate	0·10	40	436	26
"	0·01	40	580	2

Malonate in concentrations as high as 0·10 *M* has only a slight effect on transamination in the presence of *l*(+)alanine and α-ketoglutaric acid. Transamination in the presence of *l*(−)aspartic acid and α-ketoglutaric acid appears to be somewhat more sensitive to 0·10 *M* malonate. However the inhibition at this high concentration is probably non-specific, since at lower concentrations (0·01 *M*) no inhibitory effect is observed with either *l*(+)alanine or *l*(−)aspartic acid.

NaF, As_2O_3 and octyl alcohol have small inhibitory effects, while pyrophosphate, iodoacetate and bromoacetate have no appreciable effect on transamination in the presence of *l*(+)alanine and α-ketoglutaric acid.

The results with As_2O_3 and octyl alcohol are in agreement with those reported by Braunstein & Kritzmann [1937] and Kritzmann [1938]. The results with malonate do not agree with the statement made by Braunstein [1939].

Discussion

It has been pointed out in a previous section of this paper that α-ketoglutaric acid forms glutamic acid when added alone to pigeon breast muscle. Preliminary experiments show that the anaerobic glutamic acid synthesis from ammonium α-ketoglutarate is too small to account for the total glutamic acid formed. This must mean that there are NH_2-donators other than NH_3 preformed in the muscle. Among the amino-acids, *l*(+)alanine and *l*(−)aspartic acid are the only ones which could act in this manner. This suggests that these two amino-acids, like glutamic acid [Cohen, 1939], occur free in pigeon breast muscle.

The fact that the three amino-acids which alone are active in transamination are also the only ones which have specific dehydrogenases in muscle [Thunberg, 1920–1; Ahlgren, 1924; Needham, 1930; Euler *et al.* 1938; Dewan, 1938] suggests that transamination depends on the activity of a dehydrogenase during some phase of the reaction. On this basis the limitation of transamination in pigeon breast muscle to those amino-acids which possess dehydrogenases may be understood.

Summary

1. Transamination (Braunstein & Kritzmann) has been studied in pigeon breast muscle. Of 21 different α-amino-acids studied in the presence of α-ketoglutaric acid, *l*(−)aspartic acid and *l*(+)alanine are the most active in forming glutamic acid; *dl*-α-aminobutyric acid and *l*(+)valine are slightly active. None of the remaining amino-acids is appreciably active.

2. Of a series of ketonic acids tested, oxaloacetic and pyruvic acids show the greatest activity in causing the anaerobic disappearance of glutamic acid; α-ketobutyric and mesoxalic acids are slightly active; α-ketovaleric, α-ketohexoic, acetoacetic and laevulic acids are not appreciably active.

3. The data from these experiments can be explained by assuming the existence of three enzyme systems which are concerned with the following reactions:

Glutamic acid ⇌ α-ketoglutaric acid
Aspartic acid ⇌ oxaloacetic acid
Alanine ⇌ pyruvic acid

By combinations of these three systems the following reactions may be brought about:

(1) α-Ketoglutaric acid + alanine ⇌ glutamic acid + pyruvic acid.
(2) α-Ketoglutaric acid + aspartic acid ⇌ glutamic acid + oxaloacetic acid.
(3) Oxaloacetic acid + alanine ⇌ aspartic acid + pyruvic acid.

The occurrence of reaction (3) in pigeon breast muscle has not yet been conclusively demonstrated. The slight activity of certain homologues can be explained by a slight affinity for one of these enzyme systems.

4. Transamination in pigeon breast muscle is inhibited by high concentrations of cyanide. NaF, As_2O_3 and octyl alcohol have small inhibitory effects. Pyrophosphate, malonate, iodoacetate and bromoacetate have no appreciable effect.

5. Of a number of different amino compounds tested other than α-amino-acids, none has been found to be active in transamination.

6. Certain discrepancies between the results of Braunstein & Kritzmann [1938] and those reported here are discussed. The failure of these authors to use a suitable control in their experiments explains their statement that all α-amino-acids of the *l*-series are active in transamination in pigeon breast muscle.

I wish to express my thanks to Dr H. A. Krebs for his constant advice and help throughout this study.

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The effect upon mice of intraventricular injection of excitant and depressant amino acids

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A SHORT report has recently been published by Kita *et al.*[1] on the effects of intraventricular injections of some amino acids upon mice. The relative potencies of the substances which caused convulsions were similar to those of the same substances as excitants of spinal neurones of the cat[2, 3] and toad[4], and also of cortical neurones of the cat.[5] It was therefore of interest to extend the results of these workers to include other amino acids known to have strong excitatory and depressant actions on mammalian and amphibian neurones.

Small groups of 4-week-old mice of both sexes were used. The amino acids were dissolved in a mammalian Ringer solution and the range of concentration used for each substance is shown in Table 1. The solution to be tested (0·05 ml) was injected through the skull of unanaesthetised mice according to the method of Haley and McCormick.[6] Controls, in which the same quantity of a suspension of Indian Ink was similarly injected, were also carried out. Post-mortem sections performed

TABLE 1. (A) EXCITANT AMINO ACIDS

Group*	Amino Acids	No. of mice	Dose (μg/0·05 ml)	Effect
A	N-Methyl-D-aspartic acid	4	10	L
		9	2–5	+ + +
		4	1	+ (+)
B	N-Methyl-DL-aspartic acid	4	20–50	L
		6	5–10	+ + +
		5	2	+ +
D	N-Methyl-L-aspartic	4	50	+ +
		2	20	+
C	D-Homocysteic acid	8	20–50	+ + +
		2	10	+ +
C	DL-Homocysteic acid	8	25–50	+ + +
		4	10	+ (+)
E	L-Homocysteic acid	6	20–50	+
D	N-Methyl-DL-glutamic acid	4	50	+ + (+)
		2	20	+
E	D-Aspartic acid	5	50	+
E	L-Aspartic acid	5	50	+
F	L-Glutamic acid	2	150	
		6	50–100	Nil

(B) DEPRESSANT AMINO ACIDS

Group*	Amino Acids	No. of mice	Dose (μg/0·05 ml)	Effect
A[1]	3-Amino-1-propane-sulphonic acid	8	100	— — —
		4	50	— —
		4	20	—
B[1]	γ-Amino-n-butyric acid	6	150-250	— — —
		6	100	— —
		4	50	Nil
	Taurine	13	100-150	— —
		6	20–50	Nil

* The symbols A, B . . . F and A[1], B[1] are intended to convey relative ranking in decreasing order of potency. It is, however, not possible for accurate quantitative comparisons to be made from these data.

immediately after these animals had been killed with ether showed that in four out of twenty control mice some of the injected fluid was located outside the ventricles. After injection of mammalian Ringer or Indian Ink suspension, the animals tended to be subdued in their behaviour for periods of 15–60 sec, but occasionally slight twitching or incoordination of movement was noted, presumably due to trauma produced by the injection.

In large doses, amino acids known to be strong excitants of single neurones[3] produced clonic flexor convulsions followed by spasm (usually of extensor type) and death. In Table 1 the effects of these lethal doses are denoted by "L".

Lower dosages caused gross hyperactivity, with running, leaping and violent scratching at the head or body. Flexor convulsions were frequently observed as part of this response. During recovery the animal was usually subdued and its responses to being disturbed remained sluggish over long periods (10 min to over half an hour). This pattern of effects is denoted "+ + +". Still smaller doses cause moderate (+ + or +) excitation, usually with somewhat incoordinated attempts at grooming, or a continued circling around the cage at a walking pace. During recovery, which took place after 1–5 min, the animals were subdued.

Depressant amino acids produced loss of muscle tone and gross incoordination of movement. The animals were unable to right themselves if placed on their back or sides, and tended after a short period to fall asleep. Recovery from these effects, denoted "— — —", was very slow (greater than 30 min). Smaller doses of the depressants produced incoordination of movement and transient flaccidity, after which gradual recovery occurred. Lethal dosages of the depressant amino acids were not determined and although 200 μg of 3-amino-propane-sulphonic acid impaired respiration in two of the four mice tried, one of which subsequently died, this result is of doubtful significance.

The relative potencies of the amino acids in both series is very similar to that found on other preparations and by other methods of drug administration. N-Methyl-D-aspartic acid was the most potent substance, but the minimum lethal dose found for this compound was about ten times greater than that reported by Kita *et al.*[1] It is interesting also that taurine had a depressant effect not greatly lower than that of γ-amino-*n*-butyric acid. This result is in accordance with those of Curtis and Watkins[5] on cat cerebral cortical neurones, although Purpura *et al.*[7] have reported that taurine has no action on cat cortical cells.

There would appear to be little justification for the suggestion of Kita *et al.*,[1] that N-methylaspartic acid may have a transmitter function in the central nervous system. As yet, there is no report in the literature of the occurrence of N-methyl-aspartic acid in nervous tissue, nor indeed in any biological tissue. In an investigation of the free acidic amino acids of sheep brain, no N-methyl-aspartic acid was found.[8] Further, the L-form, which would be the more likely enantiomorph to occur naturally in mammals (including rodents), is not significantly stronger than either L-aspartic or L-glutamic acids, both of which occur abundantly in nervous tissue. L-Cysteic and L-homocysteic acids are also potent excitants[3] and could well be considered if an L-amino acid is found to have a role as an excitatory transmitter in the central nervous system. However, in discussing this question, Curtis and Watkins[2, 3] have been led to conclude that a transmitter function of amino acids is unlikely.

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THE EXCITATION AND DEPRESSION OF MAMMALIAN CORTICAL NEURONES BY AMINO ACIDS

BY

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Amino acids related to L-glutamic and γ-amino-*n*-butyric acid have been administered electrophoretically, and by pressure ejection, into the extraneuronal environment of single neurones in the pericruciate cortex of cats anaesthetized with allobarbitone or allobarbitone-urethane. Acidic amino acids related to glutamic acid, particularly *N*-methyl-D-aspartic acid, excited cortical neurones. Neutral amino acids related to γ-amino-*n*-butyric acid, particularly 3-amino-1-propanesulphonic acid, depressed cortical neurones. Some of the depressants blocked the antidromic invasion of Betz cells by pyramidal volleys. There are no essential differences between the sensitivities of cortical and spinal neurones towards locally administered amino acids. A transmitter function of such amino acids within the mammalian central nervous system is considered unlikely.

When ejected electrophoretically from glass micropipettes, many acidic amino acids related to glutamic acid excite neurones in the feline spinal cord (Curtis, Phillis & Watkins, 1960; Curtis & Watkins, 1960b, 1963), brain stem (Curtis & Koisumi, 1961), lateral geniculate nucleus (Curtis & Davis, 1962), thalamus (Curtis & Andersen, 1962; Andersen & Curtis, 1964a), retina (Noell, 1960), cerebral cortex (Krnjević & Phillis, 1963a), cerebellar cortex (Krnjević & Phillis, 1963a; Crawford Curtis, Voorhoeve & Wilson, 1963) and hippocampal cortex (Andersen, Crawford & Curtis, unpublished). In contrast, neutral amino acids related to γ-amino-*n*-butyric acid (GABA) are depressants (Curtis, Phillis & Watkins, 1959; Curtis & Watkins, 1960a, b, 1961; Noell, 1960; Krnjević & Phillis, 1963a). The similarity in the actions of these particular amino acids upon spinal interneurones, Renshaw cells, motoneurones, and geniculate and thalamic neurones has led to the use of the term "unspecific" when describing the excitant and depressant effects. Thus it has been considered that the actions of these two classes of amino acids are not closely related to the normal processes of synaptic transmission, although it is conceivable that some of these compounds may have a nonsynaptic regulatory function within the nervous system.

Recent experiments in which amino acids were ejected near single cortical neurones (Krnjević & Phillis, 1963a) have shown that in general the amino acid sensitivity of these cells is very similar to that of spinal interneurones. However, certain of the differences which have been reported raise doubts as to the non-

specificity of amino acid action. Whereas D-glutamic acid was approximately half to two-thirds as potent as the L-isomer as an excitant of spinal (Curtis & Watkins, 1963) and thalamic neurones (Andersen & Curtis, 1964a), cortical neurones are stated to be very much more sensitive to the L- than to the D-isomer (Krnjević & Phillis, 1961). Furthermore, *N*-methyl-D-aspartic acid, a powerful excitant of thalamic, geniculate and spinal neurones (Curtis & Watkins, 1963) has been reported to desensitize cortical neurones to subsequent administration of L-glutamic acid (Krnjević & Phillis, 1963a), a phenomenon which had not been observed elsewhere in the nervous system in the absence of a depression of nerve cell excitability following excessive depolarization (Curtis & Watkins, unpublished). Of the neutral amino acids, Krnjević & Phillis (1961, 1963a) reported that taurine and 3-amino-1-propanesulphonic acid were inactive when ejected near cortical neurones ; however, both compounds, particularly the latter, were depressants of spinal interneurones (Curtis & Watkins, 1960b, 1961).

It was felt that some of these conflicting findings may have arisen from differences in the technique of testing neuronal excitants and depressants, and in the interpretation of the observed results. Consequently the amino acid sensitivity of neurones in the pericruciate region of cats anaesthetized with allobarbitone has been reinvestigated, and the results compared with the sensitivity of spinal interneurones. It is concluded that there are no essential differences between the behaviour of these types of cells towards electrophoretically administered amino acids.

These results were presented briefly at the August (1963) meeting of the Australian Physiological Society.

METHODS

All experiments were performed upon cats anaesthetized with allobarbitone (Dial, Ciba Ltd.) or Dial Compound (Ciba Ltd.) which contained allobarbitone (100 mg/ml.) and urethane (400 mg/ml.). The usual dose was 35 to 50 mg/kg of allobarbitone injected intraperitoneally. The pyramids were exposed by partial removal of the basi-occiput, and the animal's head was then mounted in a rigid frame which could be tilted in two directions so that the pericruciate cortex could be placed approximately in a horizontal plane. The cat was suspended by a pelvic and mid-thoracic clamp with the abdomen and thorax held clear of the base of the animal frame. The preparation was maintained at $37 \pm 1°$ C by two heating pads, one of which was automatically controlled.

The pericruciate cortex was exposed by removal of the overlying bone and the posterior wall of the frontal sinus. The usual exposure was confined to one side of the skull, and extended for 1 to 1.2 cm laterally from the mid-line and 0.7 to 0.8 cm caudal to the coronal suture. After opening the dura the cortex was covered with several layers of polyethylene or cellophane sheeting, apart from a small opening left at the site of recording. During microscopic examination of the cortex the pia mater was gently removed with fine forceps from a suitable avascular area, and throughout the experiment mammalian Ringer solution (at 38° C and saturated with 95% oxygen and 5% carbon dioxide) irrigated the exposed tissue. In order to stabilize the cortex a small transparent pressure plate was applied to the site of recording without affecting the blood flow in underlying vessels (see Phillips, 1956).

Experiments were also carried out upon spinal interneurones in cats anaesthetized with pentobarbitone sodium (see Curtis & Watkins, 1960b).

The microelectrophoretic method of controlling the efflux of substances from the four drug-containing barrels of five-barrel micropipettes has been described in previous publications (Curtis & Eccles, 1958 ; Curtis & Watkins, 1960b, 1963). The overall tip diameter of the

micropipettes was 4 to 8 μ, and filling was carried out by centrifugation. Thus amino acids could be tested within a short time of preparation of the solution. This was particularly necessary when alkali (sodium hydroxide) had been added to produce a *p*H in excess of 8.5, since the *p*H fell during storage of pipettes which contained such solutions. This was presumably due to absorption of carbon dioxide from the atmosphere and to interaction between the alkali and Pyrex glass. When storage was necessary, filled micropipettes were placed in an atmosphere of nitrogen (3 to 4° C).

The concentrations of the amino acid solutions which were used are given in the Tables of this paper. When it was necessary to alter the *p*H in order to produce adequate ionization of these compounds (see Curtis & Watkins, 1960b), hydrogen ion concentrations were measured by means of a glass electrode system.

Extracellular spike responses were recorded by means of the central barrel of the five-barrel micropipette, which contained 4 to 5 M-sodium chloride solution. This was connected to a cathode follower by a small silver-silver chloride junction, and after suitable amplification the spikes were displayed on two double-beam oscilloscopes. Photographic records could be made from one of these oscilloscopes, whilst the other was used to monitor a firing frequency indicating system (Andersen & Curtis, 1964a). Essentially this consisted of a pulse generator which was triggered by the amplified spikes. The triggering level could be altered manually to discriminate between large and smaller spikes; the output pulses were observed simultaneously with the spikes and were counted by a rate meter. Thus the firing frequency of a cell was displayed continuously on a paper recorder. The use of this system ensured that for each test of an amino acid the ejecting current should be maintained until a maximum excitant or depressant effect was observed. Because of the comparatively slow response time of this recording system the plots of firing frequency (see Figs. 1 and 2) did not permit an accurate assessment of the latencies of onset and offset of amino acid-induced firing. Filmed records were necessary for this purpose.

The electrophoretic currents were measured with an accuracy of ±0.5 nA, and the terms "cationic" and "anionic" are used in this paper to describe currents which eject cations and anions respectively. One barrel of most micropipettes contained a 0.2 M solution of DL-homocysteic acid (*p*H 8–8.5). When ejected as an anion this amino acid is a powerful excitant of neurones (Curtis & Watkins, 1963) and enables ready detection of otherwise quiescent cells. Care had to be taken however that excessive amounts of this amino acid were not ejected and, in general, anionic currents of only 5 to 10 nA were allowed to flow intermittently when cells were being looked for within the cortex.

In several experiments hydrostatic pressure was used to eject amino acid solutions from the individual barrels of micropipettes which had comparatively large orifices (2.5–3.5 μ internal diameter). Silver wires within the sealed system allowed an appropriate retaining voltage to be applied to each barrel. This method of ejection was found to be unreliable, presumably because of plugging of the pipette orifices by tissue components. In some cases pressures of less than 100 mg Hg were adequate to eject sufficient DL-homocysteic acid to excite neurones; in other cases pressures as high as 350 mm Hg were ineffective, although the same cells could be readily excited when the amino acid was ejected electrophoretically as an anion from the same pipette. Observations upon the effect of a test substance ejected by pressure have been included only when either DL-homocysteic or L-glutamic acid excited neurones when ejected with pressures of less than 200 mm Hg from other barrels of the same micropipette assembly. This requirement does not however completely exclude the possibility that substances in adjacent barrels were not ejected by even higher pressures.

The amino acids used were all of analytical purity, and were either provided by Dr. J. C. Watkins (Watkins, 1962) or purchased commercially (California Corporation for Biochemical Research; British Drug Houses, Ltd.). One sample of D(−)-glutamic acid was supplied by Dr K. Krnjević, and when tested upon spinal interneurones was indistinguishable from the sample used in the present investigation.

In view of the difference between the present findings upon the potencies of D(−)- and L(+)-glutamic acid, and those of Krnjević & Phillis (1963a), the optical rotation of the samples

of these substances was kindly determined by Dr A. M. Sargeson upon 2% solutions in 5 M-hydrochloric acid, with the results

$$[\alpha]_D^{20} \text{ L}(+)\text{-glutamic acid} = +29.5°$$
$$[\alpha]_D^{20} \text{ D}(-)\text{-glutamic acid} = -30°$$

RESULTS

This investigation was confined to neurones in the pre- and postcruciate region, many of which were firing spontaneously, either in a random fashion or as spindles (Dempsey & Morison, 1943; see also Verzeano & Calma, 1954). The majority of the cells were identified as Betz cells by the responses evoked by single or tetanic stimulation of the pyramids (Phillips, 1956, 1959, 1961); these cells were found at depths of between 0.8 and 1.3 mm below the cortical surface. The relative sensitivities of pyramidal cells and of the more superficially located neurones towards excitant and depressant amino acids did not appear to be different. Thus the general term "cortical neurone" will be used throughout this report to indicate a cell which was located within 2 mm of the cortical surface, and from which spike responses could be produced by the local ejection of an excitant amino acid. The distribution of these neurones was similar to that found in other studies (see Amassian, 1961). It was usually possible to move the micropipette until the extracellular spike responses of one neurone were of the order of 250 to 500 μV (negative-positive), and so could be clearly distinguished from the smaller spikes of more distant cells. Under such circumstances the effects of the flow of current associated with electrophoretic ejection were minimal (see Curtis & Koizumi, 1961) and could readily be distinguished from amino acid actions by testing the effect of current flow through the micropipette containing sodium chloride. In some instances the spike response of a particular neurone could be identified over a distance of 50 to 100 μ, and under these conditions there was little difference between the comparative potencies of the amino acids determined at several different levels of spike size.

One difficulty common to many neurones was the change in spike shape which was observed during activation by an amino acid. Such changes, which are completely reversible, were originally observed when excitant amino acids were ejected near spinal interneurones (Curtis *et al.*, 1960), particularly with L-glutamic acid. It was proposed that the observed "depolarization block," in which the initially negative-positive spike potentials were converted either to predominantly positive spikes, or merely decreased in size before failing entirely, was associated with the change in membrane conductance and the depolarization produced by the excitant (Curtis *et al.*, 1960). In general, these alterations in spike shape of cortical neurones occurred at lower firing frequencies (20 to 60 spikes/sec) than has been observed with spinal interneurones, thalamic and geniculate neurones. As cortical neurones could be fired synaptically (or antidromically in the case of Betz cells) at higher rates than this, it was considered that the comparative ease with which depolarizing substances inactivated the spike generating mechanism might be related to disturbances in cerebral cortical circulation consequent upon the surgical procedures. Corpuscular clumping was usually evident in the smaller cortical veins, and it is possible that all the cortical neurones studied were slightly depolarized as a result

of tissue hypoxia. Deterioration in the cortical circulation and heightened sensitivity to excitant amino acids were most evident towards the end of experiments which lasted 12 to 18 hr.

Action of acidic amino acids

The difficulties which are associated with the assessment of the relative potencies of electrophoretically administered excitant amino acids have been discussed by Curtis & Watkins (1963). The most satisfactory technique is to obtain records from a large number of neurones, and to compare the maximal effects of different doses of amino acids against a reference compound (in this investigation L-glutamic acid). Reasonably consistent results were obtained for any one compound when tested upon four or five cells in each of two or three preparations. Fresh solutions, in various combinations and in fresh micropipettes, were used for each preparation. The relative potencies of amino acids were determined from the ratio of ejecting currents which produced equal firing frequencies, and therefore express the ratios of concentrations required for equal effects, a method generally accepted when the activities of agents interacting with a common receptor are compared. This method is preferable to a comparison between the effects produced by equal extraneuronal concentrations (see Krnjević & Phillis, 1963a, p. 282) as, in the latter instance, differences in response, and therefore in potency ratios, depend upon the portions of the dose/response curves which are being studied.

The range and mean potencies are given in Table 1, together with the number of separate tests. Furthermore, to facilitate comparison, the potency of these amino acids as excitants of spinal interneurones is also included (from Table 1, Curtis & Watkins, 1963). The time parameters of the excitation of cortical neurones by the various amino acids were very similar to those found for spinal neurones. The excitant action of all acidic amino acids was readily reversible, and recovery occurred even after long periods of "depolarization block" had been produced by the administration of excessive amounts of amino acid.

N-Methyl-D-aspartic acid was the most potent excitant amino acid of those tested, and had the longest duration of action after the electrophoretic current was termi-

TABLE 1

EXCITANT AMINO ACID POTENCIES

* Data from Curtis & Watkins (1963). † With solutions at *p*H 3·1, delayed excitation was observed (see text). ‡ Data from Curtis & Watkins (1960b). Sat. = saturated.

Amino acid	Concentration within micropipette (M)	*p*H of solution	Potency upon cortical neurones: Mean	Range	Tests	Potency upon spinal interneurones
N-Methyl-D-aspartic acid	0·1	8·3–8·6	6·5	2–16	24	5–12*
DL-Homocysteic acid	0·2	8·0–8·5	4	2·5–6	23	2–6*
L-Glutamic acid	2	8·0–8·2	1·0	Standard		1*
L-Aspartic acid	2	8·2–8·5	1	1–1·2	5	1*
D-Glutamic acid	2	8·0–8·3	0·7	0·5–0·8	16	0·5–0·7*
D-Aspartic acid	2	8·2–8·4	0·8	0·7–1·0	5	0·5–0·7
L-Asparagine	Sat.	3·1–4·5	Inert†		17	Inert ‡
D-Asparagine	Sat.	3 and 5	Inert		9	

nated (Curtis & Watkins, 1963). In some experiments diffusion of this substance from a 0.1 M solution was sufficient to fire neurones. When ejected with currents of 3 to 10 nA this amino acid produced a graually increasing rate of firing over 15 to 30 sec (Fig. 1), and the rate declined over a period of 2 to 10 sec after the ejection had ceased. When larger amounts of *N*-methyl-D-aspartic acid were used (currents of 12 to 30 nA) the cell increased its rate of firing more rapidly until a "depolarization block" was observed, the spike then being indistinguishable from the background noise. Simultaneously the spontaneous and antidromic spikes of the neurone were also blocked. Several seconds after the current which ejected the amino acid had been terminated, spikes often reappeared transiently for about 1 sec, presumably as portions of membrane, which had been rendered incapable of generating spikes by the amino acid, recovered and the level of depolarization remained above the threshold for spike generation.

The high potency of this compound probably explains the finding of Krnjević & Phillis (1963a) that *N*-methyl-D- and *N*-methyl-DL-aspartic acid caused a prolonged

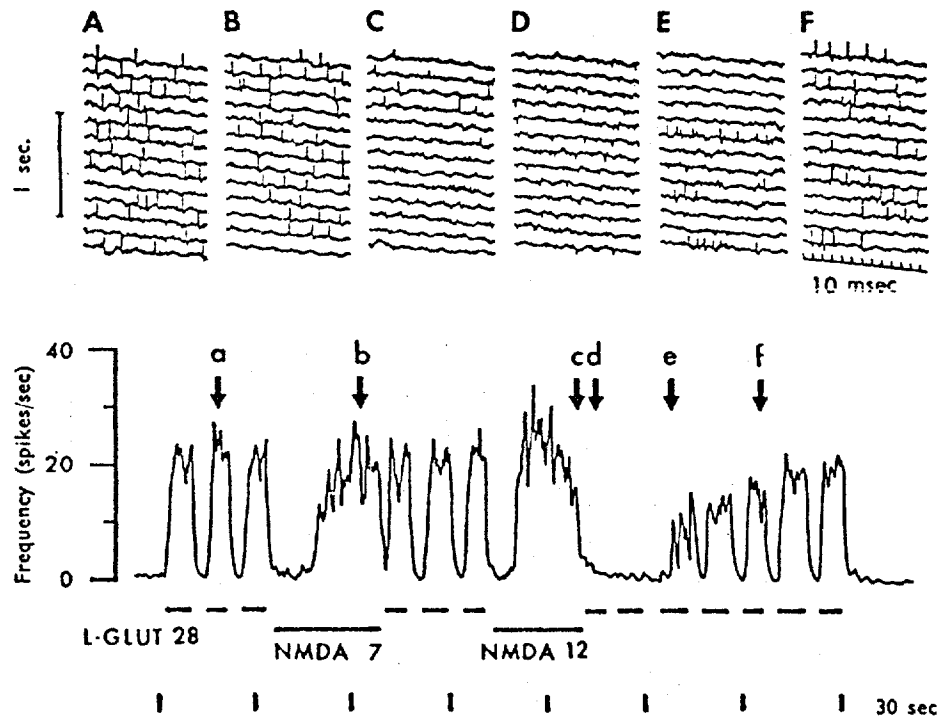

Fig. 1. Effect of *N*-methyl-D-aspartic acid (NMDA, retaining current 4nA) upon the firing of a neurone located in the postcruciate area by L-glutamic acid (L-GLUT, 28nA). In the lower half of the figure successive ejections of each of these compounds are indicated by horizontal bars (upper bars, L-glutamate ; lower bars NMDA), with the frequency of firing (spikes/sec) plotted against time. Sections (A-F) of the concurrent filmed records of the extracellular spike potential (negative upwards) are placed above this frequency record, each being representative of the spikes at times indicated by the arrows and the letters a-f (see text). Time, 10 msec for filmed records ; 1 sec. for rate of vertical film movement ; 30 sec for paper recorder.

reduction in the sensitivity of cortical neurones to L-glutamic acid. Many tests of this interaction were carried out, but the finding could be repeated only if sufficient *N*-methyl-D-aspartic acid had been administered to produce a block of neuronal spikes. The results from one such experiment are shown in Fig. 1. Initially three successive ejections of L-glutamic acid (28 nA) gave reasonably consistent firing rates of about 20 spikes/sec. Following this, the ejection of *N*-methyl-D-aspartic acid (7 nA) produced a firing frequency of 15 to 20 spikes/sec, with no change in the shape of the spike potential (compare A and B), and the firing produced by the following dose of L-glutamic acid (within 3 sec of the end of the ejection of *N*-methyl-D-aspartic acid) was unaffected. However, when a current of 12 nA was used to eject *N*-methyl-D-aspartic acid a maximum frequency of approximately 30 spikes/sec was attained, the spike was altered in shape, and the cellular responses were eventually blocked (C). Subsequently, two doses of L-glutamic acid produced only small positive spikes (D) but, 35 sec after the cessation of the *N*-methyl-D-aspartic acid ejection, L-glutamic acid again fired the cells with negative-positive spikes. Initially these spikes were small (compare E with A) and full recovery of spike size (F) was observed after 1 min.

Although in most cases the onset of complete block by *N*-methyl-D-aspartic acid was comparatively slow, with several cells and particularly with electrophoretic currents greater than 20 nA the onset was abrupt and the spikes suddenly vanished with little or no preliminary alteration in shape. Following the administration of sufficient *N*-methyl-D-aspartic acid to suppress cellular responses the usual sequence of recovery was return of antidromic invasion, restoration of spike amplitude and shape, and finally re-establishment of the sensitivity to L-glutamic acid.

Similar observations have been made upon the apparent reduction in sensitivity of neurones to L-glutamic acid by prior administration of DL-homocysteic acid and *N*-*n*-propyl-D-aspartic acid, and also to test doses of DL-homocysteic acid by *N*-methyl-D-aspartic and L-glutamic acids. Furthermore, experiments upon spinal interneurones have failed to demonstrate any depression of the firing induced by either L-glutamic or DL-homocysteic acid by a preceding dose of *N*-methyl-D-aspartic acid. In fact, as occasionally observed with cortical cells, the effectiveness of L-glutamic or DL-homocysteic acid was actually increased when these were ejected shortly after the administration of *N*-methyl-D-aspartic acid was terminated. When sufficient of the latter was ejected to produce a depolarization block of spinal interneurones the effects of subsequent doses of other excitant amino acids were reduced, as found with cortical neurones.

Several cortical cells were tested with graded doses of *N*-methyl-D-aspartic acid and it was found that there need be a difference of only 10 to 20% between currents (concentrations) which produced neither "depolarization block" nor a reduction in the sensitivity and those which did produce block and reduction in the sensitivity. It was thus concluded that the diminution in amino acid sensitivity was primarily a consequence of alterations in membrane conductance and potential, rather than of receptor desensitization due to a prolonged occupation of amino acid receptors by the *N*-alkyl excitant amino acid. This phenomenon is possibly related to the frequently observed reduction in the spontaneous discharge rate which follows

induction by amino acids of high frequency firing of spinal, thalamic and cortical neurones.

Action of depressant amino acids

Neutral amino acids related to GABA reduce the sensitivity of neurones in the cortex to depolarization by acidic amino acids, and to excitation by other means. The potency of these substances can be most readily assessed in terms of the

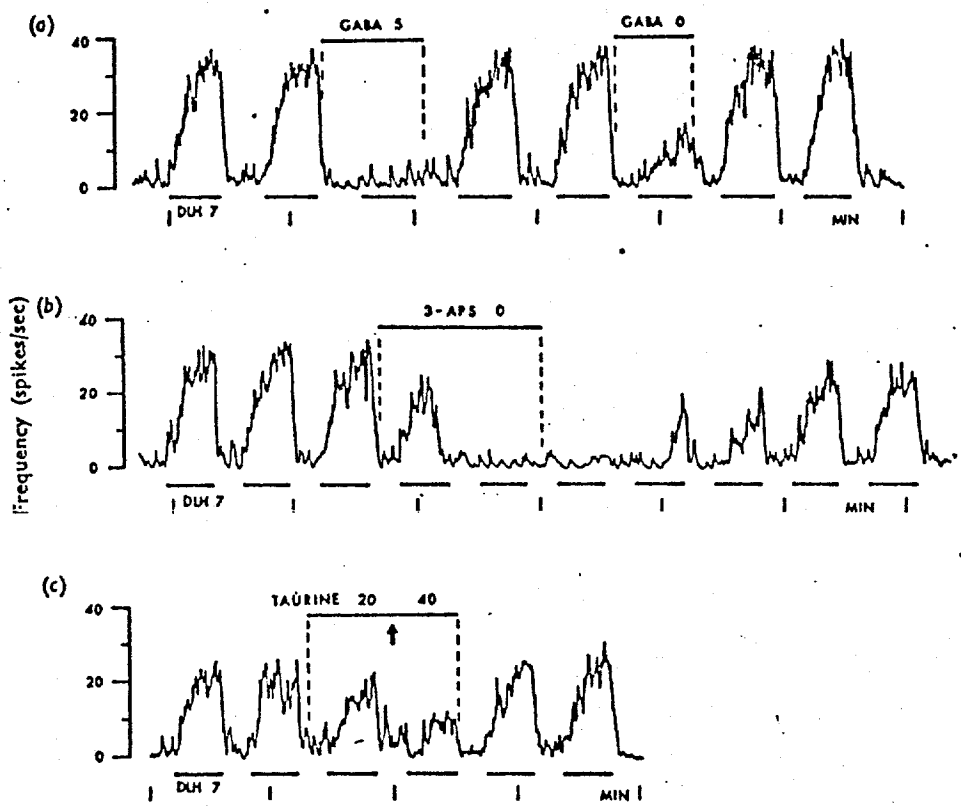

Fig. 2. (*a*) The effect of γ-amino-*n*-butyric acid (GABA) upon the frequency of the firing of a cortical neurone induced by repeated doses of DL-homocysteic acid (DLH, currents of 7nA, lower signal trace). GABA was retained by an anionic current of 20 nA and was ejected during the first interval (upper bar) by a cationic current of 5 nA. During the second interval the retaining current was terminated, allowing free diffusion of this substance from the micropipette which contained a molar solution (*p*H 2·9). Note the short duration of depressant action.
(*b*) Firing frequency of the same cortical unit as in Fig. 2*a*, showing the effect of the removal of the retaining current (anionic, 4nA) from a barrel which contained 3-amino-1-propane-sulphonic acid (3APS, 0·1M, *p*H 9·4). The lower horizontal bars again indicate repeated ejections of DLH, using currents of 7nA. The depressant action of the neutral sulphonic acid lasted for over 2 min after the retaining current was reapplied.
(*c*) The effect of taurine upon the DLH-induced firing (lower bars, 7nA) of the same cortical cell as (*a*) and (*b*). During the period of the upper horizontal bar taurine was ejected as an anion initially with a current of 20 nA which was doubled at the arrow. Time in minutes below each frequency plot.

electrophoretic current needed to produce a specified reduction in the firing rate of a cell excited by an acidic amino acid. In this series of experiments DL-homocysteic acid was used as the control excitant substance, and was ejected either continuously or at fixed intervals and for constant times (see Fig. 2, *a* to *c*). Table 2 lists the neutral amino acids used in this study, together with the concentrations and *p*Hs of the solutions. Potencies are expressed relative to that of GABA (– – –), the number of symbols indicating greater (– – – –), less (– –) and very weak (–) activity.

TABLE 2

DEPRESSANT AMINO ACID POTENCIES

The potency of cortical neurones is relative to γ-amino-*n*-butyric acid (– – –); – – – –, greater; – –, less; – very weak. *Late excitation with low *p*H solutions, see text.

Amino acid	Concentration within micropipette (M)	*p*H of solution	Potency upon cortical neurones
γ-Amino-*n*-butyric acid	1, 2	2·9–7·2	– – –
γ-Amino-β-hydroxybutyric acid	2	3·1	– –
γ-Hydroxybutyric acid	1	7	0
3-Amino-1-propanesulphonic acid	0·2	9–10·6	– – – –
Taurine	0·8	8·5–10	–
Guanidino-acetic acid	0·025	3–3·1	–
β-Guanidino-propionic acid	1	3	– –
ε-Aminocaproic acid	0·5	3·1–6·8	– *
ω-Aminocaprylic acid	0·5–1	3·0–7·7	0 *

γ-Amino-n-butyric acid (GABA). The depression of excitability, and of spontaneous firing, produced by this compound had a short latency of both onset and offset, and could be demonstrated by diffusion of GABA from a 2 M solution (Fig. 2, A) or when the amino acid was ejected electrophoretically from solutions within the range of *p*H 2.9 to 7.2 ($pK_1 = 4.23$). It is thus extremely unlikely that there is any difference between the action of GABA cations and zwitterions, as has been proposed by Kuno (1960, 1961), Muneoka (1961) and Kuno & Muneoka (1961, 1962); (see also Curtis *et al.*, 1959; Curtis & Watkins, 1960a, b; Krnjević & Phillis, 1963a).

γ-Amino-β-hydroxybutyric acid was approximately half as potent as GABA as a depressant of the firing of cortical neurones induced by DL-homocysteic acid, whereas γ-aminobutyrylcholine was a very weak depressant. The available sample of this latter compound contained GABA, and it is possible that this accounted for the depressant action which was observed.

γ-Hydroxybutyric acid. Although not an amino acid, this compound was of interest because of recent reports which indicate that systemic administration of sodium γ-hydroxybutyrate results in the depression of activity in animals and humans (Laborit, Jouany, Gerard & Fabiani, 1960; Laborit, Kind & Regil, 1961; Drakontides, Schneider & Funderburk, 1962) and in a reduction of spinal reflexes (Basil, Blair & Holmes, 1964). This compound was ejected electrophoretically as an anion, and in several tests (currents as high as 100 nA) failed to modify either

the spontaneous activity of cortical neurones, or the spikes evoked by an excitant amino acid. (Similar results were observed when this acid was administered near spinal interneurones.)

3-Amino-1-propanesulphonic acid. This compound was sufficiently potent to block the firing of many cortical neurones induced by DL-homocysteic acid merely by diffusion from solutions of 0.1 M. Indeed, considerable difficulty was experienced in controlling the diffusional efflux of this amino acid from Pyrex micropipettes, due no doubt to the high electro-osmotic mobility of this substance in aqueous solution. Under suitable conditions (0.1 to 0.2 M solution, *p*H 9 to 10, pipette orifice less than 2 μ internal diameter) this amino acid could be ejected either as an anion or as a cation, although the diffusional efflux was controlled to a large extent by a small anionic current. When ejected as a cation, 3-amino-1-propanesulphonic acid proved to be a powerful depressant of all cortical neurones upon which it was tried, and was more potent than GABA. This is illustrated in Fig. 2, B where the diffusion of 3-amino-1-propanesulphonic acid from an 0.1 M solution depressed the firing of a cortical neurone induced by DL-homocysteic acid to a greater extent than did GABA which diffused from a pipette of approximately the same internal diameter containing a 2 M solution. Furthermore, the depressant action of 3-amino-1-propanesulphonic acid was of longer duration than that of GABA. Similar results were obtained using spinal interneurones. In earlier experiments upon spinal neurones the anomalous behaviour of 3-amino-1-propanesulphonic acid under these conditions led to the use of solutions of *p*H 2.8 for ejecting neutral amino acids near interneurones (Curtis & Watkins, 1961) and in such circumstances it was also greater and more prolonged than that of GABA.

As found previously in spinal cord (Curtis & Watkins, unpublished), if micropipettes had been stored for more than a few hours, even in nitrogen and with small sealed caps upon the open upper ends of the barrels, or if concentrated solutions of *p*H lower than 8.5 were contained within pipettes which exceeded approximately 2 μ in diameter, it proved impossible to control the diffusional efflux of 3-amino-1-propanesulphonic acid. In most experiments, despite the intermittent ejection of DL-homocysteic acid from an adjacent barrel, very few neurones were located when these micropipettes were passed through cerebral or spinal tissue.

Taurine. This compound was readily administered as an anion from solutions of *p*H above 8.5. When ejected with currents of 20 to 80 nA, taurine caused depression or abolition of the response to DL-homocysteic acid of all cells which were tested (see Fig. 2, C), and although weaker than either GABA or 3-amino-1-propanesulphonic acid, taurine had an action upon cortical neurones similar to that already reported for neurones in the spinal cord (Curtis *et al.*, 1959; Curtis & Watkins, 1960a, b). The action of taurine persisted for 10 to 15 sec after the termination of the ejection, a duration which was intermediate between those of GABA and 3-amino-1-propanesulphonic acid.

Guanidino-acetic acid (glycocyamine) and β-guanidino-propionic acid. These compounds were not tested as depressants of the synaptic responses of cortical neurones, but guanidino-propionic acid was slightly more active than glcocyamine as a depressant of the firing of these cells induced by DL-homocysteic acid.

It might be expected that, because of the efficacy of synaptic excitation, the post-synaptic action of a weak depressant amino acid would not be sufficient to affect such responses, although this substance might interfere with the access of an excitant amino acid to membrane receptors (see Curtis & Watkins, 1960b) and so reduce its effectiveness. A re-examination of the action of guanidino-acetic acid upon spinal interneurones has confirmed the lack of action of this compound upon the synaptic responses of these cells, but has demonstrated a reduction in the frequency of firing produced by concurrent administration of an excitant amino acid. On the other hand, β-guanidino-propionic acid was found to be a more potent depressant since it diminished both the synaptic and amino acid evoked firing of spinal interneurones.

Other compounds related to these amino acids

ε-Aminocaproic acid and ω-amino-caprylic acid. These were found, in agreement with Krnjević & Phillis (1963a), to have "somewhat ambiguous" actions when ejected electrophoretically. Solutions of *p*H 3 were necessary to increase the degree of ionization of these compounds and so permit sufficiently large ejecting currents to be passed. Under these conditions ε-aminocaproic acid had a slight depressant action on cortical neurones but, after a latency of 15 to 90 sec, paroxysmal high frequency firing was produced by both compounds and continued for up to 15 sec after termination of the current flow. This excitatory phenomenon could not be reproduced by ejecting neutral solutions (*p*H 6.7 to 7.7) of either of these compounds by means of pressure (up to 300 mm Hg for 3 min), although in most experiments when ε-aminocaproic acid was so administered a depression of the firing of cortical neurones induced by DL-homocysteic acid was observed.

Thus the delayed excitation appears to be related to the effects of local *p*H changes produced when the cationic form of the ejected amino acid releases its extra proton on reaching the medium around the micropipette tip (see Curtis & Watkins, 1960b, p. 119). GABA and closely related short chain ω-amino acids are presumably sufficiently potent depressants to mask this delayed effect.

D- *and* L-*Asparagine.* These were inactive when administered electrophoretically (currents of 30 nA) or by pressure, unless the *p*H of the solutions was below 4. In such experiments a delayed excitation was observed with subsequent depression of the sensitivity of the cell to DL-homocysteic acid which lasted for 3 to 4 min. A similar delayed firing was observed with spinal interneurones (Curtis & Watkins, 1960b) but, as with the longer chain ω-amino acids, was considered to result from local *p*H changes.

Antidromic invasion of Betz cells

If the mode of action of GABA, and related depressants, upon cortical neurones is the same as that which has been found for spinal motoneurones, namely an increase in membrane conductance which reduces the effectiveness of depolarizing currents (Curtis *et al.*, 1959), it would be expected that the antidromic invasion of Betz cells would be depressed by these substances.

Even in the absence of spontaneous firing the safety factor for the antidromic invasion of the body and dendrites of Betz cells is extremely high (see Phillips, 1959) and it was anticipated that some difficulty would occur when attempting to test the effect of GABA upon this form of firing. This proved to be the case, and only in rare instances, using currents of the order of 100 to 200 nA, could sufficient GABA be ejected electrophoretically to block antidromic firing. In several instances GABA converted a soma-dendritic (A or SD) spike to a smaller initial segment (B) spike, but more often there was complete suppression of the soma-dendritic spike to leave a small axonal or M spike. The depression of these antidromic responses was readily measured by the "firing index," which expressed the number of soma-dendritic spikes as a percentage of ten supramaximal axonal stimuli.

3-Amino-1-propanesulphonic acid was more effective than GABA as a depressant of cortical neurones, and the action of this amino acid upon the antidromic spikes of one cell are illustrated in Fig. 3. Throughout the series the stimulus applied

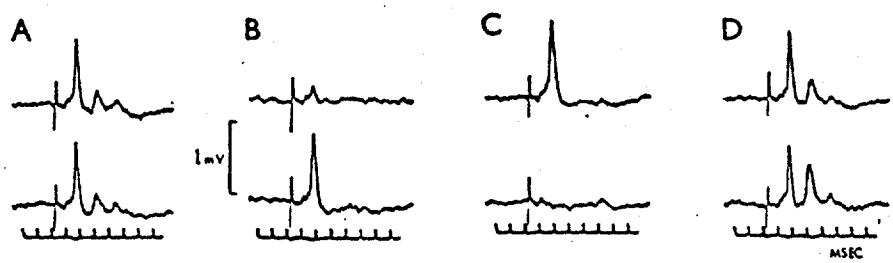

Fig. 3. The effect of 3-amino-1-propanesulphonic acid (3APS) upon the antidromic invasion of a Betz cell in the postcruciate area. Each column shows two successive responses to supramaximal pyramidal stimuli delivered at a rate of 1 shock/sec. Negativity is recorded upwards. A, control ; firing index 100%. B, approximately 10 sec after a current of 80nA began to eject 3APS ; firing 80%. C, 10 sec after this current was increased to 150nA ; firing index 50%. D, 10 sec after the electrophoretic ejection of 3APS was terminated : firing index 100%. Voltage scale, 1 mV, time scale in msec.

to the pyramids was supramaximal for the axon of this Betz cell, and the antidromic spike potential (latency 1.27 msec) was followed by a complex field potential produced by the antidromic and synaptic excitation of other nearby neurones. The firing index of this cell was initially 100% (A), but 10 sec after a cationic current of 80 nA began to eject 3-amino-1-propanesulphonic acid the index was 80% (B). When the electrophoretic current was increased to 150 nA the firing index fell to 50% (C), and full recovery to the original value was observed after the termination of the current (D). The depression, and recovery, of the field potentials generated by other nearby neurones is also apparent in this series.

DISCUSSION

The importance of the present results, and those of Krnjević & Phillis (1963a), lies in the evidence which has been obtained regarding the sensitivity of unidentified pericruciate neurones and Betz cells towards electrophoretically administered amino acids. Although this method of drug administration has some disadvantages (see

Curtis, 1964), particularly in respect to the concentrations which are attained, it is clear that when amino acids are tested by ejection from multibarrel micropipettes there are no essential differences between the amino acid sensitivity of cortical neurones and that of spinal interneurones (Curtis *et al.*, 1959, 1960; Curtis & Watkins, 1960a, b, 1961, 1963). A limited study of spinal Renshaw cells, lateral geniculate neurones (Curtis & Watkins, 1963) and neurones of the ventrobasal thalamus (Andersen & Curtis, 1964a) has also failed to reveal serious discrepancies in this pattern. This type of amino acid sensitivity is in marked contrast to the behaviour of neurones in the various regions towards acetylcholine, cholinomimetics and cholinergic blocking agents (Curtis, Phillis & Watkins, 1961; Curtis & Koizumi, 1961; Krnjević & Phillis, 1963b; Curtis, Ryall & Watkins, 1964; Crawford *et al.*, 1963) or towards indole and phenylethylamine derivatives (Curtis *et al.*, 1961; Curtis & Davis, 1962; Krnjević & Phillis, 1963c; Andersen & Curtis, 1964b).

There are, however, differences between the results reported here, and those of Krnjević & Phillis (1963a). Some of these discrepancies are no doubt due to differences in technique, and it is thus relevant that the method used to assess amino acid actions and potencies in the present investigation were identical with those used in recent studies in this laboratory upon spinal and thalamic neurones (see particularly Curtis & Watkins, 1963). In particular, the use of a rate meter and paper recorder permitted a close analysis to be made of firing frequency and a ready assessment of the maximum action for a particular current used to eject an amino acid. These refinements in technique led to a recent revision (Curtis & Watkins, 1963) of an earlier study of the action of amino acids upon spinal interneurones (Curtis & Watkins, 1960b).

The present findings on the relative potencies of the D- and L-isomers of aspartic and glutamic acid are similar to those of Krnjević & Phillis (1961, 1963a), but, although high accuracy cannot be claimed for such estimations, we do not agree with these investigators that D-glutamic acid was always "much less effective" than L-glutamic acid since the potency difference is of the order of only 0.5 to 0.8. The difference in the evaluation of these two amino acids has presumably arisen from the different methods used to assess excitant amino acid potency, as outlined in Results. The report that *N*-methyl-D-aspartic acid desensitized cortical neurones to subsequent doses of L-glutamic acid (Krnjević & Phillis, 1963a) has not been substantiated, since any such interaction between different amino acids upon cortical and spinal neurones appears to be associated with the presence of a "depolarization block" due to excessive concentrations of a powerful depolarizing agent, rather than to a specific interference of access to receptor sites. However, the slow recovery of amino acid sensitivity after the production of such a "block" (see Fig. 1) warrants additional investigation.

The failure of Krnjević & Phillis (1961, 1963a) to demonstrate a depressant action of electrophoretically administered taurine and of 3-amino-1-propane-sulphonic acid is rather surprising since, like GABA, each of these sulphonic amino acids readily depresses cortical responses when applied topically in solution to the exposed cortical surface (Curtis & Watkins, 1961; but see also Purpura, Girado, Smith, Callan & Grunfest, 1959). Furthermore, when injected intraventricularly

into mice, all three amino acids result in a diminution of spontaneous activity, a loss of righting reflexes and eventually a state resembling sleep (Crawford, 1963). Again the relative potencies of the amino acids were similar to those observed in electrophoretic studies upon cortical neurones and spinal interneurones.

In view of the inability to reproduce the delayed excitation of ω-aminocaprylic acid and asparagine by pressure ejection from neutral solutions, it cannot be stated with any degree of certainty that these amino acids excite neurones, or that the results obtained electrophoretically agree with those of Hyashi (1956), Purpura *et al.* (1959) and van Harreveld (1959) who administered amino acid solutions topically to the exposed cortex. On the other hand, if indeed these amino acids do excite central neurones, then the effects upon cortical and spinal neurones are practically identical, since the report by Curtis & Watkins (1960b) that ω-aminocaprylic acid and L-asparagine were inactive upon spinal interneurones was made on the basis that the delayed excitation was not directly associated with these amino acid molecules.

The finding that γ-amino-*n*-butyric acid, and particularly 3-amino-1-propane-sulphonic acid, block the antidromic invasion of Betz cells does not lend support to the postulate of Purpura, Girado & Grundfest (1957) and Purpura *et al.* (1959) (see also Grunfest 1958, 1960; Purpura, 1959, 1960) that GABA, and presumably also closely related amino acids, specifically block superficial excitatory axodendritic synapses. It is clear that, as with spinal motoneurones (Curtis *et al.*, 1959), GABA produces an alteration in the membrane conductance of cortical neurones (see also Bindman, Lippold & Redfearn, 1962). The difficulty which was experienced in blocking the antidromic invasion of Betz cells by pyramidal volleys was presumably associated with the high safety factor for this type of excitation, and with the limited proportion of the membrane of any one cell which was affected by the locally administered amino acid.

The failure of γ-hydroxybutyric acid to affect central neurones, both cortical and spinal, suggests that the alterations in behaviour, in the electroencephalogram and in spinal reflexes which are produced by this compound are unlikely to be due to a direct action upon central cells (Laborit *et al.*, 1960, 1961; Drakontides *et al.*, 1962; Basil *et al.*, 1964). Indeed the latent period between intravenous administration and the depression produced by γ-hydroxybutyrate (Drakontides *et al.*, 1962; Basil *et al.*, 1964) suggests that it has an indirect action, possibly as a result of interfering with the metabolism of GABA. It is pertinent to point out that a related substance, γ-butyrolactone, which also has central depressant effects (Giarman & Schmidt, 1963) does not increase the brain level of GABA. However total brain levels of GABA may not necessarily be related to the state of excitability of central neurones (see also Kamrin & Kamrin, 1961; Medina, 1963) and it may be more relevant to these studies to ascertain differences in the distribution of amino acids between intra- and extra-cellular compartments (see Curtis & Watkins, 1960a).

In conclusion, it may be stated that the actions of individual amino acids upon neurones within the spinal cord and cerebral cortex (and thalamus—Andersen & Curtis, 1964a) are very similar in both potencies and time courses. This similarity suggests either that some of these substance (or structurally related compounds)

are synaptic transmitters common to synapses upon all of these various types of neurone, or that amino acid receptors are components of the membrane of all neurones but are not directly associated with transmitter receptors, in which case the amino acid action could be considered to be nonspecific. Despite the impressive and striking action of amino acids such as GABA and L-glutamic acid, arguments against a role of either of these compounds as synaptic transmitters have already been presented (Curtis *et al.*, 1959, 1960 ; Curtis & Watkins, 1960a, 1963), and the recent findings of Ryall (1962) and of Weinstein, Roberts & Kakefuda (1963) are relevant in that the the subcellular distribution of GABA and L-glutamic acid in mammalian brain does not indicate any special aggregation of these amino acids in synaptic endings, as is found for acetylcholine (see Ryall, 1964). It is thus very probable that the action of these excitant and depressant amino acids is unrelated to the presence of specific transmitter receptor sites.

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Meat Flavor and Observations on the Taste of Glutamate and Other Amino Acids

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Author's abstract

A direct study has been made, using a taste panel, to determine the truth or falsity of the impression that monosodium glutamate has a meaty or "chickeny" taste.

Meat was investigated to arrive at the characteristics of "meaty" flavor, starting with unaged, lean beef, and including pork, lamb and chicken in the investigation. The beef was divided into juice and fiber and each of these was examined separately for flavor, in the cold state and after moderate heating. It was found that the characteristic meaty flavor which is developed on heating is derived from meat fiber rather than from juice, that it consists almost entirely of odor, and that chemically it is a mixture of hydrogen sulfide with various acids and amines, presumably split from the amino acids of the protein.

Monosodium glutamate appears to be entirely without odor when pure. Its taste has all four components: sweetness, sourness, saltiness and bitterness. In addition, glutamate has the capacity for stimulating the feeling nerves of mouth and throat to produce the sensation describable as "satisfaction."

Amino acids in general, as well as their sodium salts and their hydrochlorides, appear to be without odor. The taste of those in the neutral range of pH seems always to be sweet but usually somewhat bitter. Saltiness and sourness, which characterizes glutamic acid and its sodium salt, are exceptional tastes among the amino acids.

It is concluded that since meat flavor is predominantly odor, and pure monosodium glutamate is odorless, that meaty flavor cannot be due to, or be reproducible by, monosodium glutamate. The origin of the meaty association has been traced back to the crude glutamates formerly available, which had odor, due to protein decomposition products.

When monosodium glutamate was first produced in Japan a generation ago, it was claimed by some to produce a meaty flavor. Its developer, Ikeda, however, was more cautious and explicit and said that it had a "glutamic taste," implying that this was something new among tastes. The meaty association may have been mostly wishful thinking, for meat was a scarce and much-appreciated food, or again the claim may have been allowable within the limits of advertisers' license. At any rate, the thought that glutamate had a meaty taste came to this country along with the glutamate, and that thought still persists. Some users call glutamate "chickeny" rather than meaty while still others deny all meaty association.

In a ten-year study on "The Flavor of Meat and Meat Products" made by the United States Department of Agriculture and reported by Howe and Barbella in 1937, some tests were run on purified monosodium glutamate to find out if it were responsible for the flavor of meat. A negative conclusion was reached. To quote, "this recrystallized material has been offered to judges in solution of high molarity and also in dry form, and meat flavor has not been associated with them. It is our belief that this alleged meat flavor, if present in the original compound, must be due to the impurities present rather than to glutamic acid or its monosodium salt."

Recently, studies have been carried out at the Arthur D. Little laboratories on the flavor of meat and also on the flavor of monosodium glutamate and various amino acids, using a panel of tasters. We report herewith the highlights of the findings. For the benefit of those who may wish to go into more detail than is practicable in this paper, our studies will be published shortly as two papers, in *Food Research*.

The Flavor of Meat

In our work on meat, we sought to find what constituent of meat gives rise to flavor in cooking and to gain some idea of the nature of the flavoring substances produced. This was done on beef, then on

pork and lamb and finally on chicken. Unaged meat was used to avoid bringing in the factor of "ripening." The cooking done was always gentle, at or below 100°C. to stimulate conditions inside a roast or other sizeable piece of meat. That procedure and objective proved adequate for the present study, and pyrogenic flavor development such as occurs on the browned outside of the roast, steak or chop was left for a later study.

The first testing was carried out to determine whether juice or fiber was the source of the flavor of cooked meat. Some fresh beef of good quality was trimmed of all localized fat, it was pounded, and then was squeezed in a hydraulic press to remove the bulk of the juice. The light gray mass of squeezed fiber was then leached in cold water for several hours to remove juice not squeezed out by the press. The fiber mass was found to be odorless and tasteless, though on chewing it was found to produce a slight feeling of astringency in the mouth. On being heated even to 50°C. appreciable odor developed. This was most easily detected by chewing a piece of the warmed fiber, whereby the structure was opened up and the aroma diffused into the smelling area of the nasal chamber. After being heated to 100°C. for an hour, the fiber became strongly "meaty" and could be smelled directly by the nose from an appreciable distance. It was the "cold roast beef" type of odor, sulfury yet pleasingly fragrant. On chewing, it tasted meaty but the effect was found to be due almost entirely to aroma since it vanished when the nose was pinched.

Some juice freed of fiber was then smelled and tasted. It had weak serum-like odor of the piperidine type. It had considerable blood-like taste, in which saltiness and sweetness were distinctly evident. On being heated, this juice coagulated. It developed almost no aroma, and the taste was found to be not much different from what it was before the heating.

Fat was then smelled and tasted, raw and after heating, and likewise bone and marrow. In no instance was there appreciable meaty odor or taste, originally, or on heating.

These simple tests proved that the odor and taste of raw meat reside mostly in the juice, whereas those of cooked meat come from the fiber. Also, it was shown that the distinctive flavor of cooked meat is predominately odor.

Pieces of beef were then boiled, in plain water, in acidified water and in alkalinized water and the odors evolved were noted. Other tests made, wherein the vapors from beef were condensed by boiling in a Claisen flask, proved that hydrogen sulfide was given off and was an important constituent of meat flavor. Acidic substances were found to be evolved but were not identified in the presence of the hydrogen sulfide. Alkaline vapors included a simple low amine such as a methyl-

amine, a piperidine-like amine and probably, indole. The combination of these unpleasant substances plus possibly some neutral substances constituted the relatively pleasant odor that we know as "meaty." Since these substances were produced from fiber, which is principally protein, one might speculate that they were protein fragments, presumably produced by thermal "cracking."

Glutamic Taste

The writer, in 1932, in cooperation with L. F. Henderson, studied the taste of purified glutamic acid and its monosodium salt. We sought at that time to analyze the glutamic taste, to find out if it had some unique characteristic which made it exceptional among tastes. We made up solutions of sugar, salt, tartaric acid and caffeine and combined these in various ways in the attempt to match the taste of sodium glutamate. At about 2 thresholds of concentration of glutamate, we were able to match its taste rather well with 0.6 threshold of sweetness, 0.7 of saltiness, 0.3 of sourness and 0.9 of bitterness. At that time we felt well enough satisfied with this match that the glutamic taste was considered as operating only through the usual four kinds of taste buds.

Today, we find a "tingling feeling" factor in addition to taste. Besides the tingling feeling there is marked persistency of taste sensation. The persistent effect is present in the whole of the mouth region, including the roof of the mouth and the throat. It is difficult to describe this sensation other than to call it a "feeling of satisfaction."

What can be the cause of this glutamic effect, which is apparently independent of true taste, but which adds psychologically to the flavor of whatever has been eaten? It is suggested that monosodium glutamate may act as a functional amine which reaches and stimulates nerve endings lying within the buccal cavity. If so, this effect should be expected to be highly specific as to molecular constitution, with isomers and homologs mostly inactive, as is the case. In any event, it is proper to consider glutamate as a stimulator of the sense of feeling as well as that of taste, and the indications are that it is unique in this capacity.

Studies of Purified Glutamate

Glutamic acid has been freshly purified by a combination of carbon treatment and recrystallization to produce a fine white meal nearly or completely free from odor. This has also been done with its monosodium salt, leading to the conclusion that these pure substances have no odor.

When odoriferous lots of monosodium glutamate were treated in water solution with activated carbon, much of the impurity was adsorbed by the carbon, leaving the glutamate with much less odor. When the used carbon was washed free of glutamate solution it was observed

to give off considerable odor, implying that the adsorption was at least partially reversible. When the carbon was treated with acids, the adsorbed acids were released in considerable amount from the carbon and the bases were suppressed. When treated with caustic alkali, the bases became conspicuous and the acids were suppressed. The acidic odors released were sourish and caramel-like, suggesting caproic acid. The basic substances evolved were numerous, including a simple amine, piperidine, indole and a substance of plastery-earthy odor. Whether these substances were derived from the glutamic radical or from associated impurities is not known, but chemically they could have been derived from amino acids by "cracking" or by some other type of disintegration.

Pure odorless sodium glutamate has considerable taste value, detectable in concentrations as low as .03%, strong at .5%, but not apparently much stronger at greater concentration. By direct observation, the taste of solid glutamate appears to be only salty and sweet, whereas in solution all four components are obvious.

The taste of glutamic acid is reminiscent of that of sodium glutamate, but is much sourer. It has not been observed to produce any marked feeling reactions. As an acid it is about as sour as the same concentration of tartaric or citric acid. The pH of glutamic acid is about 3.3 and that of monosodium glutamate, 7.0, in concentrations of about 0.2%, such as might be used in foods. A titration curve between these points is of a smooth sigmoidal type with no evidence of intermediate compounds. Since the pH values of almost all foods in which glutamate is used fall between these two points, it is probable that both the mono-acid and di-acid glutamate ions (HG^- and G^{--}) are present. Due to the great buffering action of the saliva and of the tissues of the tongue, we get the glutamate taste whether glutamic acid or its monosodium salt is applied to the taste buds and therefore we cannot tell from direct observations which ion is responsible for the taste. We vote for the mono-acid ion in view of what is now known, but the grounds for choice are not too secure.

Amino Acids other than Glutamic

Examination of over a dozen amino acids of high purity, including methionine, have led to the conclusion that amino acids in general as well as their sodium salts or their hydrochlorides are without odor. Studies of the tastes of these acids were also carried out. Most of the amino acids, unlike the doubly-acid glutamic, hydroxy-glutamic and aspartic acids, gave solutions that were chemically neutral or nearly so. Where this was the case, the acid itself was used; where not, its sodium salt. Three amino acids were found to be conspicuously sweet-tasting: glycine, alanine and hydroxyproline. Nearly every other one

tested, again including methionine, was weakly to moderately sweet. It seems that sweetness of taste is a characteristic of amino acids. No saltiness of taste was noted except as previously recorded with the sodium salt of L (+) glutamic acid, so that saltiness is exceptional tastewise. Sourness likewise was found to be rare and to characterize for the most part only the dicarboxy acids and the hydrochlorides. Many amino acids were found to be bitter to some degree though none were strongly so. Bitterness in slight degree may, therefore, be considered as characteristic of amino acids. The sodium salts of the two aspartic acids, and of the D (—) glutamic acid were found to be tasteless or nearly so.

To the best of our information, the taste of L (+) glutamic acid and its monosodium salt is unique: a relatively strong taste composed of a blend of sweetness, saltiness, sourness and bitterness with a persistent tingling feeling remaining in the mouth.

The Flavor of Meat versus the Taste of Glutamate

The typical or characteristic flavor of cooked meat, as determined by direct test, has been found to consist of aroma produced by the action of heat on meat fiber. This is true of all kinds of meats including chicken. The taste of meat is slight compared with the aroma, this taste resides in the juice, and it is not significantly affected by the operation of cooking. Meat flavor is principally smelled rather than tasted.

Pure glutamate has taste but essentially no odor. In this fundamental respect it cannot substitute for meat. It is not meaty, or chickeny, and the reason for its successful use in food must be sought in some other direction.

The association of early lots of glutamate with meatiness can now be ascribed principally to the strong odor of the crude glutamate then available. The pure glutamate commercially available today is not meaty; yet it has greater value in foods, in reinforcing or accentuating their natural flavors than the old odoriferous glutamate.
References: (15) (16) (33) (35) (41).

Discussion

Dr. Crocker: The temperature in the interior of a large roast of meat never exceeds the boiling point of water. The temperature reaches 140°F. for a very rare roast, and up to 180°F. for a well-done roast. The actual cooking temperatures within the body of a piece of meat are relatively low. When you chew articles that are very low in odor, you release the aroma, it goes up to your smelling chamber by the back way and is detected. It is always interesting to find out whether odor alone is present or just taste. You pinch your nose, and at the point

of release if flavor appears in a greater amount, then it is only aroma.

Dr. Manning: Does the temperature inside a roast of beef and the length of time of cooking destroy the enzymes that would hydrolyze the proteins in the beef?

Dr. Crocker: The answer to that question is that there are no enzymes apparently involved in the production of meaty flavor.. That is treated in detail in a paper to appear in *Food Research.*

Mr. Galvin: On this soluble or liquid fraction of meat, have you done any analytical work which would indicate what the meat flavor is due to in any of these soluble fractions?

Dr. Crocker: No analytical work has been done by us. I wonder if the Meat Institute has done anything on that problem?

Dr. Kraybill: None.

Dr. Anson: In confirmation of this idea that the taste of meat is aroma, you can take cooked meat and distill it and the flavor will be distilled off. One can get very complicated in talking about analysis of taste, but if you take an ordinary person and give him a mixture in the right concentration of glutamate and salt, he is reminded of meat or chicken. What it means in a more profound analytical way, I would not know.

Dr. Manning: Would taste tests carried on in that way with articles of food left in the mouth cause that effect, or should tests be carried out by a jury in which the teeth have been cleaned by a dentist?

Dr. Crocker: We went through a great deal of effort with a few people to find out if the condition of the teeth had anything to do with it, and the conclusion was quite negative. We found also that the saliva had very little to do with it.

Mr. Nair: In connection with this question of whether glutamate has a chicken flavor we tried out a taste panel using dried chicken meat and salt versus glutamate and salt. The powder dried from chicken broth and chicken meat was suspended or dissolved in water with a definite concentration of salt (about .7%). Glutamate was dissolved in water with the same salt concentration. At low levels of flavor concentration some people identified the glutamate solution as having a fairly good chicken flavor while other panel members were uncertain whether the chicken meat suspension had a chicken flavor or not. Of course we had to cross them up a bit so they did not distinguish between the samples by observing differences in clarity of appearance. I might add that we always test such solutions with salt present to bring out glutamate or chicken flavor. We incline to the view that some people identify glutamate as chicken-like in flavor.

EFFECTS OF FEEDING INDIVIDUAL AMINO ACIDS UPON THE DISTRIBUTION OF OTHER AMINO ACIDS BETWEEN CELLS AND EXTRACELLULAR FLUID

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Amino nitrogen is present in much higher concentration in tissues than in plasma (1–3). Nevertheless injected amino acids are taken up by the cells so that the tissue concentrations are increased more than the plasma concentration (1). Glutamine is present at higher concentrations in the tissues than in the plasma of dogs (3). In contrast the sum of the concentrations of valine and leucine is not appreciably higher in the tissues than in the plasma of guinea pigs (4).

Although the tissue amino acids appear to be free by customary analytical methods, they may be present in some unstable combination which does not pass through the cell wall. Thus, administered creatine enters cells against apparent concentration gradients to form phosphocreatine (5, 6). Such unstable compounds of the amino acids might be identical with the high energy intermediates postulated in peptide bond formation (7), for example N-phosphoryl compounds. We have been interested in the possibility that the magnitude of the distribution ratios of amino acids between cells and extracellular fluids might determine whether growth or wasting occurs.

In the present communication evidence is reported that glycine is distributed according to a characteristic ratio between the cells (liver and muscle) and the extracellular fluid. Amino acids which produced high concentrations in the organism when fed produced a diminution of the distribution ratio (cells to extracellular fluid) for glycine. Similarly when high glycine levels were produced by feeding glycine, the distribution ratios for the non-glycine amino acids were reduced. Of the amino acids tested, only glutamic acid produced *increases* rather than decreases in the distribution ratios of the three categories of amino acids determined. We interpret these findings to mean that each of the amino acids, when at high concentration, competes with other amino acids for the means employed by cells for the concentration of amino acids, and that L-glutamic acid in some way contributes to the intracellular concentration of other amino acids.

EXPERIMENTAL

Guinea pigs of both sexes (300 to 550 gm., Rockland Farms) were maintained upon the Rockland guinea pig diet with greens in addition two or three times a week. Amino acids and sodium benzoate were administered by mouth to animals after a 12 hour fast, in either a neutral solution or a suspension by a dropper pipette, and washed down with water. Feedings were practically quantitative. Dosages were usually 10 to 20 millimoles of amino acid per kilo of body weight. With a view to having more or less steady absorption from the gut for nearly 3 hours, two-thirds of the total amount of amino acid was given at the beginning of the experiment, one-sixth an hour later, and one-sixth 2 hours later. The animals were sacrificed at 3 hours. Omitting the later two portions and sacrificing the animals at 2 hours did not modify greatly the effects observed with glycine.

Other experimental details have been described (8), including the determination of glycine (9) and of glutamine α-amino nitrogen and non-glutamine α-amino nitrogen, both at pH 4.7 with ninhydrin (10). The α-amino nitrogen was thus divided into three categories, that due to glutamine, that due to glycine, and that due to neither, which for convenience will be called the "residual" α-amino acid nitrogen. Glycine was recovered as well (101 $\pm$ 3 per cent) from the picric acid-lead acetate filtrates at various levels of enrichment of tissues, as from standard solutions. Corrections (small, but appreciable) were made routinely for the formaldehyde obtained in the absence of ninhydrin. The specificity of the glycine method (9, 11) is supported by the observation that the deduction of the values for glycine nitrogen from the values for the total α-amino nitrogen eliminated most of the variability of the latter values. To enhance the precision of the glycine determination, aliquots usually were selected which gave optical transmittances between 45 and 60 per cent.

The concentration of each category of nitrogen in mg. per gm. of cell water was calculated from the proportions of extracellular and intracellular water, determined according to Lowry and Hastings (12, 8). The water distribution was determined in the tissues of the control animals and of those receiving sodium benzoate or glycine, but average normal values were assumed for other animals when it became apparent that variations in water distribution were not of significant magnitude. The differences reported have been checked for statistical significance (13).

Peptides in Tissues—We have attempted to measure peptides in tissues by analyzing filtrates prepared with picric acid, followed by neutral lead acetate (10). The picric acid filtrates contained conjugated α-amino nitrogen not passing through cellophane equivalent to about 2 mg. per 100 gm. of tissue, indicating about 99.9 per cent removal of protein. The lead

acetate treatment, however, left only 0.02 mg. per cent of non-dialyzable conjugated α-amino nitrogen, so that dialysis of these filtrates was unnecessary.

The following results for conjugated α-amino nitrogen were obtained: five guinea pigs, liver 0.2 mg. per cent ($\sigma = 0.2$), muscle 3.2 mg. per cent ($\sigma = 1.1$), kidney 1.0 mg. per cent ($\sigma = 0.8$); mouse carcass 1.9 mg. per cent, mouse carcass with skin 7.0 mg. per cent.

The lead acetate precipitation, designed to remove the abundant glutathione, undoubtedly removed other peptides. From a fibrin hydrolysate, for example, containing about two-thirds of the amino acids in conjugated form, 42 per cent of the conjugated amino acid nitrogen was removed by lead acetate under conditions simulating those used in preparing tissue filtrates. One may conclude that tissues, especially muscle, contain appreciable concentrations of non-protein amino acid conjugates other than glutathione. Anfinsen and Beloff[1] have concurrently made similar observations upon the livers of rats.

Effect of Lead Acetate Treatment upon Free Amino Acids—The lead acetate filtrates were not ideal for the measurement of free amino acids but seemed preferable to glutathione-rich filtrates. About one-tenth of the α-amino acid nitrogen was lost from plasma by the treatment, and 14 per cent from a fibrin hydrolysate. The amino acids removed most extensively by lead acetate when they were added to plasma and tissue filtrates were glutamic acid (60 to 80 per cent), aspartic acid (50 to 70 per cent), and histidine (about 50 per cent), as indicated by the loss of α-amino nitrogen. Glycine, alanine, serine, phenylalanine, and proline were not lost to a measurable extent; methionine, tyrosine, and leucine suffered losses of 10 per cent or less. For many of the plasma samples, α-amino nitrogen was determined both before and after lead acetate treatment; the only plasmas showing severe losses were those obtained after histidine feeding.

RESULTS AND DISCUSSION

The glycine nitrogen of the plasma of normal guinea pigs varied for unknown reasons from 0.4 to 2.6 mg. per cent; the coefficients of variation (σ/mean) were 0.60, 0.50, and 0.52 respectively for glycine in plasma, liver cells, and muscle cells. Glycine contributed most of the variability of the amino acid concentrations of these tissues (Table I). The variations in glycine, however, *occurred together in the three tissues*, so that coefficients of correlation among them were above 0.9, or, expressed in another way, the coefficients of variation of the distribution ratios were 0.17 and 0.25.

[1] Personal communication.

The liver cell tended to contain 33 times as much glycine as the extracellular fluid; the muscle cell, 8.3 times as much. The residual amino acids showed distribution ratios of 15.7 (σ = 3.0) and 5.3 (σ = 1.1) for liver and muscle respectively. The residual amino acids were not sufficiently variable to establish that their changing values in the liver, muscle, and plasma were positively correlated. The surprisingly large proportion of the free amino acids represented by glycine (31 to 63 per cent of the non-

TABLE I

Effects of Ingestion of Benzoate (300 Mg. per Kilo) and of Glycine, Glutamate, and Aspartate (Each 20 mM per Kilo) upon Distribution of Amino Acids between Extracellular Fluid and Cells of Liver and Muscle

The mean values are given, followed by the standard deviations.

	Glutamine	Glycine		"Residual"	
	α-Amino N in cell water	α-Amino N in cell water	(Cellular)/(Extracellular)	α-Amino N in cell water	(Cellular)/(Extracellular)
	mg. per cent	*mg. per cent*		*mg. per cent*	
Controls (13)*					
Liver	5.5 ± 1.4	32 ± 16	33 ± 5	33 ± 6	15.7 ± 3.0
Muscle	5.5 ± 1.8	8.6 ± 4.5	8.3 ± 2.0	13.2 ± 2.9	5.3 ± 1.1
Benzoate (3)†					
Liver		9.0 ± 4.2	19 ± 2.4	51 ± 12	25 ± 7
Muscle		7.6 ± 3.0	16.5 ± 1.6	27 ± 14	13.5 ± 8
Glycine (4)					
Liver		129 ± 13	5.5 ± 0.7	22 ± 9	7.0 ± 2.6
Muscle		41 ± 8	1.7 ± 0.2	9.0 ± 1.8	3.0 ± 0.4
Glutamate (5)					
Liver	10.3 ± 2.1	33 ± 6.2	40 ± 3.8	49 ± 4.4	21 ± 3.7
Muscle	8.5 ± 2.4	10.0 ± 2.2	12.2 ± 2.0	21.5 ± 5.5	7.9 ± 1.1
Aspartate (3)					
Liver		37 ± 3	31 ± 2	44 ± 1.4	21 ± 3
Muscle		10.6 ± 1.7	8.8 ± 0.9	17 ± 1.8	7.9 ± 1.5

* The figures in parentheses represent the number of animals.

† Animals sacrificed 70, 80, and 120 minutes after feeding sodium benzoate.

glutamine α-amino nitrogen in liver, 19 to 56 per cent in muscle) may be related to the relatively large contribution of glycine to urinary amino acid conjugates in man (11).

Sodium Benzoate—A dose of sodium benzoate (300 mg. per kilo of body weight) which should stimulate a nearly maximum rate of synthesis of hippuric acid reduced the free glycine of liver and secondarily that of plasma, so that the distribution ratios, liver cell to plasma, and muscle cell to plasma, moved downward and upward respectively (Table I). This illustrated what happens when the withdrawal of an amino acid is

stimulated in one of the two tissues, and is to be contrasted with the effects of L-proline or DL-serine, for example, which caused both liver and muscle cells to lose glycine (see below). The results indicate a predominance of the liver over the kidneys (14) in hippuric acid synthesis in the guinea pig, the distribution ratio for glycine between kidney and plasma being little changed. Accumulation of hippuric acid in the liver (measured as conjugated amino acid nitrogen extractable by ethyl acetate) was small, in one case 1.7 mg. per cent of nitrogen.

L-Amino Acids—Of the amino acids administered (excepting DL forms) L-proline, L-histidine, glycine, and L-methionine caused large rises in plasma amino acids. These were also the amino acids giving the largest rises in

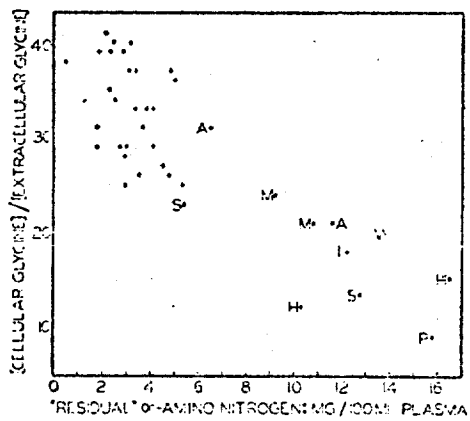

FIG. 1. Relation of the distribution ratio for glycine between liver cells and extracellular fluid, to the plasma residual α-amino acid nitrogen after the feeding of individual amino acids. *A* = DL-alanine; *H* = L-histidine hydrochloride; *M* = L-methionine; *P* = L-proline; *S* = DL-serine; *T* = DL-threonine; *V* = DL-valine. +, experiments with other amino acids; ○, fasting controls.

the amino acids of liver and muscle cells. As the glycine concentration of the plasma was increased after glycine feeding, the distribution ratios for glycine were decreased (although the absolute increase in the glycine concentration was always greater in the cells than in the plasma). Similarly, at high concentrations of residual α-amino nitrogen, the distribution ratio of this category was reduced. This would be expected if the capacity of cells for concentrating each of the amino acids is limited. But whenever large elevations of the plasma glycine were produced, the distribution ratios of the other (residual) amino acids, between the cells (liver and muscle) and the plasma, were also depressed (Table I), with an accompanying rise in plasma concentration. Similarly, whenever the plasma residual amino acids were elevated, the distribution ratio of *glycine* between liver

cells and extracellular fluid was decreased approximately in proportion (Fig. 1). The ingestion of L-proline caused the plasma glycine nitrogen to rise to the extraordinary level of 7.7 mg. per cent with the distribution ratios falling below one-third of normal. An effect upon the muscle glycine was not apparent under the conditions of the experiments, except in the case of proline.

All the other L-amino acids examined gave undetectable to moderate increases in the plasma α-amino acid nitrogen, smaller effects upon the α-amino acid nitrogen of liver cells, and no significant effect upon the distribution of glycine. The majority of the amino acids tested caused increases of the α-amino nitrogen of muscle cells of the guinea pig (*cf.* (2)). Several of the differences in plasma response to ingested amino acids recorded here have been noted before in other species (15–23).

DL-*Amino Acids*—In cases where comparisons were made (alanine, serine, leucine, and phenylalanine), the DL-amino acids elevated the plasma amino acid concentration more than the corresponding L acids. The intense effect upon glycine distribution produced by high accumulations of an amino acid was also obtained with DL-amino acids (threonine, serine, valine, and alanine), muscle as well as the liver being affected (Table II; Fig. 1).

The amino acids appear to show competitive inhibition among themselves for the means by which the cells concentrate amino acids, indicating that these means do not operate independently for each amino acid. An identical mechanism could scarcely concentrate each of the large number of amino acids and yet maintain characteristic distributions for each in the face of the variations of the amino acid mixtures presented. Thus, a high plasma glycine level reduced the distribution ratio of other amino acids much less than that of glycine itself. The slope indicated roughly by the points of Fig. 1 suggests that an increase of about 9 mg. per cent of α-amino nitrogen due to a single amino acid in the plasma (*i.e.*, about 9 times the glycine concentration) halves the ability of the liver cell to hold glycine. Protein synthesis obviously requires the presence in some form and at some limiting concentration of every amino acid (essential or non-essential) which must be built into the molecule. As a corollary, it may be inferred that, when a strongly asymmetric accumulation of amino acids is produced by feeding or injection, the ability of the cells to retain other amino acids will be handicapped and one may expect an inhibitory effect upon growth or nitrogen retention. Thus, the difficulty in producing optimum growth of rats with diets containing gelatin supplemented with amino acids, or with diets containing 5 to 6 per cent of glycine, L-proline, or DL-phenylalanine (24, 25), may well be an effect of the excessive accumulation of one or more amino acids. The amino acids producing high blood concentrations upon feeding are the ones which cause extra energy production ("heat of specific dynamic action") and in some cases extra urea excretion (16, 26).

TABLE II

Effects of Ingestion of Several Other Amino Acids upon Distribution of Amino Acids between Extracellular Fluid and Cells of Liver and Muscle

Because of the great variability in glycine concentrations the distribution ratio (glycine nitrogen per 100 gm. of cell water to 1.05 × mg. of glycine nitrogen per 100 ml. of plasma) appears more useful. For the residual α-amino acids the cellular concentration is the more useful value because of its relative constancy. Mean values followed by standard deviations are given where two or more animals are grouped.

Amino acid fed	Dosage, mg per kilo	Glycine			Residual α-amino N		
		Plasma	Liver	Muscle	Plasma	Liver	Muscle
		α-Amino N	Distribution ratio	Distribution ratio	α-Amino N	Concentration in cell water	Concentration in cell water
		mg. per cent			*mg. per cent*	*mg. per cent*	*mg. per cent*
Fasting (13 animals)		1.02	33	8.6	2.46	38	13.2
		±0.61	±5	±2.1	±0.53	±6	±2.9
L-Alanine	10	0.92	31	8.6	3.86	46	24
" *	10	0.73	26	7.1	4.76	34	22
DL-Alanine*	10	1.22	31	6.2	6.5	46	21
" *	20	1.52	21	6.4	11.6	41	31
L-Serine	12.5	1.95	28	6.5	2.92	60	20
DL-Serine*	12.5	0.82	37	8.0	3.19	62	19
"	12.5	2.4	23	5.9	5.39	64	22
DL-Serine*	20	3.1	13.5	3.4	12.8	95	21
DL-Threonine*	15	1.89	18	8.0	12.2	93	23
DL-Valine*	15	1.0	20	6.6	13.8	49	28
L-Leucine (3 animals)	10	1.24	33	9.9	4.4	34	15.2
		±0.46	±6	±1.7	±0.9	±2	±4.7
DL-Leucine*	10	0.51	39	11	6.54	35	21
L-Proline*	15	7.7	9	2.2	15.8	72	30
L-Methionine (2 animals)	10	0.85	22	9.8	10.0	83	23
		±0.05	±2	±1.0	±0.8	±2	±1
L-Phenylalanine*	10	0.81	33	8.6	3.81	42	17.0
DL-Phenylalanine*	10	0.99	36	9.7	5.60	43	17.9
L-Tyrosine	10	0.95	29	10.3	4.1	38	19.2
L-Tryptophan	10	0.77	37	11	4.8	38	18.5
L-Ornithine	6.4	0.76	37	10.9	3.12	48	20.6
L-Lysine	10	1.96	27	9.2	4.29	43	16
L-Arginine (2 animals)	10	0.94	33	10.1	2.6	48	17
		±0.15	±1	±0.3	±0.8	±7	±4
L-Histidine (2 animals)	10	1.07	13.7	9.7	13.4	76	24
		±0.12	±1.5	±0.5	±3.1	±11	±1

* Basal ration supplemented with pyridoxine, 5 mg. per kilo.

The elevation of the alanine concentration of plasma when glycine is ingested by man, and of the glycine concentration when DL-alanine is fed (13), is an additional illustration of the response of cells to high concen-

trations of an amino acid. Decreases in the plasma concentration of several amino acids in dogs after DL-methionine feeding were noted by Hier and Bergeim (21). Humans fed glycine showed increased amino acid conjugates in plasma ((11); *cf.* (17)). The conjugated amino acids of an ultrafiltrate of the plasma of a dog were doubled after a dose of L-glutamic acid which elevated the portal α-amino nitrogen to 10.7 mg. per cent.[2] High plasma concentrations of an amino acid may provoke loss of peptides as well as of free amino acids from cells.

The observations recorded here may also assist in explaining the urinary losses of amino acids in mice fed peroxide-treated proteins (27), and the production of vomiting during the infusion of DL-amino acids and of L-glutamic and L-aspartic acids (28–30).

L-*Glutamic Acid*—The failure of L-glutamic acid absorption to increase noticeably the amino nitrogen of peripheral blood was noted by Seth and Luck (16). Increases occurred in portal blood, however. In our experiments, none of the three fractions of the amino acids of cardiac plasma was increased during glutamic acid absorption by guinea pigs; in fact, the residual amino acids were decreased (means, fasting, 2.46 mg. per cent; after glutamic acid, 2.01 mg. per cent).

In a partially gastrectomized dog receiving by mouth 20 mM of L-glutamic acid (as sodium glutamate) per kilo of body weight, we observed, in collaboration with Dr. Charles Dent,[2] a net decrease in the glycine, glutamine, and residual α-amino nitrogen in both the portal and jugular plasma, following preliminary increases, especially of the residual α-amino nitrogen in the portal plasma.

The explanation, we believe, lies in an increased uptake of various amino acids by tissues when glutamate is fed. Each of the three categories of amino acids determined was increased in liver and muscle cells of guinea pigs, and each of the distribution ratios was elevated while glutamate was being absorbed. Glycine does not appear to undergo transamination with the glutamate-α-ketoglutarate system (31, 32).

After feeding L-aspartic acid, as with L-glutamic acid, the plasma concentrations were not elevated, whereas the residual amino acids in liver and muscle were increased. The distribution of glycine was unaffected, however.

It is concluded that glutamate (or α-ketoglutarate) is somehow involved in the system by which the cells concentrate the amino acids presented to them in the extracellular fluid. Whether the observed effects are related to the conversion of glutamate to α-ketoglutarate (transamination) or to the disposition of the α-ketoglutarate with resultant formation of high

[2] Unpublished results with Dr. Charles Dent.

energy phosphate compounds, or to other reactions, remains to be determined.

SUMMARY

1. Glycine represented a large and highly variable portion of the amino acids of skeletal muscle and liver of guinea pigs. The glycine of the muscle cells, liver cells, and plasma were covariant, so that fairly constant distribution ratios were maintained. The glutamine levels of these tissues were also highly variable but with little covariance among them. The concentrations of the non-glutamine, non-glycine ("residual") amino acids were moderately constant.

2. Those amino acids which upon feeding caused high plasma concentrations (L-proline, L-histidine, glycine, L-methionine, DL-threonine, DL-serine, DL-valine and DL-alanine) caused the largest increases in the amino acids of liver and muscle cells.

3. When high glycine levels were produced by feeding glycine, not only was the distribution of glycine affected but the distribution ratios of the residual amino acids (cellular to extracellular) were also reduced.

4. Those amino acids which produced high concentrations in the organism caused a reduction not only of the distribution ratios for the category to which they belong but of the glycine distribution ratios as well. Those amino acids which gave small rises in the plasma concentration had little if any effect upon glycine distribution.

5. Glutamic acid alone of the amino acids studied produced an *increase* in the distribution ratios of each of the three categories of amino acids determined. The plasma amino acids were *diminished* during glutamate absorption.

6. These observations are interpreted to mean that the various L- and D-amino acids compete with each other, although only to a restricted extent, for the means by which cells concentrate the amino acids presented to them by the extracellular fluid, and that L-glutamic acid contributes in some way to this concentrating mechanism.

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CLXXXII. TRANSAMINATION IN PIGEON BREAST MUSCLE

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D. M. NEEDHAM [1930] first observed that in pigeon breast muscle added glutamic acid disappears from the tissue while the concentration of amino groups, as measured by the Van Slyke method, remained unchanged. She concluded that the amino group of glutamic acid is transferred to "some reactive carbohydrate residue" to form a new amino-acid. Braunstein & Kritzmann [1937; 1938] succeeded in elucidating this reaction in detail. They showed that glutamic acid reacts as follows:

(1) Glutamic acid + α-ketonic acid ⇌ α-ketoglutaric acid + amino-acid. The "reactive carbohydrate residue" of Needham is thus an α-ketonic acid. The reaction is reversible and proceeds with great rapidity under suitable conditions. It is a general reaction, occurring in almost every animal tissue, and has been termed "transamination" [Schaeffer & Le Breton, 1938; Cohen, 1939; Braunstein, 1939]. The widespread occurrence and the rapid rate of transamination suggest that the reaction plays an important part in tissue metabolism, although its significance is as yet by no means clear.

The detailed study of transamination has been hampered by the lack of suitable analytical methods. The procedure used by Braunstein & Kritzmann [1937] does not appear to be very satisfactory for the authors themselves [1938] question their analytical data in several instances. Since the determination of glutamic acid synthesis in the presence of α-ketoglutaric and amino-acids is one of the ways of measuring transamination, a more reliable method for glutamic acid determination was recently worked out by the author [1939].

The preliminary experiments with this method showed that although most tissues are capable of bringing about transamination, the different tissues show great differences in detail. In muscle, for example, only three amino-acids, *l*(+) glutamic, *l*(−)aspartic and *l*(+)alanine react rapidly; two others, *dl*-α-amino-butyric and *l*(+)valine react slightly; while the other amino-acids do not react appreciably. In kidney, on the other hand, many more amino-acids react.

This paper is confined to a study of transamination in pigeon breast muscle. Experiments on the rates of transamination in this tissue and the reactivity of different amino-acids are reported.

Procedure and methods

Principle of the procedure. For the study of transamination it is desirable to separate this reaction from other reactions in which amino- and α-keto-acids take part. Many of the other reactions require molecular O_2 and they can therefore be eliminated by working under anaerobic conditions. Since it is simpler we have preferred this method to the use of inhibitors. Comparative experiments showed that the results obtained under anaerobic conditions are not essentially different from those obtained aerobically in the presence of bromo-acetate [Braunstein & Kritzmann, 1937], an inhibitor which prevents certain

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side reactions. Neither method however eliminates the side reactions completely, for glutamic acid slowly disappears anaerobically, as well as aerobically in the presence of iodoacetate, without the addition of ketonic acids. The mechanism of this reaction is not clear; it may be due to transamination to ketonic acids provided by the muscle. The rate of the reaction however is relatively slow so that it generally does not interfere with experiments on transamination of added substrates.

Transamination was studied by the determination of glutamic acid formed on the addition of α-ketoglutaric acid and amino-acids. In all experiments a blank was carried out with α-ketoglutaric acid alone to determine the glutamic acid formation from preformed NH_2-donators (NH_3 or amino-acids). The values of the "blanks" in most experiments were considerable, showing that a relatively high concentration of NH_2-donators is present in pigeon breast muscle.

An increased glutamic acid formation from added α-ketoglutaric acid and amino acids may be considered as conclusive proof of transamination. Euler *et al.* [1939] studied the disappearance of oxaloacetic acid in the presence of amino-acids and they concluded that transamination occurs when the added amino-acids increased the rate of disappearance of oxaloacetic acid. This type of experimentation may suggest that transamination takes place, but cannot provide conclusive proof. The demonstration of aspartic acid formation in this reaction would in our view be the only conclusive evidence of transamination.

Experimental procedure. Pigeon breast muscle was chilled immediately after the death of the animal and finely divided in a Latapie mincer. The minced muscle was suspended in 0·10 *M* phosphate buffer, *p*H 7·4. Unless otherwise stated the muscle suspension used in the different experiments consisted of 1 part muscle plus 7 parts phosphate buffer. The suspension (3 ml.) was pipetted into the main compartment of conical Warburg manometer flasks provided with a side arm, which contained the substrate, and a centre well which contained a stick of yellow phosphorus. The substrates were used as neutral 0·2 *M* solutions (0·4 *M* for *dl*-amino-acids) and usually added in amounts to give a final concentration of 0·017 *M*. Substrates which were soluble with difficulty were weighed directly into the side arm of the cup.

Anaerobic conditions were maintained by filling the vessels with N_2 and by the yellow phosphorus in the centre well. The vessels were shaken at 40° and unless otherwise stated the substrate was added from the side arm after 5 min. shaking. At the end of the experimental period the flasks were detached and 1 ml. 10% H_2SO_4 added to stop the reaction. The solutions were washed into graduated cylinders with 6–7 ml. distilled water, 1 ml. 10% Na_2WO_4 was added and the solutions made up to a volume of 15–20 ml. The solutions were filtered and an aliquot of the clear filtrate employed for the glutamic acid determination.

Analytical procedure. Glutamic acid was determined by the method recently described by the author [1939]. Interfering succinic acid, and malonic acid when added, were removed by ether extraction before the glutamic acid determination. The aliquot of the clear filtrate was acidified with 2 ml. 10% H_2SO_4 and extracted with ethyl ether for 2 hr. The ether was removed from the aqueous phase and the latter brought to a *p*H of approximately 5 by the addition of alkali in the presence of a drop of bromocresol purple. The glutamic acid in the aqueous phase was then determined.

The results are expressed as μl. glutamic acid (147 mg. glutamic acid being equivalent to 22,400 μl.). This procedure was used since the glutamic acid was determined as μl. O_2 (1 μl. O_2 is equivalent to 2 μl. glutamic acid).

Results

Glutamic acid formation from amino-acids and α-ketoglutaric acid

The question was first investigated as to which amino-acids react with α-ketoglutaric acid to form glutamic acid. In these experiments a relatively long incubation period of 40 min. was chosen to allow for the formation of measurable amounts of glutamic acid from slowly reacting amino-acids. The results of these experiments are listed in Table I.

Table I. *Glutamic acid formation from α-ketoglutaric acid and different amino-acids*

Substrate conc. 0·017 *M* (for *dl*-amino-acids, 0·034 *M*). N_2. Yellow phosphorus. 40°.

Amino-acid added	Period of incubation min.	μl. Glutamic acid formed: Without amino-acid	μl. Glutamic acid formed: With amino-acid	Increase in glutamic acid due to added amino-acid μl.	% of added α-ketoglutaric acid converted into glutamic acid
l(−)Aspartic acid	40	161	815	654	49
l(+)Alanine	40	96	636	543	40
l(+)Valine	40	96	220	124	9
l(−)Phenylalanine	40	96	170	74	—
l(−)Tyrosine	40	142	204	62	
l(−)Cysteine	40	94	152	56	—
d(−)Valine	40	96	149	53	—
l(−)Histidine	40	96	146	50	—
d(+)Histidine	40	96	140	44	—
l(−)Leucine	40	142	180	38	—
d(−)Alanine	40	130	160	30	—
l(+)Citrulline	40	142	168	26	—
l(+)*iso*Leucine	40	142	166	24	—
l(−)Methionine	30	138	156	18	—
l(+)Arginine	40	96	99	3	—
Glycine	40	96	95	—	—
l(+)Ornithine	40	142	104	—	—
l(−)Proline	40	142	142	—	—
d(+)Phenylalanine	40	96	92	—	—
l(−)Tryptophan	40	142	124	—	—
dl-Serine	40	142	140	—	—
dl-Lysine	30	138	113	—	—
Homologous series					
dl-Alanine	40	130	564	434	32
dl-α-Aminobutyric acid	40	130	266	136	10
dl-α-Aminovaleric acid	40	130	145	15	—
dl-α-Aminohexoic acid	40	130	150	20	—

Of the 21 different amino-acids tested, *l*(−)aspartic acid and *l*(+)alanine are the only ones which show a large glutamic acid formation; *dl*-α-aminobutyric acid and *l*(+)valine show a slight activity. None of the remaining amino-acids is significantly active.

These results differ in various points from those reported by Braunstein & Kritzmann [1938] whose experiments were carried out aerobically in the presence of bromoacetate. In order to ascertain whether the differences in the results are due to differences in experimental conditions, transamination was also studied under conditions similar to those of Braunstein & Kritzmann. It is seen from Table II that the results obtained aerobically in the presence of iodoacetate are essentially the same as those obtained anaerobically. If anything these experi-

Table II. *Glutamic acid formation from α-ketoglutaric acid and different amino-acids under aerobic conditions in presence of iodoacetate*

Substrate conc. 0·016 *M*. Iodoacetate conc. (final), 0·002 *M*. (Iodoacetate plus muscle suspension incubated 15 min. before substrate added). 1 part minced muscle plus 5 parts phosphate buffer. Reaction time, 60 min. Air.

Amino-acid added	Glutamic acid formed μl.	Increase in glutamic acid due to added amino-acid μl.	% of added α-ketoglutaric converted into glutamic acid
—	92	—	—
l(+)Valine	98	6	—
d(−)Valine	33	—	—
d(+)Histidine	98	6	—
l(−)Histidine	55	—	—
l(+)Arginine	127	35	—
l(+)Alanine	450	358	27
	No iodoacetate		
l(+)Alanine	214	122	9

ments show that the formation of glutamic acid is greater under anaerobic conditions. The inhibitory effect of iodoacetate on the aerobic disappearance of glutamic acid formed from *l*(+)alanine and α-ketoglutaric acid is seen from Table II. This is in agreement with the findings of Braunstein & Kritzmann [1937] who first reported this effect with bromoacetate.

Braunstein & Kritzmann [1938] found 14 different amino-acids to react with α-ketoglutaric acid in pigeon breast muscle, but the authors themselves question their results for 3 amino-acids. Of these amino-acids only the findings for alanine are confirmed by our experiments. From our data previously discussed it follows that the discrepancy between our results and those of Braunstein & Kritzmann cannot be attributed to a difference in experimental procedure. The different results may however be explained by the omission of a suitable control by Braunstein & Kritzmann. As can be seen from Table I α-ketoglutaric acid when added to a muscle suspension forms considerable amounts of glutamic acid in the absence of any added amino-acid. The failure of Braunstein & Kritzmann to take this into account is the chief reason for the high values obtained for all the amino-acids. An additional factor is to be found in the method used by Braunstein & Kritzmann for the determination of glutamic acid. The method employed by these workers measures the NH_2-N which is insoluble in alcohol after the addition of $Ba(OH)_2$ and thus would include aspartic acid and possibly other amino-acids. The high values for certain of the amino-acids, e.g. cystine, reported by these workers is probably due to the insolubility of these amino-acids under the conditions used for the analysis.

On the other hand, of all the amino-acids studied we find *l*(−)aspartic to be the most active. Braunstein & Kritzmann have reported no data for this amino-acid, presumably because their method does not permit the differentiation between glutamic and aspartic acids.

None of the amino-acids of the *d*-series tested was found to be active (Table I).

Effect of ketonic acids on the anaerobic disappearance of glutamic acid

Under anaerobic conditions glutamic acid slowly disappears when added to pigeon breast muscle [Needham, 1930; Braunstein & Kritzmann, 1937; Cohen, 1939]. Braunstein & Kritzmann [1937] showed that the disappearance of glutamic acid is markedly accelerated if pyruvic acid is added, and that the disappearance of glutamic acid is accompanied by the formation of equivalent

amounts of alanine. These authors thus demonstrated the reversibility of transamination.

In order to determine whether pyruvic acid can be replaced by other ketonic acids, the effects of a series of α- and other keto-acids on the anaerobic disappearance of glutamic acid were studied. If the activities of the members of the homologous series of α-ketomonocarboxylic acids are compared (Table III)

Table III. *Disappearance of added glutamic acid in the presence of ketonic acids*

Substrate conc. 0·017 *M*. N_2. Yellow phosphorus. 40°. Incubation period, 40 min.

Ketonic acid added	Glutamic acid added μl.	Glutamic acid found μl.	Glutamic acid disappeared μl.	% Glutamic acid disappeared
—	1344	1240	104	7·5
Oxaloacetic	1344	654	690	51·5
Pyruvic	1344	688	656	49
α-Ketobutyric	1344	1090	254	19
α-Ketovaleric.	1344	1180	164	12
α-Ketocaproic	1344	1190	154	11
Mesoxalic	1344	1105	239	18
Acetopyruvic	1344	1162	182	13·5
Acetoacetic	1344	1192	152	11
Laevulic	1344	1360	—	—

it is seen that pyruvic acid is the most active, α-ketobutyric acid is slightly active, while α-ketovaleric and α-ketohexoic acids are not appreciably active. These results are in keeping with the findings of the reverse reactions (Table I). Of the α-ketonic dicarboxylic acids, oxaloacetic acid shows an activity of the same order as pyruvic whilst mesoxalic shows a small activity. Acetoacetic, acetopyruvic and laevulic acids are not appreciably active. Laevulic acid appears to inhibit the small anaerobic disappearance of glutamic acid.

All the preceding observations on transamination in pigeon breast muscle can be explained on the assumption that three different enzyme systems are concerned with transamination in this tissue, one each for the three reactions:

Glutamic acid ⇌ α-ketoglutaric acid
Aspartic acid ⇌ oxaloacetic acid
Alanine ⇌ pyruvic acid

The slight activities of *l*(+)valine and *dl*-α-aminobutyric acid, on the one hand, and α-ketobutyric acid and mesoxalic acid on the other, may be explained by assuming slight affinities of *l*(+)valine, *dl*-α-aminobutyric and α-ketobutyric acids for the alanine ⇌ pyruvic acid system, and of mesoxalic for the aspartic ⇌ oxaloacetic acid system. It is well known that the affinities of other enzyme systems, for example, lactic dehydrogenase, are not strictly limited to one substrate. Homologous compounds may also react but the rate of reaction rapidly falls off as the carbon chain is lengthened. If this holds for the transaminating enzymes, then the failure of the higher homologues of alanine and pyruvic acid to react is easily understood. The fact that α-aminobutyric acid is physiologically a rare compound makes the explanation of its activity difficult on any other basis.

Glutamic acid formation from amino compounds other than α-amino-acids

A number of different amino compounds—β-, δ-, and ε-amino-acids, amines and aminopurines—were tested for glutamic acid formation in the presence of

α-ketoglutaric acid, but no significant increase was observed with any of these compounds (Table IV). These findings confirm those of Braunstein & Kritzmann [1938].

Table IV. *Glutamic acid formation from α-ketoglutaric acid and amino compounds other than α-amino acids*

Substrate conc. 0·017 *M*. N_2. Yellow phosphorus. 40°.

Amino-acid added	Period of incubation min.	Glutamic acid formed: Without amino-acid μl.	Glutamic acid formed: With amino-acid μl.
β-Alanine	30	85	114
δ-Aminovaleric	30	85	114
ε-Aminohexoic	30	85	92
dl-β-Aminobutyric	40	130	127
Adenine	40	138	95
Histamine	40	138	118
Putrescine	20	122	125
Spermine	40	122	116
Heptylamine	40	122	92

Rates of formation and disappearance of glutamic acid by transamination

The data in the previous sections are not accurate measurements of the rate of transamination, since in cases where the rate is very rapid, as with *l*(−)aspartic acid and *l*(+)alanine, the reaction comes to an equilibrium before the end of the incubation period. To determine the rate of transamination shorter periods of incubation were therefore chosen. The data from such experiments are shown in Figs. 1, 2 and 3.

From Fig. 1, it is seen that the initial rates are very rapid for *l*(−)aspartic acid and *l*(+)alanine. In the case of *dl*-α-aminobutyric acid and *l*(+)valine, the initial rates are also appreciable but the curves show an early plateau, for some unexplained reason. This is not due to a true equilibrium since the plateaux reached from both sides of the reaction lie at different levels (Fig. 2).

Under the experimental conditions employed the reaction:

(2) glutamic acid + pyruvic acid ⇌ ketoglutaric acid + alanine

has an equilibrium constant

$$\frac{[\text{glutamic}] \times [\text{pyruvic}]}{[\text{ketoglutaric}] \times [\text{alanine}]}$$

of approximately 1 (see Fig. 2). This is in agreement with the previous finding reported by Braunstein & Kritzmann [1937]. None of the remaining systems shown in Fig. 2 shows any evidence of reaching an equilibrium.

The reaction:

(3) glutamic acid + oxaloacetic acid ⇌ ketoglutaric acid + aspartic acid

reaches an equilibrium even faster than reaction (2) (Fig. 3). The equilibrium constant of this reaction is of an order similar to that of reaction (3). Owing to some side reactions in which oxaloacetic acid disappears, reaction (3) does not come to a standstill and this complicates the accurate determination of the equilibrium constant.

If the rates for reactions (2) and (3) are expressed as $Q_{\text{glutamic acid}}$,

$$\frac{\mu\text{l. glutamic acid formed}}{\text{mg. dry wt. of tissue} \times \text{hr.}},$$

values of 39 and 44 respectively are obtained. Q_{O_2} values for respiration of pigeon breast muscle under optimum conditions are of the same order.

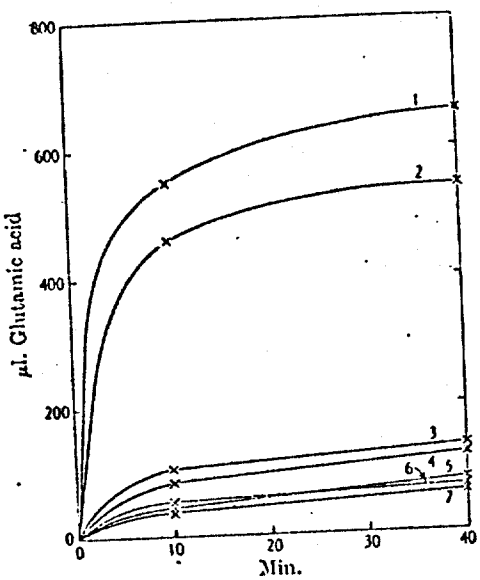

Fig. 1.

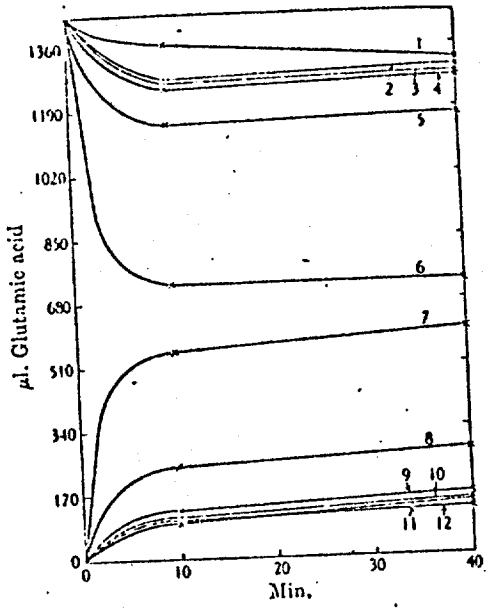

Fig. 2.

Fig. 1. Rate of glutamic acid formation from α-ketoglutaric acid and amino-acids. Substrate conc. 0·017 *M* (for *dl*-amino-acids, 0·034 *M*). N_2. Yellow phosphorus. 40°. (Corrected for blank.) Curves: 1, *l*(−)Aspartic acid + α-ketoglutaric acid; 2, *l*(+)alanine + α-ketoglutaric acid; 3, *dl*-α-aminobutyric acid + α-ketoglutaric acid; 4, *l*(+)valine + α-ketoglutaric acid; 5, *l*(+)phenylalanine + α-ketoglutaric acid; 6, *l*(−)cysteine + α-ketoglutaric acid; 7, *l*(−)tyrosine + α-ketoglutaric acid.

Fig. 2. Rate of glutamic acid formation in the presence of α-ketoglutaric acid plus amino-acids, and rate of glutamic acid disappearance in the presence of ketonic acids. Substrate conc. 0·017 *M* (*dl*-amino-acids, 0·034 *M*). N_2. Yellow phosphorus. 40°. Curves: 1, Glutamic acid; 2, glutamic acid + acetoacetic acid; 3, glutamic acid + α-ketohexoic acid; 4, glutamic acid + α-ketovaleric acid; 5, glutamic acid + α-ketobutyric acid; 6, glutamic acid + pyruvic acid; 7, α-ketoglutaric acid + *dl*-alanine; 8, α-ketoglutaric acid + *dl*-α-aminobutyric acid; 9, α-ketoglutaric acid + *dl*-α-aminovaleric acid; 10, α-ketoglutaric acid + *dl*-α-aminohexoic acid; 11, α-ketobutyric acid + *dl*-β-aminobutyric acid; 12, α-ketoglutaric acid.

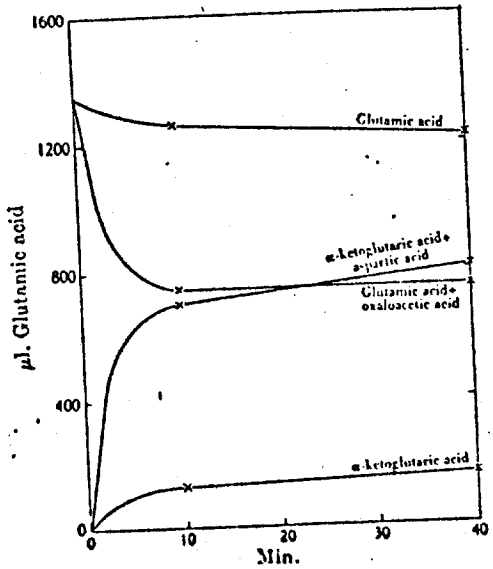

Fig. 3. Rate of glutamic acid formation in the presence of α-ketoglutaric acid plus aspartic acid, and rate of glutamic acid disappearance in the presence of oxaloacetic acid. Substrate conc. 0·017 *M*. N_2. Yellow phosphorus. 40°.

Effect of small amounts of aspartic acid on transamination

The lack of a suitable method for the determination of aspartic acid has so far prevented an investigation of the reaction:

(4) oxaloacetic acid + amino-acid $\rightleftharpoons$ aspartic acid + ketonic acid

(except when the amino-acid is glutamic acid). However, reaction (4) can be studied in an indirect way by coupling it with reaction (3). Experimentally this means the addition of α-ketoglutaric acid to the system represented by reaction (4). The balance sheet of reactions (3) and (4) is:

(5) α-ketoglutaric acid + amino-acid $\rightleftharpoons$ glutamic acid + α-ketonic acid.

Thus it should be possible to detect transamination according to reaction (4) by the addition of α-ketoglutaric acid, aspartic acid and another amino-acid to a muscle suspension. When these three substances are added, reaction (3) takes place and oxaloacetic acid is formed. Oxaloacetic acid would then be expected to react according to reaction (4), and aspartic acid would be regenerated. Since aspartic acid is expected to act in the manner of a catalyst in this reaction, small concentrations of aspartic acid should suffice to bring about an effect. With this in mind previously studied amino-acids were tested with and without the addition of aspartic acid. From the data in Table V it is seen that there is no significant

Table V. *Effect of small concentrations of aspartic acid on transamination from α-ketoglutaric acid and different amino-acids*

Final conc. of α-ketoglutaric acid and amino-acids, 0·016 *M*. Final conc. of aspartic acid, 0·0016 *M*. Substrate added after 5 min. incubation. N_2. Yellow phosphorus. 40°.

Amino-acid added	Period of incubation with substrate min.	μl. Glutamic acid formed: Without aspartic acid	μl. Glutamic acid formed: With aspartic acid	Increase in glutamic acid due to aspartic acid μl.
—	40	110	194	84
l(+)Alanine	40	520	560	40
l(+)Valine	40	164	218	54
l(−)Phenylalanine	40	140	210	70
l(+)Arginine	40	129	222	93
l(−)Leucine	40	138	241	103

increase in glutamic acid formation from the various amino-acids in the presence of aspartic acid, beyond that of aspartic acid itself. The conclusion may be drawn from these experiments that oxaloacetic acid reacts with no amino-acids other than glutamic acid and alanine. However direct proof for the reaction with alanine has not been produced.

The effect of inhibitors on transamination

The effects of different inhibitors on transamination are listed in Table VI. In these experiments the inhibitors were incubated with the muscle suspension for 15 min. before the substrate was added from the side arm.

Transamination is markedly inhibited by high concentrations of cyanide. As seen from the data the percentage inhibition is roughly proportional to the concentration of cyanide. The effect of high concentrations of cyanide suggests that the inhibition may be due to cyanohydrin formation [Green & Williamson, 1937]: however, the 30 % inhibition by 10^{-3} *M* cyanide indicates that some other basis for the inhibition may exist.

Table VI. *Effect of inhibitors on glutamic acid formation from α-ketoglutaric acid and l(+)alanine and l(−)aspartic acid*

Final substrate conc. 0·015 *M*. Inhibitor incubated with muscle suspension for 15 min. before substrates added. N_2. Yellow phosphorus. 40°.

Inhibitor	Final conc. *M*	Incubation period min.	Glutamic formed μl.	Inhibition %
	α-Ketoglutaric acid + *l*(+)alanine			
—	—	30	576	—
Cyanide	0·05	30	122	79
"	0·01	30	288	50
"	0·001	30	400	30
"	0·0001	30	505	12
Malonate	0·10	30	484	10
"	0·01	30	542	6
Pyrophosphate	0·01	30	534	7
NaF	0·02	30	490	15
Iodoacetate	0·002	30	550	5
Bromoacetate	0·002	30	526	9
As_2O_3	0·01	30	488	15
Octyl alcohol	Sat.	30	498	14
	α-Ketoglutaric acid + *l*(−)aspartic acid			
—	—	40	592	—
Malonate	0·10	40	436	26
"	0·01	40	580	2

Malonate in concentrations as high as 0·10 *M* has only a slight effect on transamination in the presence of *l*(+)alanine and α-ketoglutaric acid. Transamination in the presence of *l*(−)aspartic acid and α-ketoglutaric acid appears to be somewhat more sensitive to 0·10 *M* malonate. However the inhibition at this high concentration is probably non-specific, since at lower concentrations (0·01 *M*) no inhibitory effect is observed with either *l*(+)alanine or *l*(−)aspartic acid.

NaF, As_2O_3 and octyl alcohol have small inhibitory effects, while pyrophosphate, iodoacetate and bromoacetate have no appreciable effect on transamination in the presence of *l*(+)alanine and α-ketoglutaric acid.

The results with As_2O_3 and octyl alcohol are in agreement with those reported by Braunstein & Kritzmann [1937] and Kritzmann [1938]. The results with malonate do not agree with the statement made by Braunstein [1939].

Discussion

It has been pointed out in a previous section of this paper that α-ketoglutaric acid forms glutamic acid when added alone to pigeon breast muscle. Preliminary experiments show that the anaerobic glutamic acid synthesis from ammonium α-ketoglutarate is too small to account for the total glutamic acid formed. This must mean that there are NH_2-donators other than NH_3 preformed in the muscle. Among the amino-acids, *l*(+)alanine and *l*(−)aspartic acid are the only ones which could act in this manner. This suggests that these two amino-acids, like glutamic acid [Cohen, 1939], occur free in pigeon breast muscle.

The fact that the three amino-acids which alone are active in transamination are also the only ones which have specific dehydrogenases in muscle [Thunberg, 1920–1; Ahlgren, 1924; Needham, 1930; Euler *et al.* 1938; Dewan, 1938] suggests that transamination depends on the activity of a dehydrogenase during some phase of the reaction. On this basis the limitation of transamination in pigeon breast muscle to those amino-acids which possess dehydrogenases may be understood.

Summary

1. Transamination (Braunstein & Kritzmann) has been studied in pigeon breast muscle. Of 21 different α-amino-acids studied in the presence of α-ketoglutaric acid, *l*(−)aspartic acid and *l*(+)alanine are the most active in forming glutamic acid; *dl*-α-aminobutyric acid and *l*(+)valine are slightly active. None of the remaining amino-acids is appreciably active.

2. Of a series of ketonic acids tested, oxaloacetic and pyruvic acids show the greatest activity in causing the anaerobic disappearance of glutamic acid; α-ketobutyric and mesoxalic acids are slightly active; α-ketovaleric, α-ketohexoic, acetoacetic and laevulic acids are not appreciably active.

3. The data from these experiments can be explained by assuming the existence of three enzyme systems which are concerned with the following reactions:

Glutamic acid ⇌ α-ketoglutaric acid
Aspartic acid ⇌ oxaloacetic acid
Alanine ⇌ pyruvic acid

By combinations of these three systems the following reactions may be brought about:

(1) α-Ketoglutaric acid + alanine ⇌ glutamic acid + pyruvic acid.
(2) α-Ketoglutaric acid + aspartic acid ⇌ glutamic acid + oxaloacetic acid.
(3) Oxaloacetic acid + alanine ⇌ aspartic acid + pyruvic acid.

The occurrence of reaction (3) in pigeon breast muscle has not yet been conclusively demonstrated. The slight activity of certain homologues can be explained by a slight affinity for one of these enzyme systems.

4. Transamination in pigeon breast muscle is inhibited by high concentrations of cyanide. NaF, As_2O_3 and octyl alcohol have small inhibitory effects. Pyrophosphate, malonate, iodoacetate and bromoacetate have no appreciable effect.

5. Of a number of different amino compounds tested other than α-amino-acids, none has been found to be active in transamination.

6. Certain discrepancies between the results of Braunstein & Kritzmann [1938] and those reported here are discussed. The failure of these authors to use a suitable control in their experiments explains their statement that all α-amino-acids of the *l*-series are active in transamination in pigeon breast muscle.

I wish to express my thanks to Dr H. A. Krebs for his constant advice and help throughout this study.

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The effect upon mice of intraventricular injection of excitant and depressant amino acids

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A SHORT report has recently been published by Kita *et al.*[1] on the effects of intraventricular injections of some amino acids upon mice. The relative potencies of the substances which caused convulsions were similar to those of the same substances as excitants of spinal neurones of the cat[2, 3] and toad[4], and also of cortical neurones of the cat.[5] It was therefore of interest to extend the results of these workers to include other amino acids known to have strong excitatory and depressant actions on mammalian and amphibian neurones.

Small groups of 4-week-old mice of both sexes were used. The amino acids were dissolved in a mammalian Ringer solution and the range of concentration used for each substance is shown in Table 1. The solution to be tested (0·05 ml) was injected through the skull of unanaesthetised mice according to the method of Haley and McCormick.[6] Controls, in which the same quantity of a suspension of Indian Ink was similarly injected, were also carried out. Post-mortem sections performed

TABLE 1. (A) EXCITANT AMINO ACIDS

Group*	Amino Acids	No. of mice	Dose (μg/0·05 ml)	Effect
A	N-Methyl-D-aspartic acid	4	10	L
		9	2–5	+ + +
		4	1	+ (+)
B	N-Methyl-DL-aspartic acid	4	20–50	L
		6	5–10	+ + +
		5	2	+ +
D	N-Methyl-L-aspartic	4	50	+ +
		2	20	+
C	D-Homocysteic acid	8	20–50	+ + +
		2	10	+ +
C	DL-Homocysteic acid	8	25–50	+ + +
		4	10	+ (+)
E	L-Homocysteic acid	6	20–50	+
D	N-Methyl-DL-glutamic acid	4	50	+ + (+)
		2	20	+
E	D-Aspartic acid	5	50	+
E	L-Aspartic acid	5	50	+
F	L-Glutamic acid	2	150	
		6	50–100	Nil

(B) DEPRESSANT AMINO ACIDS

Group*	Amino Acids	No. of mice	Dose (μg/0·05 ml)	Effect
A[1]	3-Amino-1-propane-sulphonic acid	8	100	— — —
		4	50	— —
		4	20	—
B[1]	γ-Amino-n-butyric acid	6	150–250	— — —
		6	100	— —
		4	50	Nil
	Taurine	13	100–150	— —
		6	20–50	Nil

* The symbols A, B . . . F and A[1], B[1] are intended to convey relative ranking in decreasing order of potency. It is, however, not possible for accurate quantitative comparisons to be made from these data.

immediately after these animals had been killed with ether showed that in four out of twenty control mice some of the injected fluid was located outside the ventricles. After injection of mammalian Ringer or Indian Ink suspension, the animals tended to be subdued in their behaviour for periods of 15–60 sec, but occasionally slight twitching or incoordination of movement was noted, presumably due to trauma produced by the injection.

In large doses, amino acids known to be strong excitants of single neurones[3] produced clonic flexor convulsions followed by spasm (usually of extensor type) and death. In Table 1 the effects of these lethal doses are denoted by "L".

Lower dosages caused gross hyperactivity, with running, leaping and violent scratching at the head or body. Flexor convulsions were frequently observed as part of this response. During recovery the animal was usually subdued and its responses to being disturbed remained sluggish over long periods (10 min to over half an hour). This pattern of effects is denoted "+ + +". Still smaller doses cause moderate (+ + or +) excitation, usually with somewhat incoordinated attempts at grooming, or a continued circling around the cage at a walking pace. During recovery, which took place after 1–5 min, the animals were subdued.

Depressant amino acids produced loss of muscle tone and gross incoordination of movement. The animals were unable to right themselves if placed on their back or sides, and tended after a short period to fall asleep. Recovery from these effects, denoted "– – –", was very slow (greater than 30 min). Smaller doses of the depressants produced incoordination of movement and transient flaccidity, after which gradual recovery occurred. Lethal dosages of the depressant amino acids were not determined and although 200 μg of 3-amino-propane-sulphonic acid impaired respiration in two of the four mice tried, one of which subsequently died, this result is of doubtful significance.

The relative potencies of the amino acids in both series is very similar to that found on other preparations and by other methods of drug administration. N-Methyl-D-aspartic acid was the most potent substance, but the minimum lethal dose found for this compound was about ten times greater than that reported by Kita *et al.*[1] It is interesting also that taurine had a depressant effect not greatly lower than that of γ-amino-*n*-butyric acid. This result is in accordance with those of Curtis and Watkins[5] on cat cerebral cortical neurones, although Purpura *et al.*[7] have reported that taurine has no action on cat cortical cells.

There would appear to be little justification for the suggestion of Kita *et al.*,[1] that N-methylaspartic acid may have a transmitter function in the central nervous system. As yet, there is no report in the literature of the occurrence of N-methyl-aspartic acid in nervous tissue, nor indeed in any biological tissue. In an investigation of the free acidic amino acids of sheep brain, no N-methyl-aspartic acid was found.[8] Further, the L-form, which would be the more likely enantiomorph to occur naturally in mammals (including rodents), is not significantly stronger than either L-aspartic or L-glutamic acids, both of which occur abundantly in nervous tissue. L-Cysteic and L-homocysteic acids are also potent excitants[3] and could well be considered if an L-amino acid is found to have a role as an excitatory transmitter in the central nervous system. However, in discussing this question, Curtis and Watkins[2, 3] have been led to conclude that a transmitter function of amino acids is unlikely.

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THE EXCITATION AND DEPRESSION OF MAMMALIAN CORTICAL NEURONES BY AMINO ACIDS

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Amino acids related to L-glutamic and γ-amino-*n*-butyric acid have been administered electrophoretically, and by pressure ejection, into the extraneuronal environment of single neurones in the pericruciate cortex of cats anaesthetized with allobarbitone or allobarbitone-urethane. Acidic amino acids related to glutamic acid, particularly *N*-methyl-D-aspartic acid, excited cortical neurones. Neutral amino acids related to γ-amino-*n*-butyric acid, particularly 3-amino-1-propanesulphonic acid, depressed cortical neurones. Some of the depressants blocked the antidromic invasion of Betz cells by pyramidal volleys. There are no essential differences between the sensitivities of cortical and spinal neurones towards locally administered amino acids. A transmitter function of such amino acids within the mammalian central nervous system is considered unlikely.

When ejected electrophoretically from glass micropipettes, many acidic amino acids related to glutamic acid excite neurones in the feline spinal cord (Curtis, Phillis & Watkins, 1960; Curtis & Watkins, 1960b, 1963), brain stem (Curtis & Koisumi, 1961), lateral geniculate nucleus (Curtis & Davis, 1962), thalamus (Curtis & Andersen, 1962; Andersen & Curtis, 1964a), retina (Noell, 1960), cerebral cortex (Krnjević & Phillis, 1963a), cerebellar cortex (Krnjević & Phillis, 1963a; Crawford Curtis, Voorhoeve & Wilson, 1963) and hippocampal cortex (Andersen, Crawford & Curtis, unpublished). In contrast, neutral amino acids related to γ-amino-*n*-butyric acid (GABA) are depressants (Curtis, Phillis & Watkins, 1959; Curtis & Watkins, 1960a, b, 1961; Noell, 1960; Krnjević & Phillis, 1963a). The similarity in the actions of these particular amino acids upon spinal interneurones, Renshaw cells, motoneurones, and geniculate and thalamic neurones has led to the use of the term "unspecific" when describing the excitant and depressant effects. Thus it has been considered that the actions of these two classes of amino acids are not closely related to the normal processes of synaptic transmission, although it is conceivable that some of these compounds may have a nonsynaptic regulatory function within the nervous system.

Recent experiments in which amino acids were ejected near single cortical neurones (Krnjević & Phillis, 1963a) have shown that in general the amino acid sensitivity of these cells is very similar to that of spinal interneurones. However, certain of the differences which have been reported raise doubts as to the non-

specificity of amino acid action. Whereas D-glutamic acid was approximately half to two-thirds as potent as the L-isomer as an excitant of spinal (Curtis & Watkins, 1963) and thalamic neurones (Andersen & Curtis, 1964a), cortical neurones are stated to be very much more sensitive to the L- than to the D-isomer (Krnjević & Phillis, 1961). Furthermore, *N*-methyl-D-aspartic acid, a powerful excitant of thalamic, geniculate and spinal neurones (Curtis & Watkins, 1963) has been reported to desensitize cortical neurones to subsequent administration of L-glutamic acid (Krnjević & Phillis, 1963a), a phenomenon which had not been observed elsewhere in the nervous system in the absence of a depression of nerve cell excitability following excessive depolarization (Curtis & Watkins, unpublished). Of the neutral amino acids, Krnjević & Phillis (1961, 1963a) reported that taurine and 3-amino-1-propanesulphonic acid were inactive when ejected near cortical neurones ; however, both compounds, particularly the latter, were depressants of spinal interneurones (Curtis & Watkins, 1960b, 1961).

It was felt that some of these conflicting findings may have arisen from differences in the technique of testing neuronal excitants and depressants, and in the interpretation of the observed results. Consequently the amino acid sensitivity of neurones in the pericruciate region of cats anaesthetized with allobarbitone has been reinvestigated, and the results compared with the sensitivity of spinal interneurones. It is concluded that there are no essential differences between the behaviour of these types of cells towards electrophoretically administered amino acids.

These results were presented briefly at the August (1963) meeting of the Australian Physiological Society.

METHODS

All experiments were performed upon cats anaesthetized with allobarbitone (Dial, Ciba Ltd.) or Dial Compound (Ciba Ltd.) which contained allobarbitone (100 mg/ml.) and urethane (400 mg/ml.). The usual dose was 35 to 50 mg/kg of allobarbitone injected intraperitoneally. The pyramids were exposed by partial removal of the basi-occiput, and the animal's head was then mounted in a rigid frame which could be tilted in two directions so that the pericruciate cortex could be placed approximately in a horizontal plane. The cat was suspended by a pelvic and mid-thoracic clamp with the abdomen and thorax held clear of the base of the animal frame. The preparation was maintained at 37±1° C by two heating pads, one of which was automatically controlled.

The pericruciate cortex was exposed by removal of the overlying bone and the posterior wall of the frontal sinus. The usual exposure was confined to one side of the skull, and extended for 1 to 1.2 cm laterally from the mid-line and 0.7 to 0.8 cm caudal to the coronal suture. After opening the dura the cortex was covered with several layers of polyethylene or cellophane sheeting, apart from a small opening left at the site of recording. During microscopic examination of the cortex the pia mater was gently removed with fine forceps from a suitable avascular area, and throughout the experiment mammalian Ringer solution (at 38° C and saturated with 95% oxygen and 5% carbon dioxide) irrigated the exposed tissue. In order to stabilize the cortex a small transparent pressure plate was applied to the site of recording without affecting the blood flow in underlying vessels (see Phillips, 1956).

Experiments were also carried out upon spinal interneurones in cats anaesthetized with pentobarbitone sodium (see Curtis & Watkins, 1960b).

The microelectrophoretic method of controlling the efflux of substances from the four drug-containing barrels of five-barrel micropipettes has been described in previous publications (Curtis & Eccles, 1958 ; Curtis & Watkins, 1960b, 1963). The overall tip diameter of the

micropipettes was 4 to 8 μ, and filling was carried out by centrifugation. Thus amino acids could be tested within a short time of preparation of the solution. This was particularly necessary when alkali (sodium hydroxide) had been added to produce a *p*H in excess of 8.5, since the *p*H fell during storage of pipettes which contained such solutions. This was presumably due to absorption of carbon dioxide from the atmosphere and to interaction between the alkali and Pyrex glass. When storage was necessary, filled micropipettes were placed in an atmosphere of nitrogen (3 to 4° C).

The concentrations of the amino acid solutions which were used are given in the Tables of this paper. When it was necessary to alter the *p*H in order to produce adequate ionization of these compounds (see Curtis & Watkins, 1960b), hydrogen ion concentrations were measured by means of a glass electrode system.

Extracellular spike responses were recorded by means of the central barrel of the five-barrel micropipette, which contained 4 to 5 M-sodium chloride solution. This was connected to a cathode follower by a small silver-silver chloride junction, and after suitable amplification the spikes were displayed on two double-beam oscilloscopes. Photographic records could be made from one of these oscilloscopes, whilst the other was used to monitor a firing frequency indicating system (Andersen & Curtis, 1964a). Essentially this consisted of a pulse generator which was triggered by the amplified spikes. The triggering level could be altered manually to discriminate between large and smaller spikes; the output pulses were observed simultaneously with the spikes and were counted by a rate meter. Thus the firing frequency of a cell was displayed continuously on a paper recorder. The use of this system ensured that for each test of an amino acid the ejecting current should be maintained until a maximum excitant or depressant effect was observed. Because of the comparatively slow response time of this recording system the plots of firing frequency (see Figs. 1 and 2) did not permit an accurate assessment of the latencies of onset and offset of amino acid-induced firing. Filmed records were necessary for this purpose.

The electrophoretic currents were measured with an accuracy of ±0.5 nA, and the terms "cationic" and "anionic" are used in this paper to describe currents which eject cations and anions respectively. One barrel of most micropipettes contained a 0.2 M solution of DL-homocysteic acid (*p*H 8–8.5). When ejected as an anion this amino acid is a powerful excitant of neurones (Curtis & Watkins, 1963) and enables ready detection of otherwise quiescent cells. Care had to be taken however that excessive amounts of this amino acid were not ejected and, in general, anionic currents of only 5 to 10 nA were allowed to flow intermittently when cells were being looked for within the cortex.

In several experiments hydrostatic pressure was used to eject amino acid solutions from the individual barrels of micropipettes which had comparatively large orifices (2.5–3.5 μ internal diameter). Silver wires within the sealed system allowed an appropriate retaining voltage to be applied to each barrel. This method of ejection was found to be unreliable, presumably because of plugging of the pipette orifices by tissue components. In some cases pressures of less than 100 mg Hg were adequate to eject sufficient DL-homocysteic acid to excite neurones, in other cases pressures as high as 350 mm Hg were ineffective, although the same cells could be readily excited when the amino acid was ejected electrophoretically as an anion from the same pipette. Observations upon the effect of a test substance ejected by pressure have been included only when either DL-homocysteic or L-glutamic acid excited neurones when ejected with pressures of less than 200 mm Hg from other barrels of the same micropipette assembly. This requirement does not however completely exclude the possibility that substances in adjacent barrels were not ejected by even higher pressures.

The amino acids used were all of analytical purity, and were either provided by Dr. J. C. Watkins (Watkins, 1962) or purchased commercially (California Corporation for Biochemical Research; British Drug Houses, Ltd.). One sample of D(−)-glutamic acid was supplied by Dr K. Krnjević, and when tested upon spinal interneurones was indistinguishable from the sample used in the present investigation.

In view of the difference between the present findings upon the potencies of D(−)- and L(+)-glutamic acid, and those of Krnjević & Phillis (1963a), the optical rotation of the samples

of these substances was kindly determined by Dr A. M. Sargeson upon 2% solutions in 5 M-hydrochloric acid, with the results

$$[\alpha]_D^{20} \text{ L}(+)\text{-glutamic acid} = +29.5°$$
$$[\alpha]_D^{20} \text{ D}(-)\text{-glutamic acid} = -30°$$

RESULTS

This investigation was confined to neurones in the pre- and postcruciate region, many of which were firing spontaneously, either in a random fashion or as spindles (Dempsey & Morison, 1943; see also Verzeano & Calma, 1954). The majority of the cells were identified as Betz cells by the responses evoked by single or tetanic stimulation of the pyramids (Phillips, 1956, 1959, 1961); these cells were found at depths of between 0.8 and 1.3 mm below the cortical surface. The relative sensitivities of pyramidal cells and of the more superficially located neurones towards excitant and depressant amino acids did not appear to be different. Thus the general term "cortical neurone" will be used throughout this report to indicate a cell which was located within 2 mm of the cortical surface, and from which spike responses could be produced by the local ejection of an excitant amino acid. The distribution of these neurones was similar to that found in other studies (see Amassian, 1961). It was usually possible to move the micropipette until the extracellular spike responses of one neurone were of the order of 250 to 500 μV (negative-positive), and so could be clearly distinguished from the smaller spikes of more distant cells. Under such circumstances the effects of the flow of current associated with electrophoretic ejection were minimal (see Curtis & Koizumi, 1961) and could readily be distinguished from amino acid actions by testing the effect of current flow through the micropipette containing sodium chloride. In some instances the spike response of a particular neurone could be identified over a distance of 50 to 100 μ, and under these conditions there was little difference between the comparative potencies of the amino acids determined at several different levels of spike size.

One difficulty common to many neurones was the change in spike shape which was observed during activation by an amino acid. Such changes, which are completely reversible, were originally observed when excitant amino acids were ejected near spinal interneurones (Curtis *et al.*, 1960), particularly with L-glutamic acid. It was proposed that the observed "depolarization block," in which the initially negative-positive spike potentials were converted either to predominantly positive spikes, or merely decreased in size before failing entirely, was associated with the change in membrane conductance and the depolarization produced by the excitant (Curtis *et al.*, 1960). In general, these alterations in spike shape of cortical neurones occurred at lower firing frequencies (20 to 60 spikes/sec) than has been observed with spinal interneurones, thalamic and geniculate neurones. As cortical neurones could be fired synaptically (or antidromically in the case of Betz cells) at higher rates than this, it was considered that the comparative ease with which depolarizing substances inactivated the spike generating mechanism might be related to disturbances in cerebral cortical circulation consequent upon the surgical procedures. Corpuscular clumping was usually evident in the smaller cortical veins, and it is possible that all the cortical neurones studied were slightly depolarized as a result

of tissue hypoxia. Deterioration in the cortical circulation and heightened sensitivity to excitant amino acids were most evident towards the end of experiments which lasted 12 to 18 hr.

Action of acidic amino acids

The difficulties which are associated with the assessment of the relative potencies of electrophoretically administered excitant amino acids have been discussed by Curtis & Watkins (1963). The most satisfactory technique is to obtain records from a large number of neurones, and to compare the maximal effects of different doses of amino acids against a reference compound (in this investigation L-glutamic acid). Reasonably consistent results were obtained for any one compound when tested upon four or five cells in each of two or three preparations. Fresh solutions, in various combinations and in fresh micropipettes, were used for each preparation. The relative potencies of amino acids were determined from the ratio of ejecting currents which produced equal firing frequencies, and therefore express the ratios of concentrations required for equal effects, a method generally accepted when the activities of agents interacting with a common receptor are compared. This method is preferable to a comparison between the effects produced by equal extraneuronal concentrations (see Krnjević & Phillis, 1963a, p. 282) as, in the latter instance, differences in response, and therefore in potency ratios, depend upon the portions of the dose/response curves which are being studied.

The range and mean potencies are given in Table 1, together with the number of separate tests. Furthermore, to facilitate comparison, the potency of these amino acids as excitants of spinal interneurones is also included (from Table 1, Curtis & Watkins, 1963). The time parameters of the excitation of cortical neurones by the various amino acids were very similar to those found for spinal neurones. The excitant action of all acidic amino acids was readily reversible, and recovery occurred even after long periods of "depolarization block" had been produced by the administration of excessive amounts of amino acid.

N-Methyl-D-aspartic acid was the most potent excitant amino acid of those tested, and had the longest duration of action after the electrophoretic current was termi-

TABLE 1

EXCITANT AMINO ACID POTENCIES

* Data from Curtis & Watkins (1963). † With solutions at *p*H 3·1, delayed excitation was observed (see text). ‡ Data from Curtis & Watkins (1960b). Sat. = saturated.

Amino acid	Concentration within micropipette (M)	*p*H of solution	Potency upon cortical neurones: Mean	Range	Tests	Potency upon spinal interneurones
N-Methyl-D-aspartic acid	0·1	8·3–8·6	6·5	2–16	24	5–12*
DL-Homocysteic acid	0·2	8·0–8·5	4	2·5–6	23	2–6*
L-Glutamic acid	2	8·0–8·2	1·0	Standard		1*
L-Aspartic acid	2	8·2–8·5	1	1–1·2	5	1*
D-Glutamic acid	2	8·0–8·3	0·7	0·5–0·8	16	0·5–0·7*
D-Aspartic acid	2	8·2–8·4	0·8	0·7–1·0	5	0·5–0·7
L-Asparagine	Sat.	3·1–4·5	Inert†		17	Inert ‡
D-Asparagine	Sat.	3 and 5	Inert		9	

nated (Curtis & Watkins, 1963). In some experiments diffusion of this substance from a 0.1 M solution was sufficient to fire neurones. When ejected with currents of 3 to 10 nA this amino acid produced a graually increasing rate of firing over 15 to 30 sec (Fig. 1), and the rate declined over a period of 2 to 10 sec after the ejection had ceased. When larger amounts of *N*-methyl-D-aspartic acid were used (currents of 12 to 30 nA) the cell increased its rate of firing more rapidly until a "depolarization block" was observed, the spike then being indistinguishable from the background noise. Simultaneously the spontaneous and antidromic spikes of the neurone were also blocked. Several seconds after the current which ejected the amino acid had been terminated, spikes often reappeared transiently for about 1 sec, presumably as portions of membrane, which had been rendered incapable of generating spikes by the amino acid, recovered and the level of depolarization remained above the threshold for spike generation.

The high potency of this compound probably explains the finding of Krnjević & Phillis (1963a) that *N*-methyl-D- and *N*-methyl-DL-aspartic acid caused a prolonged

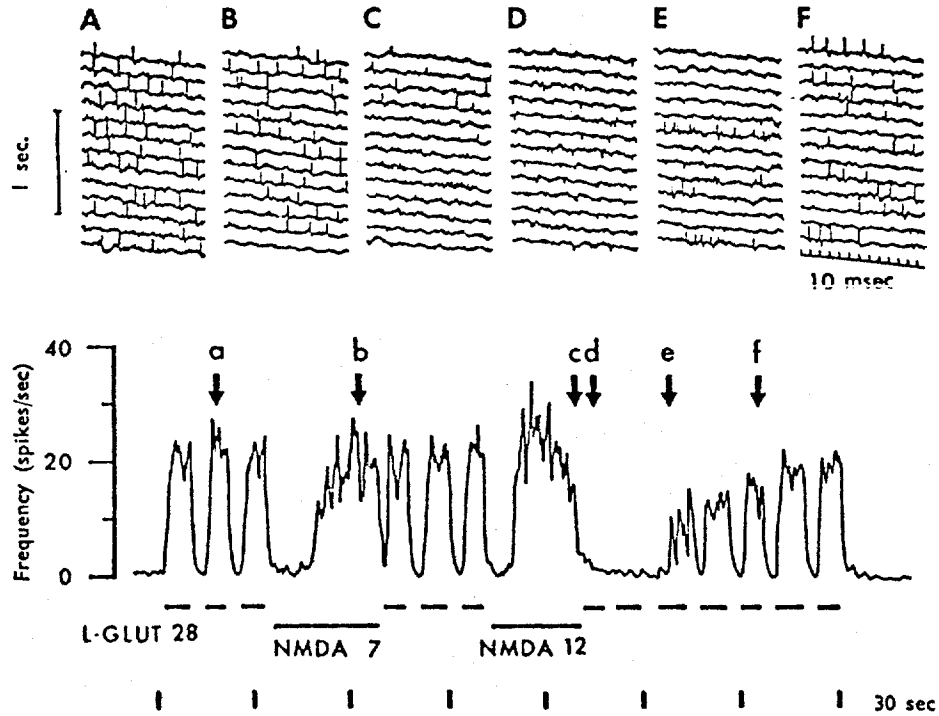

Fig. 1. Effect of *N*-methyl-D-aspartic acid (NMDA, retaining current 4nA) upon the firing of a neurone located in the postcruciate area by L-glutamic acid (L-GLUT, 28nA). In the lower half of the figure successive ejections of each of these compounds are indicated by horizontal bars (upper bars, L-glutamate ; lower bars NMDA), with the frequency of firing (spikes/sec) plotted against time. Sections (A-F) of the concurrent filmed records of the extracellular spike potential (negative upwards) are placed above this frequency record, each being representative of the spikes at times indicated by the arrows and the letters a-f (see text). Time, 10 msec for filmed records ; 1 sec for rate of vertical film movement ; 30 sec for paper recorder.

reduction in the sensitivity of cortical neurones to L-glutamic acid. Many tests of this interaction were carried out, but the finding could be repeated only if sufficient *N*-methyl-D-aspartic acid had been administered to produce a block of neuronal spikes. The results from one such experiment are shown in Fig. 1. Initially three successive ejections of L-glutamic acid (28 nA) gave reasonably consistent firing rates of about 20 spikes/sec. Following this, the ejection of *N*-methyl-D-aspartic acid (7 nA) produced a firing frequency of 15 to 20 spikes/sec, with no change in the shape of the spike potential (compare A and B), and the firing produced by the following dose of L-glutamic acid (within 3 sec of the end of the ejection of *N*-methyl-D-aspartic acid) was unaffected. However, when a current of 12 nA was used to eject *N*-methyl-D-aspartic acid a maximum frequency of approximately 30 spikes/sec was attained, the spike was altered in shape, and the cellular responses were eventually blocked (C). Subsequently, two doses of L-glutamic acid produced only small positive spikes (D) but, 35 sec after the cessation of the *N*-methyl-D-aspartic acid ejection, L-glutamic acid again fired the cells with negative-positive spikes. Initially these spikes were small (compare E with A) and full recovery of spike size (F) was observed after 1 min.

Although in most cases the onset of complete block by *N*-methyl-D-aspartic acid was comparatively slow, with several cells and particularly with electrophoretic currents greater than 20 nA the onset was abrupt and the spikes suddenly vanished with little or no preliminary alteration in shape. Following the administration of sufficient *N*-methyl-D-aspartic acid to suppress cellular responses the usual sequence of recovery was return of antidromic invasion, restoration of spike amplitude and shape, and finally re-establishment of the sensitivity to L-glutamic acid.

Similar observations have been made upon the apparent reduction in sensitivity of neurones to L-glutamic acid by prior administration of DL-homocysteic acid and *N*-*n*-propyl-D-aspartic acid, and also to test doses of DL-homocysteic acid by *N*-methyl-D-aspartic and L-glutamic acids. Furthermore, experiments upon spinal interneurones have failed to demonstrate any depression of the firing induced by either L-glutamic or DL-homocysteic acid by a preceding dose of *N*-methyl-D-aspartic acid. In fact, as occasionally observed with cortical cells, the effectiveness of L-glutamic or DL-homocysteic acid was actually increased when these were ejected shortly after the administration of *N*-methyl-D-aspartic acid was terminated. When sufficient of the latter was ejected to produce a depolarization block of spinal interneurones the effects of subsequent doses of other excitant amino acids were reduced, as found with cortical neurones.

Several cortical cells were tested with graded doses of *N*-methyl-D-aspartic acid and it was found that there need be a difference of only 10 to 20% between currents (concentrations) which produced neither "depolarization block" nor a reduction in the sensitivity and those which did produce block and reduction in the sensitivity. It was thus concluded that the diminution in amino acid sensitivity was primarily a consequence of alterations in membrane conductance and potential, rather than of receptor desensitization due to a prolonged occupation of amino acid receptors by the *N*-alkyl excitant amino acid. This phenomenon is possibly related to the frequently observed reduction in the spontaneous discharge rate which follows

induction by amino acids of high frequency firing of spinal, thalamic and cortical neurones.

Action of depressant amino acids

Neutral amino acids related to GABA reduce the sensitivity of neurones in the cortex to depolarization by acidic amino acids, and to excitation by other means. The potency of these substances can be most readily assessed in terms of the

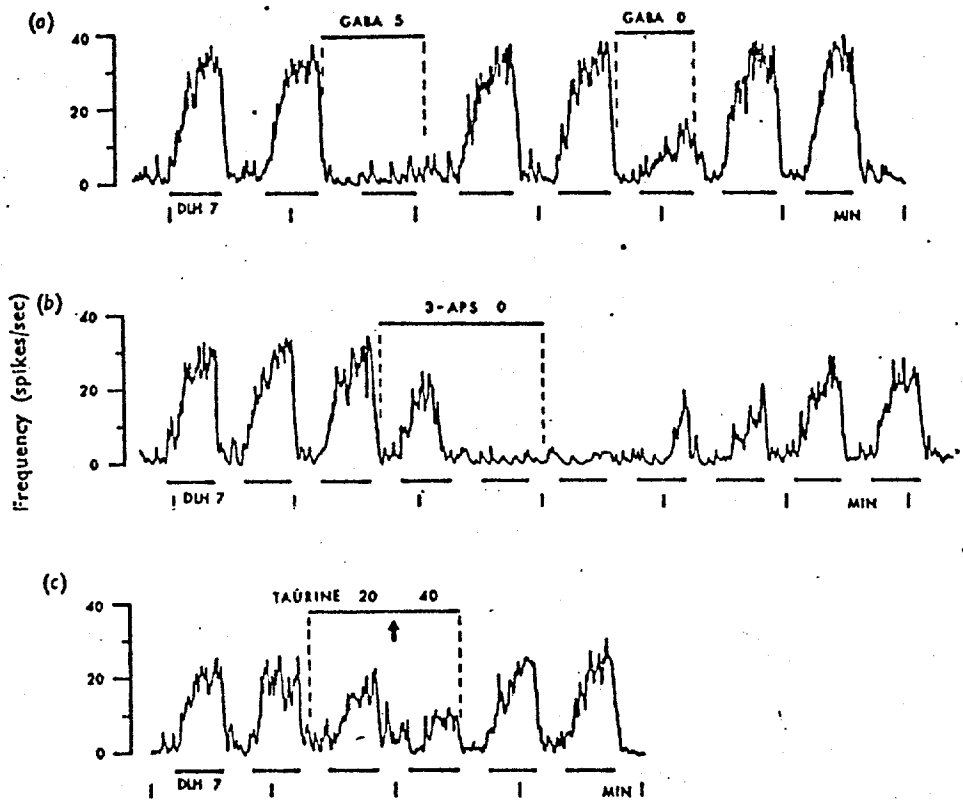

Fig. 2. (*a*) The effect of γ-amino-*n*-butyric acid (GABA) upon the frequency of the firing of a cortical neurone induced by repeated doses of DL-homocysteic acid (DLH, currents of 7nA, lower signal trace). GABA was retained by an anionic current of 20 nA and was ejected during the first interval (upper bar) by a cationic current of 5 nA. During the second interval the retaining current was terminated, allowing free diffusion of this substance from the micropipette which contained a molar solution (*p*H 2·9). Note the short duration of depressant action.
(*b*) Firing frequency of the same cortical unit as in Fig. 2*a*, showing the effect of the removal of the retaining current (anionic, 4nA) from a barrel which contained 3-amino-1-propane-sulphonic acid (3APS, 0·1M, *p*H 9·4). The lower horizontal bars again indicate repeated ejections of DLH, using currents of 7nA. The depressant action of the neutral sulphonic acid lasted for over 2 min after the retaining current was reapplied.
(*c*) The effect of taurine upon the DLH-induced firing (lower bars, 7nA) of the same cortical cell as (*a*) and (*b*). During the period of the upper horizontal bar taurine was ejected as an anion initially with a current of 20 nA which was doubled at the arrow. Time in minutes below each frequency plot.

electrophoretic current needed to produce a specified reduction in the firing rate of a cell excited by an acidic amino acid. In this series of experiments DL-homocysteic acid was used as the control excitant substance, and was ejected either continuously or at fixed intervals and for constant times (see Fig. 2, *a* to *c*). Table 2 lists the neutral amino acids used in this study, together with the concentrations and *p*Hs of the solutions. Potencies are expressed relative to that of GABA (– – –), the number of symbols indicating greater (– – – –), less (– –) and very weak (–) activity.

TABLE 2

DEPRESSANT AMINO ACID POTENCIES

The potency of cortical neurones is relative to γ-amino-*n*-butyric acid (– – –) ; – – – –, greater; – –, less ; – very weak. *Late excitation with low *p*H solutions, see text.

Amino acid	Concentration within micropipette (M)	*p*H of solution	Potency upon cortical neurones
γ-Amino-*n*-butyric acid	1, 2	2·9–7·2	– – –
γ-Amino-β-hydroxybutyric acid	2	3·1	– –
γ-Hydroxybutyric acid	1	7	0
3-Amino-1-propanesulphonic acid	0·2	9–10·6	– – – –
Taurine	0·8	8·5–10	–
Guanidino-acetic acid	0·025	3–3·1	–
β-Guanidino-propionic acid	1	3	– –
ε-Aminocaproic acid	0·5	3·1–6·8	– *
ω-Aminocaprylic acid	0·5–1	3·0–7·7	0 *

γ-Amino-n-butyric acid (GABA). The depression of excitability, and of spontaneous firing, produced by this compound had a short latency of both onset and offset, and could be demonstrated by diffusion of GABA from a 2 M solution (Fig. 2, A) or when the amino acid was ejected electrophoretically from solutions within the range of *p*H 2.9 to 7.2 ($pK_1 = 4.23$). It is thus extremely unlikely that there is any difference between the action of GABA cations and zwitterions, as has been proposed by Kuno (1960, 1961), Muneoka (1961) and Kuno & Muneoka (1961, 1962); (see also Curtis *et al.*, 1959 ; Curtis & Watkins, 1960a, b ; Krnjević & Phillis, 1963a).

γ-Amino-β-hydroxybutyric acid was approximately half as potent as GABA as a depressant of the firing of cortical neurones induced by DL-homocysteic acid, whereas γ-aminobutyrylcholine was a very weak depressant. The available sample of this latter compound contained GABA, and it is possible that this accounted for the depressant action which was observed.

γ-Hydroxybutyric acid. Although not an amino acid, this compound was of interest because of recent reports which indicate that systemic administration of sodium γ-hydroxybutyrate results in the depression of activity in animals and humans (Laborit, Jouany, Gerard & Fabiani, 1960 ; Laborit, Kind & Regil, 1961 ; Drakontides, Schneider & Funderburk, 1962) and in a reduction of spinal reflexes (Basil, Blair & Holmes, 1964). This compound was ejected electrophoretically as an anion, and in several tests (currents as high as 100 nA) failed to modify either

the spontaneous activity of cortical neurones, or the spikes evoked by an excitant amino acid. (Similar results were observed when this acid was administered near spinal interneurones.)

3-Amino-1-propanesulphonic acid. This compound was sufficiently potent to block the firing of many cortical neurones induced by DL-homocysteic acid merely by diffusion from solutions of 0.1 M. Indeed, considerable difficulty was experienced in controlling the diffusional efflux of this amino acid from Pyrex micropipettes, due no doubt to the high electro-osmotic mobility of this substance in aqueous solution. Under suitable conditions (0.1 to 0.2 M solution, *p*H 9 to 10, pipette orifice less than 2 μ internal diameter) this amino acid could be ejected either as an anion or as a cation, although the diffusional efflux was controlled to a large extent by a small anionic current. When ejected as a cation, 3-amino-1-propanesulphonic acid proved to be a powerful depressant of all cortical neurones upon which it was tried, and was more potent than GABA. This is illustrated in Fig. 2, B where the diffusion of 3-amino-1-propanesulphonic acid from an 0.1 M solution depressed the firing of a cortical neurone induced by DL-homocysteic acid to a greater extent than did GABA which diffused from a pipette of approximately the same internal diameter containing a 2 M solution. Furthermore, the depressant action of 3-amino-1-propanesulphonic acid was of longer duration than that of GABA. Similar results were obtained using spinal interneurones. In earlier experiments upon spinal neurones the anomalous behaviour of 3-amino-1-propanesulphonic acid under these conditions led to the use of solutions of *p*H 2.8 for ejecting neutral amino acids near interneurones (Curtis & Watkins, 1961) and in such circumstances it was also greater and more prolonged than that of GABA.

As found previously in spinal cord (Curtis & Watkins, unpublished), if micropipettes had been stored for more than a few hours, even in nitrogen and with small sealed caps upon the open upper ends of the barrels, or if concentrated solutions of *p*H lower than 8.5 were contained within pipettes which exceeded approximately 2 μ in diameter, it proved impossible to control the diffusional efflux of 3-amino-1-propanesulphonic acid. In most experiments, despite the intermittent ejection of DL-homocysteic acid from an adjacent barrel, very few neurones were located when these micropipettes were passed through cerebral or spinal tissue.

Taurine. This compound was readily administered as an anion from solutions of *p*H above 8.5. When ejected with currents of 20 to 80 nA, taurine caused depression or abolition of the response to DL-homocysteic acid of all cells which were tested (see Fig. 2, C), and although weaker than either GABA or 3-amino-1-propanesulphonic acid, taurine had an action upon cortical neurones similar to that already reported for neurones in the spinal cord (Curtis *et al.*, 1959; Curtis & Watkins, 1960a, b). The action of taurine persisted for 10 to 15 sec after the termination of the ejection, a duration which was intermediate between those of GABA and 3-amino-1-propanesulphonic acid.

Guanidino-acetic acid (glycocyamine) and β-guanidino-propionic acid. These compounds were not tested as depressants of the synaptic responses of cortical neurones, but guanidino-propionic acid was slightly more active than glcocyamine as a depressant of the firing of these cells induced by DL-homocysteic acid.

It might be expected that, because of the efficacy of synaptic excitation, the post-synaptic action of a weak depressant amino acid would not be sufficient to affect such responses, although this substance might interfere with the access of an excitant amino acid to membrane receptors (see Curtis & Watkins, 1960b) and so reduce its effectiveness. A re-examination of the action of guanidino-acetic acid upon spinal interneurones has confirmed the lack of action of this compound upon the synaptic responses of these cells, but has demonstrated a reduction in the frequency of firing produced by concurrent administration of an excitant amino acid. On the other hand, β-guanidino-propionic acid was found to be a more potent depressant since it diminished both the synaptic and amino acid evoked firing of spinal interneurones.

Other compounds related to these amino acids

ε-Aminocaproic acid and ω-amino-caprylic acid. These were found, in agreement with Krnjević & Phillis (1963a), to have "somewhat ambiguous" actions when ejected electrophoretically. Solutions of *p*H 3 were necessary to increase the degree of ionization of these compounds and so permit sufficiently large ejecting currents to be passed. Under these conditions ε-aminocaproic acid had a slight depressant action on cortical neurones but, after a latency of 15 to 90 sec, paroxysmal high frequency firing was produced by both compounds and continued for up to 15 sec after termination of the current flow. This excitatory phenomenon could not be reproduced by ejecting neutral solutions (*p*H 6.7 to 7.7) of either of these compounds by means of pressure (up to 300 mm Hg for 3 min), although in most experiments when ε-aminocaproic acid was so administered a depression of the firing of cortical neurones induced by DL-homocysteic acid was observed.

Thus the delayed excitation appears to be related to the effects of local *p*H changes produced when the cationic form of the ejected amino acid releases its extra proton on reaching the medium around the micropipette tip (see Curtis & Watkins, 1960b, p. 119). GABA and closely related short chain ω-amino acids are presumably sufficiently potent depressants to mask this delayed effect.

D- *and* L-*Asparagine.* These were inactive when administered electrophoretically (currents of 30 nA) or by pressure, unless the *p*H of the solutions was below 4. In such experiments a delayed excitation was observed with subsequent depression of the sensitivity of the cell to DL-homocysteic acid which lasted for 3 to 4 min. A similar delayed firing was observed with spinal interneurones (Curtis & Watkins, 1960b) but, as with the longer chain ω-amino acids, was considered to result from local *p*H changes.

Antidromic invasion of Betz cells

If the mode of action of GABA, and related depressants, upon cortical neurones is the same as that which has been found for spinal motoneurones, namely an increase in membrane conductance which reduces the effectiveness of depolarizing currents (Curtis *et al.*, 1959), it would be expected that the antidromic invasion of Betz cells would be depressed by these substances.

Even in the absence of spontaneous firing the safety factor for the antidromic invasion of the body and dendrites of Betz cells is extremely high (see Phillips, 1959) and it was anticipated that some difficulty would occur when attempting to test the effect of GABA upon this form of firing. This proved to be the case, and only in rare instances, using currents of the order of 100 to 200 nA, could sufficient GABA be ejected electrophoretically to block antidromic firing. In several instances GABA converted a soma-dendritic (A or SD) spike to a smaller initial segment (B) spike, but more often there was complete suppression of the soma-dendritic spike to leave a small axonal or M spike. The depression of these antidromic responses was readily measured by the "firing index," which expressed the number of soma-dendritic spikes as a percentage of ten supramaximal axonal stimuli.

3-Amino-1-propanesulphonic acid was more effective than GABA as a depressant of cortical neurones, and the action of this amino acid upon the antidromic spikes of one cell are illustrated in Fig. 3. Throughout the series the stimulus applied

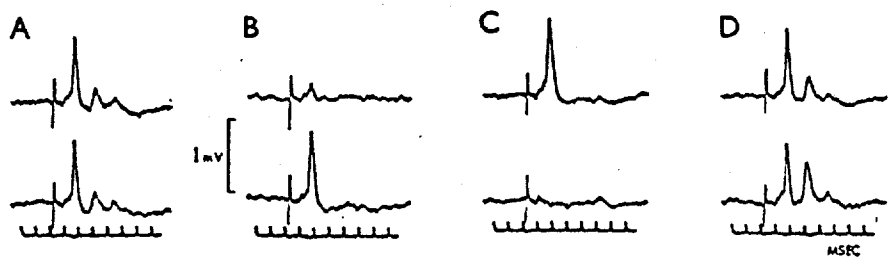

Fig. 3. The effect of 3-amino-1-propanesulphonic acid (3APS) upon the antidromic invasion of a Betz cell in the postcruciate area. Each column shows two successive responses to supramaximal pyramidal stimuli delivered at a rate of 1 shock/sec. Negativity is recorded upwards. A, control ; firing index 100%. B, approximately 10 sec after a current of 80nA began to eject 3APS ; firing 80%. C, 10 sec after this current was increased to 150nA ; firing index 50%. D, 10 sec after the electrophoretic ejection of 3APS was terminated : firing index 100%. Voltage scale, 1 mV, time scale in msec.

to the pyramids was supramaximal for the axon of this Betz cell, and the antidromic spike potential (latency 1.27 msec) was followed by a complex field potential produced by the antidromic and synaptic excitation of other nearby neurones. The firing index of this cell was initially 100% (A), but 10 sec after a cationic current of 80 nA began to eject 3-amino-1-propanesulphonic acid the index was 80% (B). When the electrophoretic current was increased to 150 nA the firing index fell to 50% (C), and full recovery to the original value was observed after the termination of the current (D). The depression, and recovery, of the field potentials generated by other nearby neurones is also apparent in this series.

DISCUSSION

The importance of the present results, and those of Krnjević & Phillis (1963a), lies in the evidence which has been obtained regarding the sensitivity of unidentified pericruciate neurones and Betz cells towards electrophoretically administered amino acids. Although this method of drug administration has some disadvantages (see

Curtis, 1964), particularly in respect to the concentrations which are attained, it is clear that when amino acids are tested by ejection from multibarrel micropipettes there are no essential differences between the amino acid sensitivity of cortical neurones and that of spinal interneurones (Curtis *et al.*, 1959, 1960; Curtis & Watkins, 1960a, b, 1961, 1963). A limited study of spinal Renshaw cells, lateral geniculate neurones (Curtis & Watkins, 1963) and neurones of the ventrobasal thalamus (Andersen & Curtis, 1964a) has also failed to reveal serious discrepancies in this pattern. This type of amino acid sensitivity is in marked contrast to the behaviour of neurones in the various regions towards acetylcholine, cholinomimetics and cholinergic blocking agents (Curtis, Phillis & Watkins, 1961; Curtis & Koizumi, 1961; Krnjević & Phillis, 1963b; Curtis, Ryall & Watkins, 1964; Crawford *et al.*, 1963) or towards indole and phenylethylamine derivatives (Curtis *et al.*, 1961; Curtis & Davis, 1962; Krnjević & Phillis, 1963c; Andersen & Curtis, 1964b).

There are, however, differences between the results reported here, and those of Krnjević & Phillis (1963a). Some of these discrepancies are no doubt due to differences in technique, and it is thus relevant that the method used to assess amino acid actions and potencies in the present investigation were identical with those used in recent studies in this laboratory upon spinal and thalamic neurones (see particularly Curtis & Watkins, 1963). In particular, the use of a rate meter and paper recorder permitted a close analysis to be made of firing frequency and a ready assessment of the maximum action for a particular current used to eject an amino acid. These refinements in technique led to a recent revision (Curtis & Watkins, 1963) of an earlier study of the action of amino acids upon spinal interneurones (Curtis & Watkins, 1960b).

The present findings on the relative potencies of the D- and L-isomers of aspartic and glutamic acid are similar to those of Krnjević & Phillis (1961, 1963a), but, although high accuracy cannot be claimed for such estimations, we do not agree with these investigators that D-glutamic acid was always "much less effective" than L-glutamic acid since the potency difference is of the order of only 0.5 to 0.8. The difference in the evaluation of these two amino acids has presumably arisen from the different methods used to assess excitant amino acid potency, as outlined in Results. The report that *N*-methyl-D-aspartic acid desensitized cortical neurones to subsequent doses of L-glutamic acid (Krnjević & Phillis, 1963a) has not been substantiated, since any such interaction between different amino acids upon cortical and spinal neurones appears to be associated with the presence of a "depolarization block" due to excessive concentrations of a powerful depolarizing agent, rather than to a specific interference of access to receptor sites. However, the slow recovery of amino acid sensitivity after the production of such a "block" (see Fig. 1) warrants additional investigation.

The failure of Krnjević & Phillis (1961, 1963a) to demonstrate a depressant action of electrophoretically administered taurine and of 3-amino-1-propane-sulphonic acid is rather surprising since, like GABA, each of these sulphonic amino acids readily depresses cortical responses when applied topically in solution to the exposed cortical surface (Curtis & Watkins, 1961; but see also Purpura, Girado, Smith, Callan & Grunfest, 1959). Furthermore, when injected intraventricularly

into mice, all three amino acids result in a diminution of spontaneous activity, a loss of righting reflexes and eventually a state resembling sleep (Crawford, 1963). Again the relative potencies of the amino acids were similar to those observed in electrophoretic studies upon cortical neurones and spinal interneurones.

In view of the inability to reproduce the delayed excitation of ω-aminocaprylic acid and asparagine by pressure ejection from neutral solutions, it cannot be stated with any degree of certainty that these amino acids excite neurones, or that the results obtained electrophoretically agree with those of Hyashi (1956), Purpura *et al.* (1959) and van Harreveld (1959) who administered amino acid solutions topically to the exposed cortex. On the other hand, if indeed these amino acids do excite central neurones, then the effects upon cortical and spinal neurones are practically identical, since the report by Curtis & Watkins (1960b) that ω-aminocaprylic acid and L-asparagine were inactive upon spinal interneurones was made on the basis that the delayed excitation was not directly associated with these amino acid molecules.

The finding that γ-amino-*n*-butyric acid, and particularly 3-amino-1-propane-sulphonic acid, block the antidromic invasion of Betz cells does not lend support to the postulate of Purpura, Girado & Grundfest (1957) and Purpura *et al.* (1959) (see also Grunfest 1958, 1960; Purpura, 1959, 1960) that GABA, and presumably also closely related amino acids, specifically block superficial excitatory axodendritic synapses. It is clear that, as with spinal motoneurones (Curtis *et al.*, 1959), GABA produces an alteration in the membrane conductance of cortical neurones (see also Bindman, Lippold & Redfearn, 1962). The difficulty which was experienced in blocking the antidromic invasion of Betz cells by pyramidal volleys was presumably associated with the high safety factor for this type of excitation, and with the limited proportion of the membrane of any one cell which was affected by the locally administered amino acid.

The failure of γ-hydroxybutyric acid to affect central neurones, both cortical and spinal, suggests that the alterations in behaviour, in the electroencephalogram and in spinal reflexes which are produced by this compound are unlikely to be due to a direct action upon central cells (Laborit *et al.*, 1960, 1961; Drakontides *et al.*, 1962; Basil *et al.*, 1964). Indeed the latent period between intravenous administration and the depression produced by γ-hydroxybutyrate (Drakontides *et al.*, 1962; Basil *et al.*, 1964) suggests that it has an indirect action, possibly as a result of interfering with the metabolism of GABA. It is pertinent to point out that a related substance, γ-butyrolactone, which also has central depressant effects (Giarman & Schmidt, 1963) does not increase the brain level of GABA. However total brain levels of GABA may not necessarily be related to the state of excitability of central neurones (see also Kamrin & Kamrin, 1961; Medina, 1963) and it may be more relevant to these studies to ascertain differences in the distribution of amino acids between intra- and extra-cellular compartments (see Curtis & Watkins, 1960a).

In conclusion, it may be stated that the actions of individual amino acids upon neurones within the spinal cord and cerebral cortex (and thalamus—Andersen & Curtis, 1964a) are very similar in both potencies and time courses. This similarity suggests either that some of these substance (or structurally related compounds)

are synaptic transmitters common to synapses upon all of these various types of neurone, or that amino acid receptors are components of the membrane of all neurones but are not directly associated with transmitter receptors, in which case the amino acid action could be considered to be nonspecific. Despite the impressive and striking action of amino acids such as GABA and L-glutamic acid, arguments against a role of either of these compounds as synaptic transmitters have already been presented (Curtis *et al.*, 1959, 1960 ; Curtis & Watkins, 1960a, 1963), and the recent findings of Ryall (1962) and of Weinstein, Roberts & Kakefuda (1963) are relevant in that the the subcellular distribution of GABA and L-glutamic acid in mammalian brain does not indicate any special aggregation of these amino acids in synaptic endings, as is found for acetylcholine (see Ryall, 1964). It is thus very probable that the action of these excitant and depressant amino acids is unrelated to the presence of specific transmitter receptor sites.

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THE EFFECTS OF EXCESS AMINO ACIDS ON THE GROWTH OF THE YOUNG RAT[1]

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High levels of single amino acids in the diet of rats beginning at 21 days produced a decreased food intake and a severe growth depression. These effects were dependent upon the kind and the concentration of amino acid supplemented. L-methionine caused the most severe growth depression while L-phenylalanine, L-tryptophan and L-cystine were also severely toxic. Less toxic were L-histidine, L-lysine and L-tyrosine. All other amino acids tested had only a slight effect on growth or none at all. Growth depression was attributed both to a depressed food intake and to a specific toxic effect of the amino acid. A direct correlation was found between the toxicity of the dietary amino acid and its concentration in the blood.

INTRODUCTION

Inborn errors of amino acid metabolism in humans are characterized by a) the presence in plasma and/or urine of a single amino acid or its metabolites in excessive concentrations and b) usually mental retardation of varying degree. No direct cause-effect relationship between the excessive amino acid and the mental aberration has been defined. These observations have stimulated attempts at developing animal model systems in which at least some of the biochemical counterparts of the diseased state can be reproduced. An initial step would be to correlate growth rate with high single amino acid environments. Although extensive study has been made on the relationship between excess phenylalanine and the clinical syndrome phenylketonuria in laboratory animals (Auerbach *et al.*, 1958; Wang and Waisman, 1961; Waisman and Harlow, 1965; Woolley and van der Hoeven, 1964; Boggs and Waisman, 1964; Kerr and Waisman, 1967), the effects of large excesses of other single amino acids have not been studied as methodically. Sauberlich (1961), Almquist (1951), Harper

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(1964) and Harper *et al.* (1966) have reported decreased growth rates in rats fed low protein diets supplemented with some excess amino acids. Further knowledge of these effects, especially on normal diets, would seem to be of particular importance if we are to enhance our understanding of the biochemical abnormalities encountered with the aminoacidemias induced by inborn errors of amino acid metabolism.

The present report describes the effects of 17 individual amino acids on the growth of the young rat under otherwise normal conditions.

Methods

Male albino rats (Sprague-Dawley strain, Holtzman Rat Company, Madison, Wisconsin) were obtained at 20 days of age and housed in individual wire-bottomed cages. The animals were allowed water *ad libitum* and fed ground laboratory chow, 24% protein content (Ralston-Purina Company, St. Louis, Missouri), for 24 hours prior to any experiment. Animals which did not gain weight normally during this first day were discarded. The acceptable rats were then placed in experimental groups by simple random distribution and fed diets in which the amino acids were added to the ground laboratory chow in the percentages (by weight) indicated in Table 1. All amino acids (Nutritional Biochemicals, Cleveland, Ohio) used in this study were the L-isomers. To minimize diurnal variation, a light-dark cycle of three hours was maintained throughout all experiments (Wang and Waisman, 1961). Controls were fed ground chow *ad libitum* or restricted to food intakes of assay animals. Daily records of the animals' food consumption and weight were kept for three weeks.

Amino acids used for injection experiments were dissolved in water or 0.05 N sodium bicarbonate with gentle heating when required. All intraperitoneal injections were made using a total volume of 2.0 ml. or less. Table 2 shows the amount injected per 55 g. rat. Sham injections of water or .05 N sodium bicarbonate served as controls. All animals were killed by decapitation after one hour of the light cycle. The blood plasma was collected and frozen in buffer prior to analysis for various amino acids by ion-exchange chromatography (Spackman *et al.*, 1958).

Results

Some effects of feeding single excess amino acids to young rats are presented in Table 1. Growth responses were dependent upon the

TABLE 1

GROWTH OF YOUNG RATS FED DIETS SUPPLEMENTED WITH EXCESS SINGLE L-AMINO ACIDS

Supplements added to basal diet[1]	Total weight gain g/3 wk[2]	Total food intake g/3 wk	Wt gain/g food consumed	Gain in weight as % control	% survival/expt
None	134 ± 11	254 ± 18	.53	100	100
+ 1% L-methionine	125 ± 9	245 ± 17	.51	93	100
+ 3% L-methionine	37 ± 3	150 ± 9	.25	28	100
+ 5% L-methionine[3]	−4 ± 0.3	103 ± 6	−.04	—	60
+ 5% L-phenylalanine	108 ± 11	218 ± 13	.50	81	100
+ 7% L-phenylalanine	38 ± 4	177 ± 14	.21	28	100
+10% L-phenylalanine	−8 ± 1	99 ± 7	−.08	—	10
+ 5% L-cystine	111 ± 9	244 ± 16	.45	83	100
+ 7% L-cystine	38 ± 4	163 ± 9	.23	28	100
+ 8% L-cystine[4]	18 ± 3	123 ± 8	.15	13	67
+ 5% L-tryptophan	128 ± 11	250 ± 22	.51	96	100
+ 7% L-tryptophan	55 ± 9	200 ± 16	.28	41	100
+10% L-tryptophan[5]	30 ± 3	205 ± 11	.15	22	100
+ 5% L-histidine	115 ± 13	190 ± 17	.61	86	100
+ 8% L-histidine	64 ± 7	153 ± 14	.42	48	100
+10% L-histidine	52 ± 5	132 ± 9	.39	39	100
+ 7% L-tyrosine	106 ± 18	227 ± 22	.47	79	100
+10% L-tyrosine	39 ± 11	162 ± 13	.24	29	33
+ 7% L-lysine	114 ± 11	276 ± 21	.41	85	100
+10% L-lysine	53 ± 4	168 ± 11	.32	40	67
+ 7% Glycine	130 ± 16	264 ± 23	.49	97	100
+10% Glycine	110 ± 9	240 ± 19	.46	82	100
+ 7% L-proline	130 ± 12	257 ± 17	.51	97	100
+10% L-proline	125 ± 11	250 ± 22	.50	93	100
+ 7% L-valine	130 ± 11	258 ± 17	.50	97	100
+10% L-valine	110 ± 9	230 ± 11	.48	82	100
+ 7% L-arginine	130 ± 13	290 ± 28	.45	97	100
+10% L-arginine	110 ± 8	288 ± 25	.38	82	100
+ 7% L-alanine	128 ± 4	250 ± 17	.51	96	100
+10% L-alanine	126 ± 7	247 ± 22	.51	94	100
+ 7% L-serine	128 ± 17	275 ± 23	.47	96	100
+10% L-serine	121 ± 11	261 ± 31	.46	90	100
+ 7% L-aspartic acid	130 ± 9	285 ± 22	.46	97	100
+10% L-aspartic acid	128 ± 12	280 ± 28	.46	96	100
+ 7% L-glutamic acid	130 ± 11	254 ± 17	.51	97	100
+10% L-glutamic acid	132 ± 10	248 ± 22	.53	99	100
+ 7% L-isoleucine	134 ± 11	271 ± 20	.49	100	100
+10% L-isoleucine	130 ± 13	270 ± 25	.48	97	100
+ 7% L-leucine	135 ± 12	294 ± 22	.46	100	100
+10% L-leucine	130 ± 13	290 ± 27	.45	97	100

[1] The number of rats fed each supplemented diet was eighteen.

[2] Initial weight of rats was 55 ± 3 g. Numbers are average ± standard error of the mean.

[3] Levels of more than 5% of L-methionine produced death in all animals in less than one week.

[4] Levels of more than 8% of L-cystine produced death in all animals by three weeks.

[5] An arbitrary maximum of 10% supplemented amino acid was used.

particular amino acid fed and also on its concentration in the diet. Methionine caused the most severe growth depression and produced early death at levels far below those of the other amino acids tested on a weight or molar basis. Tryptophan, phenylalanine and cystine

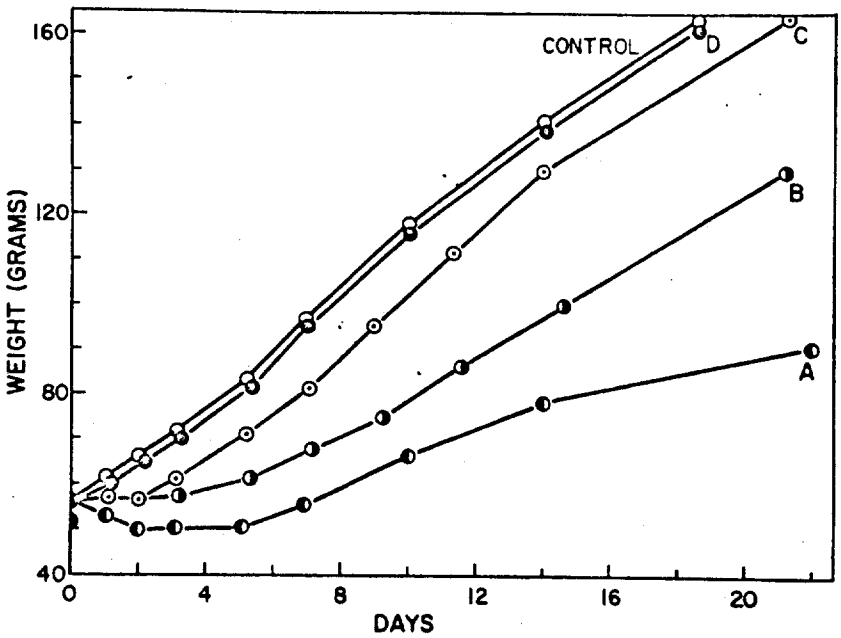

FIGURE 1

Growth of weanling rats on diets containing excess single amino acids.
Control—Ground laboratory chow
A—Chow diet supplemented with 3% of methionine, 7% of cystine, 7% of phenylalanine or 10% of tryptophan
B—Chow diet supplemented with 7% of tryptophan, 10% of lysine, 8% of histidine or 8% of tyrosine
C—Chow diet supplemented with 7% of either glycine, valine, proline or arginine
D—Chow diet supplemented with 7% of either alanine, glutamic acid, aspartic acid, leucine, isoleucine or serine
All animals were those described in Table 1.

also produced a severe growth depression and the higher concentrations fed resulted in death within several weeks. Histidine, lysine and tyrosine were moderately toxic while all the remaining amino acids tested had only a slight effect on growth or none at all. Figure 1 illustrates typical growth responses of animals fed the amino acid diets.

During the first week the growth depression was most severe but the animals adapted to the diets after this time. For the toxic amino acids growth resumed after one week and for the least toxic approximately after three days despite the animals' continuous consumption of the same diet.

In general, the most toxic amino acids in terms of poor growth caused the largest decrease in food intake. The decreased food intake was not proportional to the growth depression in all cases, however, but nearly so. The food intake of the animals fed the non-toxic amino acids was slightly increased over controls. Growth of animals fed diets supplemented with 3% of methionine or 7% of phenylalanine was in both cases depressed to 28% of that of controls yet the total food consumed was 150 and 177 g., respectively. Growth of rats fed diets supplemented with 7% of tryptophan, 10% of lysine or 10% of histidine was 40% of controls for each diet yet respective food intakes were 200, 168 and 132 g. Similar differences were noted with other amino acids especially between low levels of the toxic amino acids and higher levels of the non-toxic amino acids. In general, larger intakes were needed for the same weight gains when more toxic amino acids were fed. This would indicate that there was an effect of the amino acid *per se* on the growth of the weanling rat even though the most significant factor producing the growth depression would appear to be the reduction in food intake, whether due to its taste, metabolic effects or some other reason.

Animals fed excess tyrosine developed severe eye and paw lesions, an effect which has been previously described (Harper *et al.*, 1966) but which was most noticeable with the 10% tyrosine diet. When lower levels were fed, these lesions would also develop but disappeared after several weeks, even though the animals were consuming the same diet. Animals fed histidine consistently developed very large livers which were of a lighter color than normal livers. Lower weight gain was the only other physical change observed when the other amino acids were fed.

When newly weaned rats were injected with single large doses of individual amino acids daily for three days, they showed a pattern of toxicity similar to that observed in animals fed the same amino acids (Table 2). Loss of weight and death occurred with high amounts of nearly all of the amino acids injected. This cannot be due to de-

TABLE 2

EFFECT OF DAILY INJECTION OF INDIVIDUAL AMINO ACIDS ON GROWTH AND SURVIVAL OF WEANLING RATS

Amino acid injected[1]	Amount injected per day[2] mmoles	mg	Weight gain g/72 hours[3]	Food Consumption g/72 hrs.	Wt gain/g food consumed	% Survival 72 hours
Control (ad lib)	—	—	11.0 ± 1.0	21.0 ± 1.7	.52	100
Controlled feeding	—	—	3.0 ± 1.0	15.0	.20	100
	—	—	0 ± 0.5	9.0	—	100
	—	—	− 9.0 ± 1.0	3.0	− .30	100
	—	—	−16.0 ± 1.0	0	—	100
L-tyrosine	0.14	25	11.3 ± 0.9	23.1 ± 3.1	.49	100
	0.20	37	5.0 ± 0.5	18.1 ± 1.4	.28	100
	0.28	50	− 3.0 ± 0.2	10.4 ± 1.4	− .29	50
	0.38	67	—	—	—	0
L-tryptophan	0.25	50	6.1 ± 1.1	19.2 ± 1.7	.32	100
	0.37	75	− 0.7 ± 0.4	13.0 ± 2.2	− .05	100
	0.49	100	− 8.0 ± 0.7	4.7 ± 1.1	−1.70	33
L-methionine	0.30	45	13.4 ± 0.8	24.3 ± 2.9	.55	100
	0.60	90	− 3.4 ± 0.2	12.3 ± 1.1	− .28	100
	0.74	110	− 4.8 ± 0.8	10.7 ± 1.5	− .45	67
	0.90	134	—	—	—	0
L-phenylalanine	0.30	50	8.7 ± 0.7	23.0 ± 3.3	.38	100
	0.60	100	8.8 ± 2.1	22.3 ± 3.4	.39	100
	0.90	150	4.9 ± 0.5	18.0 ± 2.2	.27	100
	1.20	200	—	—	—	0
L-histidine	0.63	120	10.0 ± 2.0	18.5 ± 2.3	.54	100
	0.94	180	2.6 ± 0.9	14.8 ± 1.5	.18	100
	1.10	210	—	—	—	0
L-lysine	0.82	120	10.0 ± 2.3	20.7 ± 2.1	.48	100
	1.64	240	− 3.0 ± 0.8	12.0 ± 1.6	− .25	67
	2.05	300	− 5.5 ± 1.1	12.0 ± 2.1	− .46	33
Glycine	2.13	160	13.4 ± 3.1	24.2 ± 1.7	.55	100
	2.67	200	12.0 ± 1.1	24.5 ± 3.4	.49	100
	3.20	240	—	—	—	0
L-arginine	0.57	100	12.0 ± 1.5	26.0 ± 2.2	− .46	100
	1.15	200	5.7 ± 0.8	20.0 ± 2.3	.29	100
L-valine	1.15	135	12.6 ± 1.1	24.4 ± 2.8	.52	100
	1.92	225	3.0 ± 0.5	15.0 ± 2.4	.20	100
L-proline	1.39	160	10.4 ± 0.9	24.7 ± 3.4	.42	100
	2.78	320	10.5 ± 1.4	23.5 ± 2.6	.45	100
L-glutamic acid	0.82	120	9.0 ± 1.1	25.0 ± 4.5	.36	100
	1.64	240	6.5 ± 0.7	24.5 ± 3.6	.27	67
	1.90	280	—	—	—	0
L-alanine	1.09	150	10.0 ± 1.6	21.0 ± 3.6	.48	100
	2.80	250	6.3 ± 0.7	19.7 ± 2.9	.32	100
	3.37	300	5.5 ± 0.9	14.0 ± 2.2	.39	67
L-serine	1.90	200	10.0 ± 1.3	24.7 ± 3.4	.40	100
	2.86	300	6.3 ± 0.8	19.0 ± 2.3	.33	67
	3.33	350	—	—	—	0
L-leucine	0.76	100	11.0 ± 1.0	24.0 ± 2.2	.46	100
	1.27	167	6.0 ± 2.3	22.0 ± 1.7	.27	100

[1] All injections were made intraperitoneally on three successive days. The number of rats in each group was nine.

[2] Injection volumes were 2.0 ml or less; the highest concentration shown was soluble in this volume.

[3] Initial weight of rats was 55 ± 3 g. Numbers are average ± standard error of the mean.

crease in food consumption, which was also observed, since completely starved rats were able to survive for longer periods (at least four or five days), and rats fed the reduced food intakes noted in Table 2 survived for periods up to several weeks. Results differed from those in the feeding experiments in that tyrosine was the most toxic of the injected amino acids, 25 mg. or more producing severe growth depression or death. The lower toxicity after feeding could be due to relatively slow intestinal absorption (Table 3). Tryptophan is also relatively more toxic after injection than when fed, amounts of 50 mg.

TABLE 3
AMINO ACID CONCENTRATION IN PLASMA OF WEANLING RATS FED DIETS CONTAINING EXCESS SINGLE AMINO ACIDS

Supplement added to basal diet	Amino Acid Content[1] Blood plasma, 48 hr on diet	
	μmoles/ml	Fold increase over normal
+3% L-methionine	3.05 ± .28	31
+7% L-phenylalanine	1.70 ± .09	21
+7% L-tryptophan	.46 ± .37	23
+7% L-tyrosine	1.97 ± .20	25
+8% L-histidine	3.58 ± .18	24
+7% L-lysine	3.55 ± .22	6
+7% L-valine	5.00 ± .28	21
+7% Glycine	5.08 ± .21	10
+7% L-proline	2.53 ± .11	8
+7% L-arginine	.88 ± .07	4
+7% L-serine	1.74 ± .09	5
+7% L-leucine	.77 ± .27	4

[1] Numbers refer to the concentration of the particular amino acid which is supplemented in the diet.

or higher producing growth depression and death. Methionine, the most toxic of the fed amino acids, was also very toxic upon injection, amounts over 110 mg. producing death within 72 hours. By comparison, nearly twice the amount of phenylalanine, histidine and lysine were required to produce severe growth depression and death. The other amino acids tested were less toxic. Injection of high amounts of valine, proline and serine were required to produce even a slight growth depression, illustrating the relatively non-toxic influence of these compounds, a finding also observed in the feeding experiments. Difficulty in solubilizing some of the amino acids prevented their administration at very high concentrations.

Although death can occur very rapidly after injection of very large doses of amino acids due to high ammonia production (du Ruisseau

et al., 1956), this was clearly not the cause in our experiments. The dosages we administered were far below the reported LD_{50} levels (Gullino *et al.*, 1956) and the effects we observed were cumulative over several days rather than immediate. In addition, no significant elevation in blood ammonia was observed.[2]

A direct correlation was found between the toxicity of the dietary amino acid and its concentration in the blood (Table 3). Thus methionine, the most toxic amino acid, showed a higher blood concentration (31-fold above normal) than any of the lesser toxic amino acids. The non-toxic amino acids were present at much lower concentrations (only about four-fold above normal). Valine was apparently an exception, being present in very high concentrations (20-fold above normal). Lysine was also present in low concentrations relative to its toxicity but it was excreted in high concentrations in the urine.[2]

Discussion

The addition of a single amino acid in excess to an otherwise normal diet has variable effects on the young rat depending upon the kind of amino acid fed and its concentration in the diet. To summarize briefly, the amino acids can be divided into four groups relative to the concentration required to depress growth at least 50% : 1) very toxic (less than 7% of diet); methionine, cystine, phenylalanine, tryptophan 2) toxic (7% to 10% of diet); histidine, tyrosine, lysine 3) slightly toxic (greater than 10% of diet but some effect below this); glycine, proline, valine, arginine and 4) non-toxic (no effect at any concentration tested); serine, aspartic acid, glutamic acid, isoleucine, leucine and alanine. The most striking consequence which is observed immediately after placing the animal on the supplemented diet is an inhibition of growth. Depending upon the concentration of the amino acid, however, the animal will ultimately adapt to the diet and gain weight despite consumption of the same diet; the more toxic the amino acid is, the longer will be the time required to adapt. There appears to be little correlation between the toxic effect elicited by an amino acid and the type of functional group present although our data indicate that all of the amino acids will give a toxic response if they are provided at sufficiently high levels. Greater toxicity was ob-

[2] Daniel, R. G., and Waisman, H. A. Unpublished observations.

served, however, with those amino acids which are essential for rat growth. Thus the metabolic fate of the excess amino acid could suggest a common basis for all amino acid toxicities.

Rats fed excess amino acids in low protein diets (Sauberlich, 1961; Harper *et al.*, 1966) will respond differently from those fed an otherwise normal diet. Some amino acids, *e.g.* leucine, aspartic acid, isoleucine and valine, will induce a significant growth depression when present in low-protein (6% casein) diets but were not toxic in our experiments even at higher levels. Inasmuch as average daily food intakes were comparable, the higher protein intake resulted in a decreased toxicity of the above amino acids. However, the toxic effects elicited by methionine, phenylalanine, tyrosine, tryptophan, histidine and lysine were comparable to those on a low-protein diet. Therefore the higher protein intake had no effect. Thus the catabolic pathway and ultimate fate of the individual amino acid itself would appear to be the significant factor producing the growth depression rather than severe amino acid imbalance.

Concurrent with growth depression induced by excess amino acid feeding, a decrease in the food consumption of the animal also occurred and this too depended upon the kind and concentration of the amino acid fed. No explanation is available on how an excess of an amino acid depresses total food intake or whether or not it is a secondary effect accompanying a basic underlying mechanism. Reduction in food intake as a result of this imbalance might reflect a protective mechanism which discourages the animal from ingesting more of the excess amino acid which would increase the deleterious effects. This decreased food intake has been attributed by Kumpta and Harper (1962) to an abnormal amino acid composition of the blood plasma as a result of the single excess amino acid. It is apparent from our data that plasma levels of the amino acid being fed can be significantly elevated and the extent of this elevation correlates directly with the growth depression and decreased food consumption. However, experiments in which a single amino acid is injected demonstrate a specific toxic effect of that amino acid *per se* which is not entirely related to the decreased food consumption. Inasmuch as injection of a specific amino acid in large amounts would presumably elevate the plasma level of that amino acid to higher concentrations than feeding the same amount over a period of time (this is indeed the case with

methionine[3] and histidine[2] injection),[2] and because food consumption does not correspondingly decrease, no direct correlation between the two exists. Significantly no other plasma amino acids were altered to any considerable extent except that which was being fed.[2]

It is our contention, based on the data presented here and by others, that depression of growth following excess amino acid feeding is due to two factors, one being the reduced food intake and the second to the effects of the amino acid itself. The latter may in turn be due to several circumstances. First, a disruption of the normal balance of free amino acid pools may prevent normal protein or other macromolecule synthesis. Secondly, toxic metabolites could be formed from some of the individual amino acids and these may produce toxicity. This is apparently unrelated to blood ammonia levels since these do not reach toxic levels when amino acids are fed or injected at the toxic concentrations of amino acids we have used. Lastly, there may be severe disruption or antagonism of the cellular transport of other amino acids. Experiments in which the amino acid is injected rather than fed have demonstrated severe toxic effects (frequently death) which cannot be accounted for by reduced food consumption alone. These differences are not readily apparent when the excess amino acid is being fed for a longer period due to the competing effect of adaptation to the diet.

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Metabolism of glutamic acid in the perfused rabbit heart*

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DOELL, RUTH G. AND JAMES M. FELTS. *Metabolism of glutamic acid in the perfused rabbit heart.* Am. J. Physiol. 197(1): 138–140. 1959.—The metabolism of C^{14}-labeled glutamic acid by the isolated perfused heart was studied. Glutamic acid is readily assimilated by the normally beating heart and in the course of an hour's perfusion about 22% is oxidized to $C^{14}O_2$. Approximately 3.6% of the administered radioactivity is converted to glutamine. The utilization of glucose by the preparation is variable and not correlated with the concentration of glucose in the perfusate.

GLUTAMIC ACID has long been known to occupy an important position in the metabolic pathways of most tissues. It is present, along with its amide analogue glutamine, in large quantities in the free amino acid pool of many tissues (1). Tigerman (2) has reported an exceptionally high concentration of free glutamine in heart muscle; in addition, a relatively high concentration of glutaminase has been found in cardiac muscle as compared with skeletal muscle (3). Since glutamine has now been shown to play a role in the removal of ammonia in brain tissue (4) it was of interest to determine if this amino acid could have a similar function in the heart. For this study we have chosen the isolated perfused heart in order to maintain the tissue in as functional a condition as possible, with its circulatory system intact.

Although glutamic acid has been reported to enter less readily into cells than does glutamine (1) we have not found an appreciable permeability barrier to glutamic acid in the isolated perfused heart. On the contrary we have found that glutamic acid is readily assimilated and actively metabolized in this preparation.

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EXPERIMENTAL PROCEDURE

Perfusion method. Albino rabbits maintained on a standard Purina laboratory chow diet were used in this series of experiments. The animals were stunned by a blow on the head and the heart rapidly removed, cannulated via the aorta, flushed with Chenoweth's solution (5) and placed in the perfusion system, a modification of that designed by Miller (6). In this apparatus, maintained at 37°, the perfusion fluid is circulated by means of a syringe-activated pump. It is pumped from the main reservoir chamber in which the heart is enclosed to a smaller reservoir at such a height that a constant pressure of 40 cm of water is maintained at the heart. From this reservoir a part of the perfusate passes through the heart but the greater part passes via an overflow system into the aerating chamber, then back to the main reservoir.

After an equilibration period of 10 minutes, radioactive L-glutamic acid[3] or L-glutamine was added to the 75 cc of fresh Chenoweth's solution contained in the perfusion system and allowed to circulate for 5 minutes to ensure even distribution of the radioactivity. A sample of the perfusion fluid was then taken for determination of glucose and radioactivity. The perfusate was recirculated through the heart for a period of 1 hour. During this time it was in contact with a continuous stream of gas (a 95% O_2, 5% CO_2 mixture) which, after reaching partial equilibrium with the gases of the perfusion fluid, was passed through NaOH absorption towers for recovery of CO_2. At the end of the experimental period the heart was removed from the apparatus, homogenized in 50 cc of water in a Waring Blendor and aliquots taken for determination of radioactivity.

Determination of radioactivity in glutamic acid and glutamine. Samples of the perfusate and heart homogenate were heat denatured to coagulate protein and the amino acids separated by the paper chromoatographic method of

[3] L-Glutamic acid evenly labeled with C^{14} was obtained from Nuclear Chicago Corp. Ten or twenty μc (approximately 0.16 or 0.32 mg) was added in 1 or 2 cc of H_2O in each experiment (see table 1).

Kay *et al.* (7) using phenol:water or *n*-butanol:glacial acetic acid:water (4:1:5) as solvents. The strips were then sprayed with ninhydrin, the appropriate areas eluted in 71% ethanol and the color density read in a spectrophotometer. Duplicate strips were analyzed for radioactivity, by using a chromatogram scanner.

Determination of radioactivity in CO_2. The $C^{14}O_2$ was determined as $BaCO_3$ (8), counted in a Geiger counter, corrected for mass absorption and expressed as percentage of the administered radioactivity.

Glucose determination in the perfusate. The level of glucose in the perfusion fluid was determined by the glucose oxidase method.[4]

RESULTS AND DISCUSSION

The uptake of glucose from the perfusion medium varied from 0 to 17 mg and was not dependent upon the level of glucose in the perfusate nor on the apparent activity of the muscle. This finding is in agreement with results recently reported by Wintersheid *et al.* (9). The results are recorded in table 1.

Because glutamine had been reported to enter more easily into cells than does glutamic acid (1) our first experiments were carried out with radioactive glutamine. We found, however, that relatively little glutamine was removed from the perfusate by the heart (unpublished experiments) and so we turned our attention to glutamic acid as a substrate.

Glutamic acid was rapidly removed from the perfusate by the heart although the amount varied considerably from one experiment to another (table 1). This variability did not seem to be related to the size of the heart but may possibly have been dependent upon the rate of flow through the heart, which, although not measured, varied noticeably from preparation to preparation. In the course of 1 hour an average of 48% of the administered radioactivity was taken up from the perfusion medium and 22% was oxidized to $C^{14}O_2$ (table 2). The amount of glutamic acid-C^{14} appearing as $C^{14}O_2$ was not altered when the concentration of glucose was raised from 180 mg % to 360 mg %; nor did this increase in glucose concentration affect the uptake of glutamic-acid from the perfusate by the heart.

At the end of the hour's perfusion there was about 8.3% of the administered radioactivity present intracellularly as glutamic acid and about 3.6% as intracellular glutamine (table 3). Since there is a large pool of free glutamic acid in the heart, (amounting on the average to about 2.5 mg) which severely reduces the specific activity of the C^{14}-glutamic acid and since the average uptake of C^{14}-glutamic acid was 48%, this represents the conversion of approximately .044 mg of glutamic acid to glutamine or 1.7% of the glutamic acid pool per hour.

[4] 'Glucostat' reagent: Worthington Biochemicals Corp., Freehold, N. J.

TABLE 1. *Uptake of Glucose and Glutamic Acid by Isolated Perfused Rabbit Heart*

Exp. No.	Wt. of Heart	Heart Rate	Amount of Substrate Added	Perfusate Glucose Conc.	Uptake of Glucose	Uptake of Glutamic Acid, % of Radioactivity Administered
	gm	*beats/min.*	*mg*	*mg %*	*mg*	
1	7.5	175	0.32	180	8	54
3	5.6	175	0.32	180	4	32
5	4.0	175	0.16	180	4	45
7	5.3	170	0.16	180	5	55
2	4.6	170	0.32	360	3	63
4	4.9	170	0.32	360	0	40
6	5.1	165	0.16	360	17	54
8	4.7	160	0.16	360	13	44

TABLE 2. *Oxidation of Glutamic Acid by the Isolated Perfused Rabbit Heart*

Exp. No.	Perfusate Glucose Concentration, mg %	Radioactivity in CO_2, % of Administered Dose
1	180	24
3	180	19
5	180	22
7	180	28
2	360	16
4	360	18
6	360	27
8	360	22

TABLE 3. *Conversion of Glutamic Acid to Glutamine by Isolated Perfused Rabbit Heart*

Exp. No.	Intracellular Glutamic Acid Conc.	Intracellular Glutamine Conc.	Radioactivity in Intracellular:		Final Perfusate Glutamine Conc.
			Glutamic acid	Glutamine	
	mg/gm	*mg/gm*			*mg %*
1	0.40	0.58	8.3%	4.3%	2.2
2	0.45	0.76	7.6	3.2	2.2
3	0.41	0.63	9.1	3.4	2.0
4	0.41	0.72	9.2	3.4	2.4
5	0.60	0.87			2.0
6	0.56	0.91			2.0
7	0.49	0.90			2.0
8	0.53	1.25			2.0

Several ninhydrin-positive substances which were released in considerable amounts into the perfusate were detected by chromatography but were not further identified. Glutamic acid was not released in detectable amounts, but during the course of the perfusion glutamine was continually released into the medium. Radioactivity in glutamine in the perfusate was quite low due to the large volume of perfusate which it entered. However, the actual amount of glutamine released is not negligible as can be seen from the values given in table 3. On an average this release of glutamine amounted to

1.4 mg/hr. or about 30% of the total pool. Since the heart is normally in contact with blood plasma containing about 100 mg % of glutamine it is not possible to assign a physiological function to this rather large release of glutamine. Coupled with the considerable conversion of glutamic acid to glutamine, however, a function such as ammonia removal is possible.

L-Glutamine-C^{14} was kindly supplied to us by Dr. A. Meister, Biochemistry Department, Tufts University School of Medicine.

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Chronic Toxicity and Teratology Studies of L-Monosodium Glutamate and Related Compounds. A. G. Ebert, Growth Sciences Center, International Minerals and Chemical Corporation. (Sponsor: R. J. Weir.)

Two year feeding studies of L-monosodium glutamate (L-MSG), DL-monosodium glutamate (DL-MSG) and L-glutamic acid (L-GA) were carried out using Sprague-Dawley rats (75 animals of either sex/group + 150 controls) and C-57 black mice (100/group, all males + 200 controls). Rats received diets containing 0.1% or 0.4% of test compound; mice 1% or 4%. Possible adverse effects on reproduction and the teratogenic potential of L-MSG were measured in rabbits (New Zealand White variety) following 2-3 weeks of feeding at 0.1%, 0.825% and 8.25% of diet. There were 22-24 does/group and 16 males/group, all having sired or produced one litter prior to test compound feeding. Test compound feeding was continued in pregnant rabbits throughout gestation.

In rats, there were no differences between treated and control groups with respect to weight gains, food intake, hematology, gross and histopathology at any of the 3 intervals studied (241, 567 and 681 days). Survival rates at 2 years averaged 59% over all groups (range 53-63%). Gross abnormalities, seen with increasing age, included a white film over the cornea and a high incidence of fibrous tumors. Presumed tumor incidence at completion of the 730 day test averaged 40.1% across all treatments, 42.4% in controls. The most common microscopic lesion, seen in all groups, was a low grade inflammatory response in lungs,

kidneys and spleen.

In mice, however, only a single benign adenoma was found in controls or all L-MSG, DL-MSG, and L-GA treated animals. Gross and histopathology suggested renal effects in DL-MSG treated animals. Other findings were within normal limits with the exception of low two year survival rates (20% overall) due to the combatant nature of this strain of mouse.

L-MSG, at all levels studied, was without deleterious effects on reproduction of rabbits with respect to number of fetal resorptions, birth rate, survival rate at weaning, presence of internal and external abnormalities and skeletal development.

Mr. Chairman:

In the year since the last meeting of this Society, considerable attention has been drawn, largely in the lay press, to a diverse group of compounds well known to many toxicologists. I am referring to food additives and, in particular those items classified as Generally Recognized As Safe, that is on the GRAS list. Among the remarks made against the efficacy and safety of a number of these compounds were the claims that L-monosodium glutamate or MSG caused the transient neuromuscular episode in humans commonly referred to as the Chinese Restaurant Syndrome and, that the compound induced lesions in the developing central nervous system of a variety of laboratory animal species. Further, it was incorrectly stated at Congressional Hearings, that MSG had been accepted because of its natural character and long history of use rather than being toxicologically evaluated, and that such research had, in fact, not been carried out. In reply to numerous inquiries from a variety of concerned individuals we have briefly described in a number of trade and popular publications several of these supposedly non-existent studies. Chronic toxicity studies on MSG sponsored by our corporation were completed in 1952 but had, in fact, not been reported in the archival literature. We thought, therefore, it might be of interest to attendees of this meeting to briefly review results of three of these

older and previously unpublished studies on MSG, if for no other reason than to serve as a background to current studies on MSG the results of which will be reported at this meeting and elsewhere.

1st Slide Please

Three studies will be described. Two, 2-year feeding studies were carried out by A. D. Little & Co. in 1950 - 1952. Three compounds were evaluated: L-monosodium glutamate monohydrate, which is the material used as a food flavor enhancer: the monohydrate of the racemic mixture: and the L-form of the corresponding acid.

For each compound studied there were 75 rats per level +150 controls. Feeding began at 3 months of age, markedly older than the weanling age now commonly used as the starting point to begin lifetime feeding studies. Similarly, 100 male mice/level/ compound were studied beginning at 6 weeks of age.

In rats, dietary intake was at the level of 0.1% and 0.4% of total feed, equivalent to 50 or 200 mg/Kg/day, as the former represents the most common use level of MSG. The latter covers the higher use levels.

Mice received diets at 10X the concentration used in the rat studies, namely, 1% and 4% of diet, equivalent to a dose of 1.65 or 6.60 g/Kg/day. This study was designed

in part to give data at levels far greater than those used in food and also because MSG was of interest at the time as a therapeutic agent that might improve the IQ of mentally retarded children. Dietary levels of 1% to 4% roughly approximated the therapeutic regimen under evaluation which included doses of up to 45 grams per child per day.

At Hazelton laboratories in 1966, a teratology study was carried out in rabbits after feeding L-MSG at levels of 0.1% to 8.25%. This was the equivalent of up to 2.5 g/Kg/day. Males and females were fed 3 weeks prior to mating and females throughout gestation. Negative and positive controls (Thalidomide 100 mg/Kg/day) given from day 8 to 16 of gestation, were also studied. There were 40 animals per group.

Hematologic examinations were made on randomly selected rats and mice from each test group in the first two studies. The typical hematologic determinations of erythrocyte and total and differential leukocyte counts, plus hemoglobin were carried out. All values were within normal limits with the possible exception of a slight increase in monocytes especially in older animals. This finding was ascribed to low grade infections in older animals and will be seen again later.

Clinical Chemistries and organ function tests, often

characteristic of contemporary chronic toxicity studies are absent from these studies primarily because these techniques were yet to be developed when this research was conducted. As to results:

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Body weight determinations were made at 10 intervals throughout the 2 year course of the rat feeding study. No differences were observed in the period following approximately 200 days of test compound feeding, however each of the test compounds, $_{L}$-MSG, $_{DL}$-MSG & $_{L}$-GA, induced weight increases at the time intervals earlier than 200 days. As can be seen from the top figures, the growth response occured primarily in male rats. A separate short feeding study was therefore carried out in weanling rats to determine if growth acceleration was associated with increased food consumption. Feeding of each of the test compounds at the 0.4% level resulted in increased consumption of food throughout the 42 day course of the food consumption study.

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At 6 months and one year, survival rates ranged from 93 to 98%. There was no effect on survival rates that could be attributed to any of the test compounds at any of the time intervals. At the conclusion of the study survival rates ranged from 53 to 63%.

Animals that expired during the course of the study were subjected to gross and histopathologic examination except where prevented by autolysis or cannibalism. At several intervals, asymptomatic representatives from each test group were sacrificed for gross and histopathologic study, as were all survivors at the end of the 2 year study.

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Any abnormal appearing organ or tissue was preserved for microscopic evaluation. Organs and tissues routinely studied included: trachea, thyroid, esophagus, stomach, intestine, heart, lungs, liver, kidney, spleen and gonads. Conspicuous by their absence, according to modern protocols, were brain, thymus from young animals, muscle and fat samples. Organs and tissues grouped under "other" included reproductive tracts, adrenals and urinary bladder.

There was no predisposition to toxic manifestations in a given organ in any of the groups. As illustrated, gross and histopathologic findings of visceral organs were largely associated with the lungs, kidneys and spleen. On histopathologic examination, lungs showed alveolar fibrosis, lymphocytic accumulation around the bronchi, and other evidence of low grade infections in both control and treated rats. Kidneys were also characterized by evidence of low grade, chronic infection including dilated renal tubules, localized nephritis and foci of congestion in the cortex.

Recall that evidence of low grade infection was reported in the hematologic findings. Spleen involvement, included congestion and disruption of the cellular architecture, characteristic of old animals. Necrosis and general erosion of gut epithelia was observed. A low incidence of abscesses and isolated zones of hepatic necrosis was also observed. Two cases of cirrhosis were reported in the 0.1% $_{DL}$-MSG group.

Throughout the second year of the study a high incidence of localized swellings referred to as "tumors" was observed throughout all groups. The actual and percentage incidence of these swellings in each test group are illustrated in the last column. Although subsequent gross and microscopic examination indicated that a number of the swellings were cyst-like, containing a milky exudate, (that is, not verified as tumors), all swellings were referred to as presumed tumors. The highest incidence was observed in the 0.1% $_{L}$-MSG rats, however a dose response effect was not observed as the 3rd lowest incidence was in the 0.4% $_{L}$-MSG rats. The percentage incidence of swellings across all test rats averaged 40.1%, slightly lower than the 42.4% observed in controls.

There was a marked sex-linked difference in the locations and type of swellings. The incidence in female rats was

4 times that of males. In females the incidence of swellings on the ventral surface was about 10X that observed on the dorsal. However in males they were essentially evenly distributed. In females the growths were associated with mammary gland proliferation. No growths were observed within the visceral cavity of the rats.

Drs. Grey and Fogg, the toxicologist and pathologist involved in this study microscopically examined 90 of these growths. They reported that all but 3 of the tumors were benign. Forty-nine of the tumors were adenomas, 11 were fibrosarcomas, 10 were mixed and 6 were fibraodenomas. As with the incidence of total tumors, no type of tumor predominated in a given treatment group. There were 3 carcinomas, 1 in the 0.1% racemic mixture group, 2 in controls.

Although this effect could be explained on the basis of a natural phenomenon in the strain of rat used, it was deemed advisable to repeat the study using a species of animal resistant to the formation of spontaneous tumors but sensitive to known chemical carcinogens.

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The C-57 black mouse was therefore chosen and the dietary concentrations increased to 1% and 4% of each test compound. As in the studies with rats, survival rates and

values for hematologic observations were similar between treated and control mice. Body weight data were not obtained. Organs routinely examined were trachea, thyroid, submaxillary glands, lungs, heart, stomach, liver, intestine, spleen, pancreas, kidneys and skin. As in the rat studies, brain, thymus and muscle samples were not obtained. The incidence of gross pathologic findings is illustrated. Histopathologic findings in visceral organs were similar to the findings in rats. Evidence of low grade infection and tissue degeneration characteristic of the old animal was again seen across all groups.

Of greatest interest, in light of the results in rats, was the incidence of swellings at the end of the 2 year feeding. Six of the eight swellings found were, in fact, lumps under the skin in the region of the genitals and were external to the body wall, The report suggested that the swellings were traumatic, induced by fighting in the colony, because the mice were caged in groups. Microscopic examination indicated the swellings were cysts filled with viscid-yellow material. A single case of thyroid enlargement was reported.

The single tumor to be reported was not detected on gross examination but was discovered in a lung prepared for routine histopathologic study. The growth was defined as a benign adenoma and it occurred in the 1% $_{DL}$-MSG group.

As to the teratology study, breeding data are illustrated

on the next slide. Recall that L-MSG was studied at three levels the equivalent of from 30 mg/Kg to 2.5 g/Kg/day. Means ± standard errors plus totals for each of the parameters are illustrated. There were no differences between the various L-MSG treated groups in numbers of implantation sites, number of resorptions, litter sizes, incidence of still-births, or birth weights. Somewhat surprisingly, Thalidomide at 100 mg/Kg/day also was without effect.

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Not less that 48 pups/treatment were cleared and stained with KOH, glycerin and alizarin for study of any skeletal deformities. Both live and stillborn pups were examined. The highest incidence of effects on skeletal development included missing or small 5th sternbrae and the presence of additional rib formation seen throughout all groups. The report states that these findings occured at approximately the same incidence reported as the norm for this animal. In one litter of rabbits on 8.25% MSG, 3 animals showed a retardation of closing of the cranial suture. As no other litter at this dosage showed any sign of this effect it was considered to be not compound induced. Thalidomide was again without effect, possibly due to an insufficient dose.

In Summary.

$_L$-MSG monohydrate, $_{DL}$-MSG monohydrate and $_L$-glutamic acid were evaluated in 2 year feeding studies in rats and mice. In rats, each of the test compounds produced a slight acceleration of growth over negative controls early in the study, but not after 200 days. Findings on mortality rates, hematology, gross and histopathology were within normal limits. A high incidence of swellings was observed in treated and control rats during the second year of the study. A number of these growths proved to be benign adenomas suggesting the need for further research. Such research was completed in a carcinogen sensitive strain of mouse utilizing 10X the dietary concentration that had been utilized in the research on rats. Only a single neoplasia was observed. In a third study $_L$-MSG at dietary levels of up to 8.25% was without teratogenic effect in rabbits.

I would comment in conclusion on one other aspect of these studies. Following the report in Science suggesting that subcutaneously administered MSG at doses of 0.5 to 4 g/Kg to 2 - 9 day old mice induced lesions in the central nervous system and the subsequent interpretation by the lay press that MSG constituted a "Peril in Pregnancy" we asked Dr. Voelker of Hazelton to examine the brains of the formalin preserved pups that had been delivered by Caesarian

section in the teratology study just described. This work was completed using 10 pups from each dose level of MSG. Brains were paraffin imbedded and stained with H & E. Transverse sections were examined at the level of the hypothalamic nuclei and pituitary. It is to be emphasized that the protocols differed. In rabbits, MSG had been orally administered to the mothers so that pups received glutamate via placental circulation, whereas in mice MSG was subcutaneously administered to the newborn pups. Nevertheless, Dr. Voelker was convinced that the method he employed was adequate to reveal any lesions in that area of the rabbit brain reportedly effected in studies with mice. Evidence of neuronal necrosis or other pathologic alterations was not apparent in any of the MSG treated or control animals. The only pathology finding was hydrocephaly in pups from thalidomide treated does.

I am indebted to Dr. Kensler of A. D. Little, Drs. Holsing and Voelker of Hazelton and Dr. Weir now at Bionetics for their assistance in providing additional background information to the formal reports just described.

BREEDING DATA

Rabbits

TEST COMPOUND	# PREGNANT	# IMPLANTATION SITES	# RESORPTIONS	# ALIVE	# STILLBIRTHS	MEAN LITTER WT. (g)
Neg.Control	21/24	8.0±3.2 168	0.9±0.1 19	6.3±3.2 132	0.8±0.9 17	44.3±9.4
Thalidomide 100 mg/Kg/Day (Day 18-24)	18/22	8.2±2.3 148(6)*	0.5±0.7 8	6.8±2.3 109	1.0±1.2 25	41.3±6.2
L-MSG 0.1%	19/24	9.5±1.6 181	0.6±1.0 12	8.4±1.9 160	0.5±0.9 9	38.2±6.5
L-MSG 0.825%	20/24	8.7±2.0 173	0.7±1.0 14	7.6±2.1 151	0.4±0.7 8	39.6±8.9
L-MSG 8.25%	18/24	9.3±1.6 168	0.4±0.7 8	8.0±1.6 144	0.9±1.2 16	40.5±5.5

(*No. unaccounted for)

SUMMARY OF STUDIES

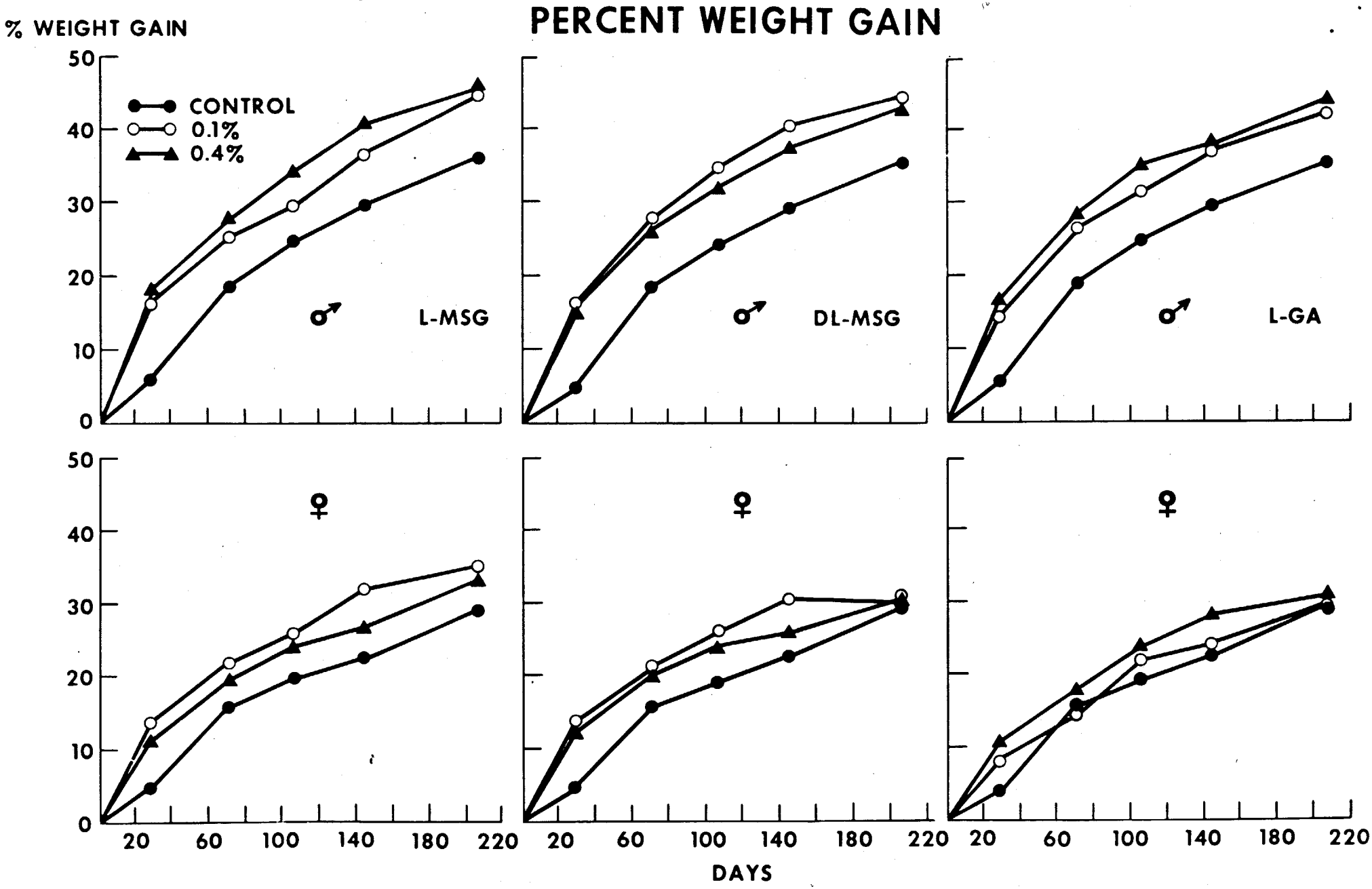
COMPOUND	ANIMALS	LEVELS IN DIET (%)	TEST	LABORATORY
L-monosodium glutamate·H_2O DL-monosodium glutamate·H_2O L-glutamic acid	Rats, Sprague-Dawley n = 75/level ♂ & ♀ + 50 controls begin 3 months	0.1 (50 mg/Kg/Day)[1] 0.4 (200 mg/Kg/Day)	2 yr feeding	A.D. Little, Inc. (1950-1951)
	Mice, C-57 Black (Jackson) n = 100/level ♂ + 100 controls begin 6 weeks	1 (1.65 g/Kg/Day)[2] 4 (6.60 g/Kg/Day)	2 yr feeding	A.D. Little, Inc. (1951-1952)
L-monosodium glutamate	Rabbits, New Zealand White n = 40 + 40 controls ♂ + ♀	0.1 (30 mg/Kg/Day)[3] 0.825 (250 mg/Kg/Day) 8.25 (2.5 g/Kg/Day)	Teratology (6-7 wk feeding)	Hazleton Labs.Inc. (1966)

[1] Assuming 200 g rat eating 10 g food/day.

[2] Assuming 30 g mouse eating 5 g/day.

[3] Assuming 2 Kg rabbit eating 60 g/day.

PERCENT WEIGHT GAIN

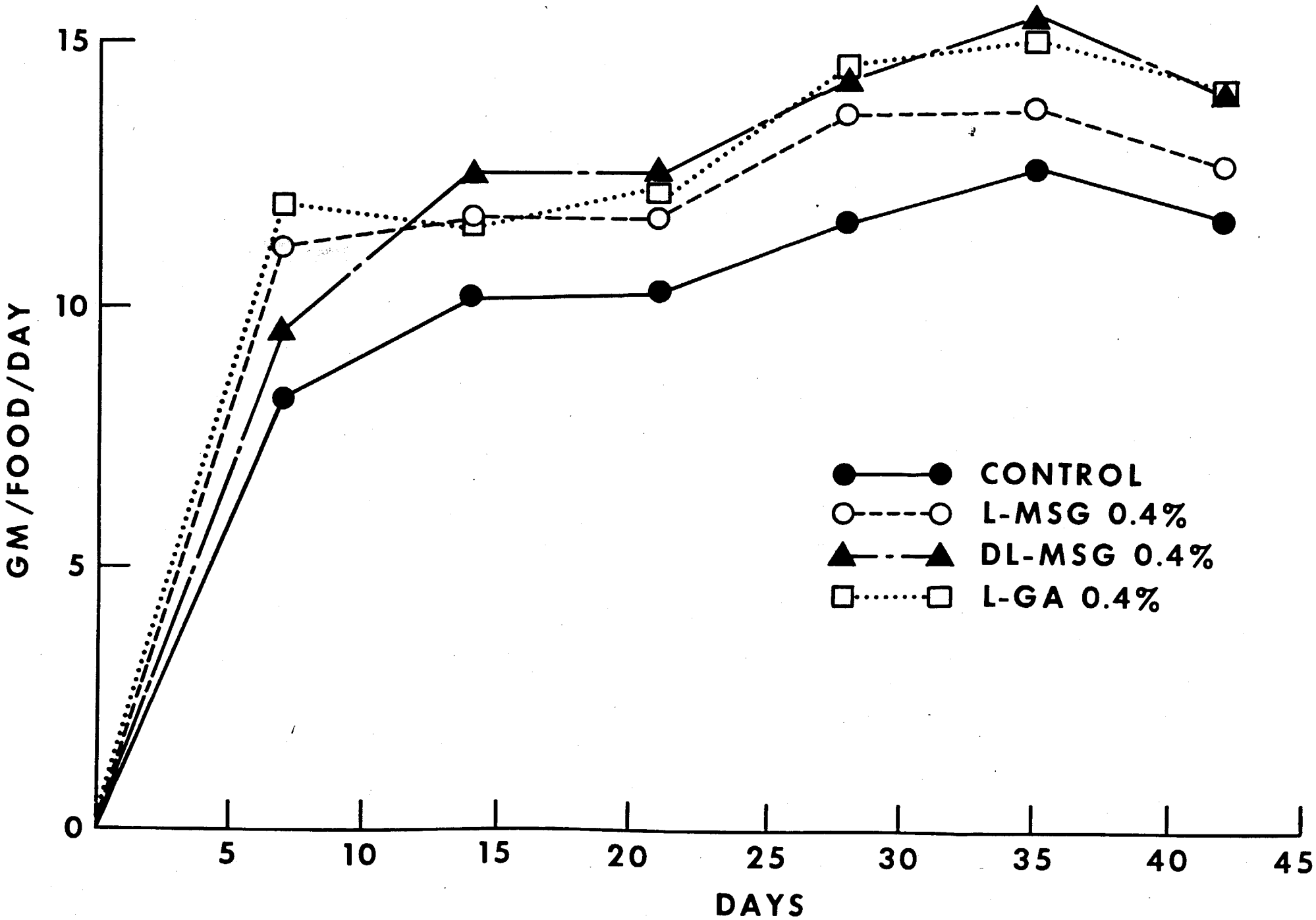


DISTRIBUTION OF GROSS AND MICROSCOPIC ABNORMALITIES AT TWO YEARS

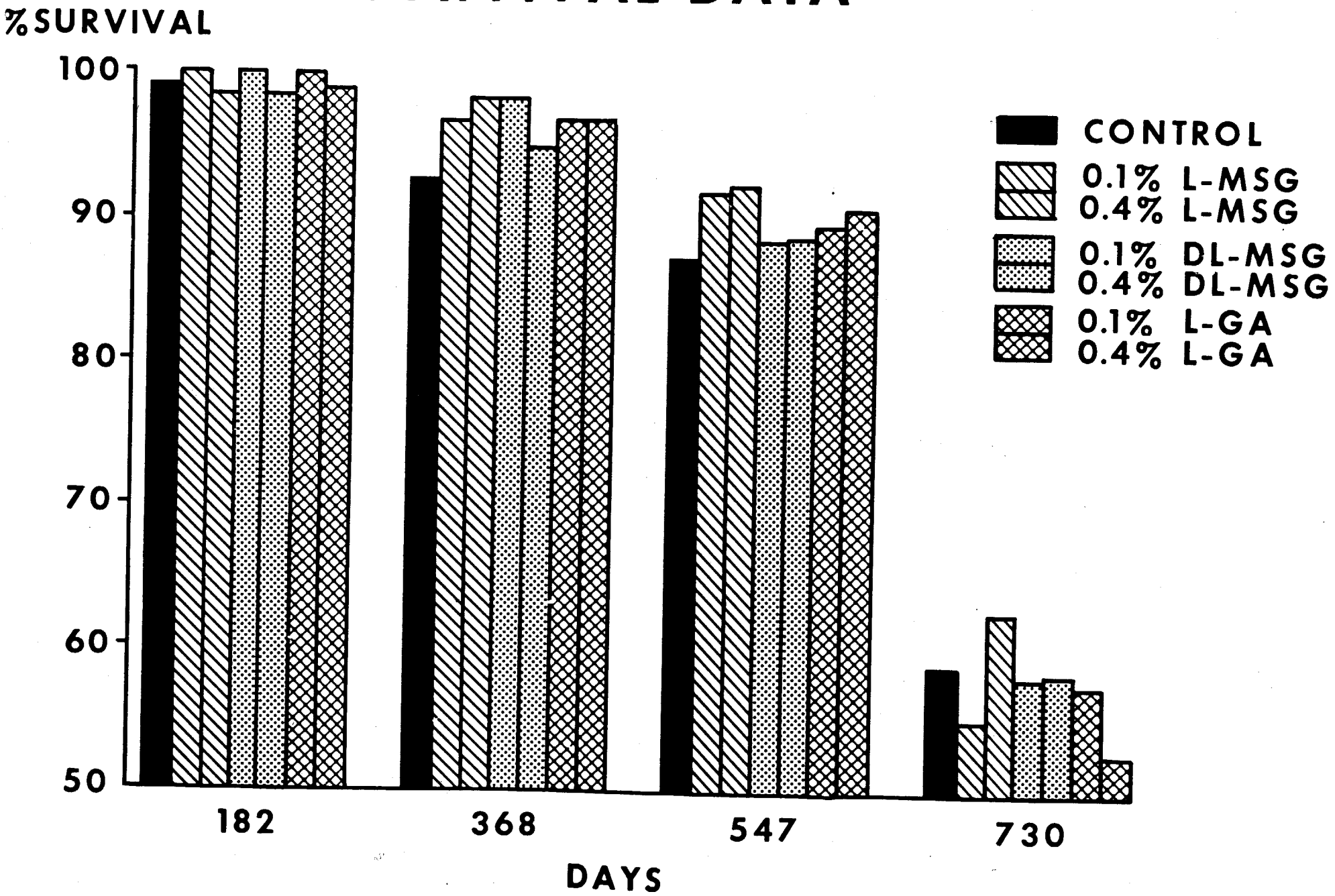
Mice

TEST GROUP		# ANIMALS	# SECTIONS	LUNGS	KIDNEYS	SPLEEN	GUT	LIVER	TESTES	OTHER	"TUMORS"
L-MSG	1%	25	76	9	5	8	3	4	1	3	0
	4%	23	84	4	5	5	0	3	4	1	3
DL-MSG	1%	23	105	5	6	4	0	4	3	0	0
	4%	27	103	5	7	5	0	4	4	0	2
L-GA	1%	20	105	6	4	9	2	5	3	3	0
	4%	29	118	7	1	4	0	2	1	1	0
Control		55	207	11	7	8	0	9	6	4	3

FEED INTAKE - RATS



SURVIVAL DATA



DISTRIBUTION OF GROSS AND MICROSCOPIC ABNORMALITIES AT TWO YEARS

Rats

TEST GROUP		# ANIMALS	# SECTIONS	LUNGS	KIDNEYS	SPLEEN	GUT	LIVER	OTHER	"TUMORS"
L-MSG	0.1%	63	154	18	13	12	3	4	1	41 (54.7)
	0.4%	75	162	9	11	8	6	2	3	30 (39.6)
DL-MSG	0.1%	65	190	7	7	7	1	3	3	22 (29.0)
	0.4%	69	173	10	13	9	1	4	2	26 (34.4)
L-GA	0.1%	73	236	10	7	8	8	0	2	31 (40.3)
	0.4%	73	163	14	9	12	6	3	1	32 (42.6)
Control		102	305	14	10	5	4	1	1	64 (42.4)

SKELETAL STAINING

Rabbits

TEST COMPOUND	# CLEARED & STAINED ALIVE	DEAD	SKELETAL ABNORMALITY	NUMBER
Neg.Control	46	9	Rib fusion Spinal curvature Missing 5th Sternbrae Small 5th Sternbrae Additional (13th) rib	1 2 6 27 27
Thalidomide 100 mg/Kg/Day (Day 18-24)	36	6	Missing 5th Sternbrae Small 5th Sternbrae Additional (13th) rib	4 22 6
L-MSG 0.1%	49	5	Rib fusion Missing 5th Sternbrae Small 5th Sternbrae Additional (13th) rib	1 4 44 25
L-MSG 0.825%	45	3	Missing 5th Sternbrae Small 5th Sternbrae Additional (13th) rib	10 26 15
L-MSG 8.25%	46	3	Missing 5th Sternbrae Small 5th Sternbrae Additional (13th) rib Retarded closure of cranial suture	11 34 27 3

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TOXICOLOGICAL EVALUATION OF SOME EXTRACTION SOLVENTS AND CERTAIN OTHER SUBSTANCES

The content of this document is the result of the deliberations of the Joint FAO/WHO Expert Committee on Food Additives which met in Geneva, 24 June - 2 July 1970[1]

Food and Agriculture Organization of the United Nations

World Health Organization

[1] Fourteenth rep of the Joint FAO/WHO Expert Committee on Food Additives, FAO Nutrition Meetings Report Series, in press; Wld Hlth Org. techn. Rep. Ser., in press.

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INTRODUCTION

The monographs contained in this paper are the result of the deliberations of the Joint FAO/WHO Expert Committee on Food Additives which met in Geneva from 24 June to 2 July 1970. Since the report[1] of this meeting also contains comments on groups of as well as individual substances considered, these monographs should be read in conjunction with the report.

Any new information and comments relating to the biological data and their evaluation should be addressed to: Food Additives Unit, World Health Organization, Avenue Appia, Geneva, Switzerland.

Specifications on the food additives considered have been prepared, and these will be issued in a separate document entitled "Specifications for the Identity and Purity of Some Extraction Solvents and Certain Other Substances".[2] Comments relating to specifications should be addressed to: Food Policy and Food Science Service, Nutrition Division, FAO, Rome, Italy.

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[1] 14th Report of the Joint FAO/WHO Expert Committee on Food Additives: FAO Nutrition Meetings Report Series, in press; Wld Hlth Org. techn. Rep. Ser., in press.

[2] FAO Nutrition Meetings Report Series, 49B WHO/Food Add./70 (in preparation)

ALLOCATION OF ACCEPTABLE DAILY INTAKES

It is generally recognized that all chemicals are toxic to animals and man if large enough amounts are administered. Even those so-called innocuous substances, when given in excessive doses, may induce untoward effects as a result of various non-specific actions, e.g. physical obstruction of the G.I. tract, alteration of osmotic pressure and nutritional imbalance. A limit on the daily intake of a substance is therefore essential for the protection of the health of the consumer. The Committee therefore followed previous practice where appropriate in allocating acceptable daily intakes to the food additives considered.

An unconditional ADI was allocated only to those substances for which the biological data available included either the results of adequate short-term and long-term toxicological investigations or information on the biochemistry and metabolic fate of the compound or both.

A conditional ADI was allocated for specific purposes arising from special dietary requirements.

A temporary ADI was allocated when the available data were not fully adequate to establish the safety of the substance and it was considered necessary that the additional evidence be provided within a stated period of time. If the further data requested do not become available within the stated period, it is possible that the temporary ADI will be withdrawn by a future Committee.

Whereas all the ADIs are subject to periodic review, especially when additional data are available, reconsideration of temporary ADIs is obligatory after the date specified. The decision arrived at will depend on the information available at the time of review. It is certainly not intended that the temporary status of the ADI for a food additive will continue to be maintained indefinitely.

For those additives for which the available information was grossly inadequate to establish safety, no ADI was allocated.

On the other hand, the Committee took cognizance of the fact that the level of use of certain food additives is limited by good manufacturing practice and that they possess a very low toxicity. For these additives the Committee established no limit except as based on good manufacturing practice.

TOXICOLOGICAL EVALUATIONS:
MISCELLANEOUS FOOD ADDITIVES AND CONTAMINANTS[a]

Substance	Acceptable daily intake for man[1] (mg/kg body-weight)
Brominated vegetable oils[b]	No ADI
Carrageenan[c]) Furcellaran[c])	0-50[2]
Cyclamates, calcium and sodium[d]	No ADI[3]
Copper and cupric sulfate[e]	No ADI[4]
Ethyl maltol[e]	0-2
Food grade mineral oil[e]	Use limited by good manufacturing practice
Mercurial compounds	No ADI
Monosodium L-glutamate[e]	0-120[5]
Oleoresins of paprika[e]	Self limiting as a spice
Phosphoric acid and phosphates[f]	0-30[6]
Tin and stannous chloride[e]	No ADI[4]

[a] Specifications are also available for gelatine and sodium caseinate.

[b] Identification test only.

[c] Specifications available (Annex 1, ref. 21).

[d] Specifications available (Annex 1, ref. 16; also Section 5.3.1(b)).

[e] Specifications available (see p. 3).

[f] Specifications available (see Annex 1, ref. 7).

[1] Unconditional ADIs unless otherwise indicated.

[2] As carrageenan or furcellaran, or the sum of both. (There was a printing error in the thirteenth report (Annex 1, ref. 19) but the monographs (Annex 1, ref. 20) are correct).

[3] Use in the management of diabetes and gross obesity not considered in this evaluation.

[4] For evaluation see section 5.3.3.

[5] Additional to the amount naturally occurring in the diet. Applicable to the general population except infants under one year of age.

[6] Conditional acceptable daily intake 30-70 mg/kg. Both the unconditional and conditional ADIs include the amount occurring in the diet.

TOXICOLOGICAL EVALUATIONS:
FILTRATION AIDS AND RELATED SUBSTANCES

Substances	Acceptable daily intake for man (mg/kg body-weight)
Activated vegetable carbon[a] (food grade)	No limit except for good manufacturing practice
Asbestos	Decision postponed[c]
Tannins (food grade)[b]	
Derived from Peruvian tara	0-0.6[d]
Derived from Turkish aleppo, Chinese tara, and Sicilian sumac	0-0.3[d]

[a] Specification available (see p. 6).

[b] Tentative specifications available (see p. 6) when used as a flocculant.

[c] See the fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives: FAO Nutrition Meetings Report Series, ; Wld Hlth Org. techn. Rep. Ser. (in press).

[d] Temporary ADI.

TOXICOLOGICAL EVALUATIONS: EXTRACTION SOLVENTS

1. Substances considered

Acetone	Petroleum hydrocarbon fractions (heptane and hexane)
1,2-Dichlorethane	Propan-2-ol
Dichloromethane	1,1,2-Trichloroethylene
Ethanol	
Methanol	

2. Evaluations

(a) The evaluations for these solvents, with the exception of trichloroethylene as a caffeine extractant and ethanol, are tentative, and are subject to re-evaluation when the relevant data become available (see section 4.3.1).

(b) These solvents should be used only in accordance with good manufacturing practice, in the expectation that this will result in minimal residues.

(c) With 1,2-dichloroethane and 1,1,2-trichloroethylene, care must be taken to avoid the formation of toxic interaction products with certain treated foods.

3. Specifications available for all these solvents with the exception of hexane and heptane, for which only tentative specifications were prepared.

Toxicological Evaluation of Some Extraction Solvents and Certain Other Substances" FAO Nutrition Meetings, Report Series No. 48A FAO, 1970

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 creatinine of free glutamic acid and 200 µg/mg creatinine 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., 1954; 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 3.5 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 (Christenson 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 D-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 incoordinated 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, 1952) and in vivo (Peraino & Harper, 1962). Further removal of excess portal glutamate and alanine 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 1050 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 SGT, 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. (Ose[illegible] et al., 1970). In another study, mice, 5-9 days old, received a single [illegible] 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 (330 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 at 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., 1948).

Intravenous glutamic acid (100 mg/kg) produces vomiting (Madden et al., 1945).

Intravenous solutions of 2.9 per cent. monosodium glu[illegible]e 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 electroshock (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 50 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, li[illegible] size and body-weight of offspring, and histopathology of major organs (excluding 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 5-6 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 es 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 / [illegible] 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. agai[illegible] 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 infant.

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MONOSODIUM L-GLUTAMATE*

SYNONYMS	Sodium glutamate, MSG
CHEMICAL NAMES	Monosodium L-glutamate monohydrate
EMPIRICAL FORMULA	$C_5H_8O_4NNa.H_2O$
STRUCTURAL FORMULA	$HOOC\ CH(NH_2)\ CH_2CH_2COONa - H_2O$
MOLECULAR WEIGHT	187.13
DEFINITION	Monosodium L-glutamate contains not less than 99% of $C_5H_8O_4NNa.H_2O$ and conforms to the following specification.
DESCRIPTION	White, practically odourless crystals or crystalline powder, with a characteristic taste.
USES	Flavour enhancer

IDENTIFICATION TESTS

A. Solubility

Water:	freely soluble
Ethanol:	sparingly soluble
Ether:	practically insoluble

B. Positive test for amino acid: to 1 ml of a 1 in 30 solution add 1 ml of ninhydrin T.S. and 100 mg of sodium acetate, and heat in a boiling water bath for 10 minutes. An intense violet-blue colour is formed.

C. Positive tests for sodium.

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* Replaces tentative specifications given earlier in "Specifications for the Identity and Purity of some Food Colours, Emulsifiers, Stabilizers, Anti-caking Agents and Certain Other Substances". FAO Nutrition Meetings Report Series No. 46B; WHO/Food Add./70.37, Rome 1970.

PURITY TESTS

Loss on drying:	Not more than 0.5% (97-99°, 5 h)
pH:	pH of a 1 in 20 solution is between 6.7 and 7.2
Specific rotation- $[\alpha]_D^{20}$	+ 24.8 to + 25.3° (10 g in 100 ml of 7% hydrochloric acid, 200 mm tube)
Chloride:	Not more than 0.2%
Arsenic:	Not more than 2 mg/kg
Lead:	Not more than 5 mg/kg
Heavy metals:	Not more than 10 mg/kg

ASSAY

Dissolve about 250 mg accurately weighed, in 100 ml of glacial acetic acid. A few drops of water may be added prior to the addition of the acetic acid to effect faster dissolution of the sample. Titrate with 0.1 N perchloric acid in glacial acetic acid, determining the end-point potentio-metrically. Each ml of 0.1 N perchloric acid is equivalent to 9.356 mg of $C_5H_3O_4NNaH_2O$.

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TECHNICAL REPORT SERIES No. 488

Evaluation of Food Additives

Fifteenth Report of the Joint FAO/WHO Expert Committee on Food Additives

This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the World Health Organization or of the Food and Agriculture Organization of the United Nations.

GENEVA
1972

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Annex 3

ADDITIVES IN BABY FOODS

Report of an FAO/WHO Meeting

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FAO/WHO MEETING ON ADDITIVES IN BABY FOODS

Rome, 14-16 June 1971

Members :

Dr W. T. C. Berry, Principal Medical Officer, Nutrition, Department of Health and Social Security, London, England

Dr P. S. Plias, Principal Medical Officer, Toxicology, Department of Health and Social Security, London, England (*Chairman*)

Mr G. Loggers, Public Health Inspector, Ministry of Social Affairs and Public Health, Leidschendam, Netherlands

Mr H. P. Mollenhauer, Chief, International Food Affairs Section, Federal Ministry of Youth, Family and Health, Bad Godesberg, Federal Republic of Germany (*Vice-Chairman*)

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1. INTRODUCTION

Following a recommendation of the First Joint FAO/WHO Conference on Food Additives[1] a series of annual meetings of the Joint FAO/WHO Expert Committee on Food Additives have been convened. Since the sixth meeting, the Expert Committee has been given the task of evaluating the safety of individual food additives following the principles and procedures laid down earlier. However, in the case of additives in baby food special considerations are needed because the detoxicating mechanisms that are effective in the more mature individual may be ineffective in the baby. Baby foods should be prepared without food additives whenever possible. Where the use of a food additive becomes necessary in baby foods, great caution should be exercised regarding both the choice of additive and its level of use.

A WHO Scientific Group on Procedures for Investigating Intentional and Unintentional Food Additives, which met in July 1966, considered that "... there are circumstances in which the benefit to the baby arising from the inclusion of some additive, for example a preservative, in its diet may greatly outweigh any possible hazard to health". With reference to unintentional contaminants, it stressed that "the diet of babies is likely, under present-day conditions, to contain traces of pesticide and other residues".[2]

Following a recommendation made in the tenth report of the Joint FAO/WHO Expert Committee on Food Additives,[3] the present Meeting was convened by FAO and WHO to study the special problems arising from exposure of infants and young children to food additives. It was opened by Dr M. Autret, Director, Nutrition Division, FAO, on behalf of the Directors-General of FAO and WHO.

2. GENERAL CONSIDERATIONS

2.1 Scope

The task of the Meeting was confined to the consideration of additives that are either not nutrients or not added to baby foods primarily for nutritional purposes. In addition to intentional additives, the Meeting also considered pesticide residues and other contaminants in baby foods. A

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[1] *Wld Hlth Org. techn. Rep. Ser.*, 1956, No. 107.

[2] *Wld Hlth Org. techn. Rep. Ser.*, 1967, No. 348, p. 11.

[3] *FAO Nutrition Meetings Report Series*, 1967, No. 43; *Wld Hlth Org. techn. Rep. Ser.*, 1967, No. 373.

baby food was defined by the Meeting as any food that is directly or by implication represented as being intended for feeding to infants and young children.

2.2 Principles for the use of intentional food additives

2.2.1 *Food for children up to 12 weeks*

On developmental grounds an arbitrary distinction may be made between children aged less than 12 weeks and older children. It is likely that the detoxicating mechanisms, the permeability of certain tissues, and other protective mechanisms of the infant aged up to 12 weeks may not have developed to a point where they are able to cope with substances that present no problem to the adult. There is little evidence regarding the age of maturation of detoxicating mechanisms, particularly as regards individual variability. However, it may be assumed that by the end of the twelfth week most of the necessary protective mechanisms have developed. Few additives have been investigated in relation to their effects on very young children. It is therefore prudent that foods intended for infants under 12 weeks should contain no additives at all. Such food would include infant formulas (and other milk-based preparations), cereal-based baby foods, and "strained" foods and fruit juices. Certain cereal-based baby foods, "strained" foods, and "junior" foods that are intended for older babies may contain additives and should be adequately labelled to ensure that they are not consumed by infants under 12 weeks.

It was accepted by the Meeting that in practice there may be certain exceptions on technological grounds to the exclusion of food additives from food for infants under 12 weeks. The use of food additives may be justified, for example, to increase shelf life, to ensure adequate sterilization by promoting homogenization, or to maintain consistency and texture in order to ensure safe and acceptable use. However, appeal to the eye or organoleptic acceptability to the mother, as opposed to the infant, does not constitute justification. The Meeting also accepts that in some countries with special difficulties—for example, of storage or supply—additives not otherwise acceptable may confer advantages that outweigh their potential hazards.

2.2.2 *Foods for children over 12 weeks*

The availability of "junior" and "strained" foods confers an advantage in that the infant may be given a diet more varied and therefore often nutritionally more satisfactory than it would otherwise receive.

Although after 12 weeks detoxicating and other protective mechanisms may be adequately developed, it must be remembered that, in terms of caloric intake per kilogram of body weight, young children consume up

to three times as much as adults. This factor needs to be borne in mind when considering levels of additives in foods designed for young children.

2.2.3 *Standards of safety of additives*

The Meeting considered that, particularly for infants under 12 weeks, toxicological investigations should be more extensive and include evidence of safety to young animals. Technological justification should be given less weight than in the case of foods intended for older babies or adults. Details are given in section 3.

2.3 Pesticides and contaminants

The above considerations on intentional additives also apply to pesticide residues and other contaminants. Ideally, pesticide residues and other contaminants, including environmental pollutants, should be absent, but it is recognized that no food, not even that intended for babies aged less than 12 weeks, can be expected to be completely free from such substances. There are situations where some contamination cannot be avoided, for example from carry-over or from equipment.

The establishment of acceptable residue levels of pesticides or other contaminants likely to be present in milk and cereals for infant foods should be based on toxicological evaluation in very young animals. Until such results are available, baby foods should be manufactured from raw materials selected and processed in such a way that only minimal residues are present in the final product.

2.4 Other general considerations

The Meeting expressed concern that in addition to foods specifically intended for babies other foods mainly intended for adults may be eaten by children. In particular, processed dairy products, bread, and biscuits, all of which may contain additives, are often fed to babies instead of cereal-based foods specifically prepared for infants. The Meeting draws the attention of the appropriate expert bodies of FAO and WHO to this problem.

3. TECHNOLOGICAL CONSIDERATIONS

The Meeting was aware that any approval of additives at present would be of a temporary nature until the requirements for toxicological testing as laid down in section 4.2 have been met. The raw materials should be selected so as to contain a minimum of contaminants, as discussed in section 2.3. On individual groups of additives the following conclusions were reached.

3.1 Chemical preservatives

These should not be used in baby food except in special circumstances as specified in paragraph 2.2.1. Raw materials treated with nitrites or nitrates should be avoided.

3.2 Food colours

In keeping with the principles laid down in section 2, these should not be used for the express purpose of colouring.

3.3 Emulsifiers, stabilizers, and thickeners

These are necessary for a variety of baby foods. Experience from some countries suggests that starch is suitable for thickening and stabilizing purposes for infant formulas.[1] Lecithin, monoglycerides and diglycerides appear to be suitable for emulsification purposes. Gelatin and sodium and calcium caseinates, which are also used, are not regarded as additives.

3.4 Flavouring agents and flavour enhancers

These are not necessary in infant formulas. However, babies over 12 weeks of age may be capable of distinguishing flavours. All baby foods should be prepared in such a way that their natural flavour is retained, if desired. In certain processes, however, such as sterilization or deaeration, it is not always possible to retain the original flavour. In such cases, the restoration of desirable natural flavours may be justified. For the manufacture of some composite foods, including food ingredients from novel sources, the addition of natural flavours from edible products may also be justified. On the other hand, there appears to be no technological justification for flavour enhancers. Although salt may be regarded as a flavouring agent, sodium and chloride are necessary nutrients in infant formulas. If sodium chloride is added to other classes of baby foods for taste purposes, it should be limited to 0.25% by weight of the product to be consumed (see also section 5.5).

3.5 Antioxidants

Some antioxidants are needed for a variety of baby foods. Experience shows that, under favourable climatic conditions, a normally acceptable

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[1] See the fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives, *FAO Nutrition Meetings Report Series*, 1971, No. 48; *Wld Hlth Org. techn. Rep. Ser.*, 1971, No. 462.

shelf-life can be maintained for some foods solely by the use of such naturally occurring antioxidants as tocopherols and ascorbic acid, or their appropriate esters. Packaging under nitrogen or carbon dioxide may be required in certain cases.

3.6 Buffers and pH regulators

A variety of suitable chemical compounds are available.

4. TOXICOLOGICAL CONSIDERATIONS

4.1 Vulnerability of very young infants

Although breast feeding is recognized to be the commonest form in which the newborn infant receives its food supply in the early weeks of life, comparatively few babies are still breast fed at 6 weeks of age in the more highly developed countries. Many infants have by then experienced contact with infant formulas as substitutes for milk, and some may receive their entire food supply in that form from the day of birth. Furthermore, cereal-based infant foods and other classes of infant foods, such as strained foods and supplementary infant foods, may have been introduced at various ages and provide an appreciable proportion of the caloric intake for infants between 3 and 6 months. Thereafter, the child is increasingly fed from the family meals, while cereal consumption tends to wane and canned strained foods and " junior " convenience foods often become more important constituents of the diet.

Very young infants are especially vulnerable to foreign chemicals because the mechanisms that provide protection against these substances are absent or not fully developed. Although the evidence for this derives mainly from studies with drugs rather than with food additives, it is likely that such very young infants are less efficient than older children in metabolizing some food additives and may therefore accumulate them to excessive levels. If this occurs at a time when sensitivity to toxic effects is critical because of the delicately balanced growth and differentiation processes, there may be deleterious consequences that may not appear until much later in the child's development. Very young infants may also differ from older children in relation to physiological barriers protecting sensitive tissues, such as the blood-brain barrier or the protective barriers for retinal or lens tissue.

The known differences between the very young infant and the older child include the following : low gastric acid secretion, different biochemical capabilities of the gastrointestinal tract, inability to digest and absorb certain

substances, deficiency in methaemaglobin reductase systems, diminished renal excretory processes at both glomerular and tubular sites.

4.2 Toxicological testing

After reviewing the guidance given in the various FAO/WHO publications for the toxicological testing of intentional and unintentional food additives, it was considered that modification of some of the recommended procedures was necessary. Before a food additive is regarded as safe for use in food intended for infants up to 12 weeks of age, the toxicological studies should be extended to include animals in the corresponding period of life. There is, however, a lack of precise information relating to this developmental period in both animals and the human infant. It is difficult to recommend precise toxicological testing procedures until more basic research has been undertaken. There are also difficulties in selecting the appropriate species. In these circumstances short-term studies should be conducted in several species and should include the oral administration of the additive under test, at suitable dose levels, to newly born animals up to and including the end of the weaning period. This type of exposure is necessary in order to discover whether the existing species variation seriously affects the handling of the food additive by the test animals and to compensate partly for the serious lack of knowledge in this area both in animals and men.

When lifespan studies and multigeneration studies are carried out, they should be extended to include the oral administration of the food additive at suitable dose levels to a proportion of animals from the day of birth throughout the pre-weaning period. These animals should then be observed for the required period as laid down for the usual lifespan and multigeneration tests. Multigeneration tests should be carried out for at least 3 generations. If there are any indications of heritable mutagenic effects these tests should be followed by specific studies. Some members of the filial generations should be exposed to the additive for their lifespan. The enhanced sensitivity of the newborn of several species to certain carcinogenic agents is particularly relevant in this regard. It is also necessary to place greater emphasis in future on observations of the behavioural developments of animals used for toxicological testing, particularly in long-term studies. Because of uncertainties still existing in this field, the accumulation of experimental information is of paramount importance for the evaluation of the results in terms of human health hazard.

The Meeting emphasized the importance of the recommendations made by the WHO Scientific Group on Procedures for Investigating Intentional and Unintentional Food Additives with regard to the desirability of supplementing animal studies on additives by investigations in man. It considered that this applies specially to additives to be used in infant foods.

5. SPECIFIC FOOD ADDITIVES

Certain food additives in respect of which specific queries had been raised are considered below. These considerations do not imply that any greater significance should be accorded to these additives than to others not specially considered.

5.1 Modified starches

Certain modified starches were stated in the thirteenth report of the Joint FAO/WHO Expert Committee on Food Additives [1] to be equivalent to normal products of digestion. These included starches treated by physical means, acid-treated starches, enzyme-treated starches, and alkali-treated starches. The Meeting considered that there could be no objection to their being used as additives in foods for infants beyond 12 weeks of age.

5.2 Lactic acid

This food additive was re-evaluated at the thirteenth meeting of the Joint FAO/WHO Expert Committee [1] and the previous restriction on the use of the D-enantiomorph in the diet of very young infants was upheld. The evidence for this decision appears in the appropriate monograph.[2] No additional evidence has come forward to indicate a need to change the opinion expressed in the ninth report of the Joint FAO/WHO Expert Committee that only L-lactic acid be used in the preparation of infant foods.[3]

5.3 Nitrates and nitrites

There are wide variations in the occurrence of nitrates and nitrites in vegetables and certain prepared meat products. These substances are of special importance because of the extreme sensitivity of the human infant to the induction of methaemoglobinaemia. Because the mechanism of methaemoglobin reduction is poorly developed and because of the increased liability of fetal erythrocytes to methaemoglobin formation, during the first 2–3 months of life there is a greater risk of adverse reactions from absorbed nitrite. This nitrite is derived essentially from bacterial

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[1] *FAO Nutrition Meetings Report Series*, 1970, No. 46; *Wld Hlth Org. techn. Rep. Ser.*, 1970, No. 445.

[2] *FAO Nutrition Meetings Report Series*, No. 40 A, B, C; WHO/Food Add/67.29.

[3] *FAO Nutrition Meetings Report Series*, 1966, No. 40; *Wld Hlth Org. techn. Rep. Ser.*, 1966, No. 339.

activity in the gastrointestinal tract, a process that occurs particularly readily in the very young infant because of the inadequacy of acid production in the stomach. It is therefore recommended that every effort should be made to reduce to a minimum the total nitrate and nitrite intake of infants. It is also necessary to consider the possibility of nitrosamine formation *in vivo* following simultaneous ingestion of nitrite and amine-containing foods. Almost all the nitrosamines hitherto examined in animal tests have been shown to possess a carcinogenic potential. In view of the particular sensitivity of the young infant to these toxic agents this is a further reason for restricting nitrate and nitrite intake to a minimum. It has also been shown that the presence of nitrate in water used in the processing of canned food may lead to excessive concentrations of tin with possible consequent deleterious effects.

5.4 Phosphates and calcium

The level of phosphates in the diet must be considered in relation to the calcium intake. There is a wide latitude for dietary variations in calcium content without significant toxicological effects and the calcium contributed from food additives is unlikely to make a substantial impact on total intake. The Meeting agreed that the calcium-phosphorus ratio in a baby food should not be less than 1 : 1.2. This provision should be made to apply at least to infant formulas and cereal-based baby foods.

The toxicological aspects of phosphates as food additives were reviewed in the seventh report of the Joint FAO/WHO Expert Committee.[1] The contribution to the phosphate load from the carry-over of phosphates used as additives in certain ingredients of infant formulas should be allowed for when calculating the general adjustment of the calcium-phosphorus ratio in infant foods.

5.5 Sodium compounds

The investigation of various infant foods and infant formulas has revealed a considerable variation in the total amounts of sodium, depending largely on the combination of the foods. There is some evidence, both from animal experiments and from epidemiological studies in man, of a correlation between sodium intake and the appearance of hypertension. Restriction in the sodium content by limiting the amount of salt to be added to infant food is therefore recommended (see section 3).

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[1] *FAO Nutrition Meetings Report Series*, 1964, No. 35 ; *Wld Hlth Org. techn. Rep. Ser.*, 1964, No. 281.

6. RECOMMENDATIONS

(1) Fundamental research should be carried out on methods of toxicological testing of substances intended for use as additives in infant foods, special attention being given to the problem of differences in pre-weaning development between the test animals and man, thus permitting more reliable predictions of toxicity to the human infant.

(2) WHO should promote toxicological studies of those additives, the use of which is essential in baby foods.

(3) As new pertinent information becomes available, the safety of additives in all foods taken by infants should be reassessed.

(4) A more thorough investigation should be made of the standards and criteria used for assessing the technological justification for the use of additives used in baby food.

(5) In evaluating the safety of food additives, the possibility should be borne in mind that certain classes of foods containing additives may be consumed by infants as well as by adults.

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THIRD JOINT FAO/WHO CONFERENCE ON FOOD ADDITIVES AND CONTAMINANTS — FAO/WHO/C/INF/73.3

Geneva, 22-26 October 1973 — ORIGINAL: ENGLISH

TOXICOLOGICAL EVALUATION OF CERTAIN FOOD ADDITIVES WITH A REVIEW OF GENERAL PRINCIPLES AND OF SPECIFICATIONS

SEVENTEENTH REPORT OF THE JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

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Monographs containing summaries of relevant biological data and toxicological evaluations will be issued separately by FAO and WHO under the title:

Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers and thickening agents

FAO Nutrition Meetings Report Series, No.

WHO Food Additives Series, No. 5

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JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES
Geneva, 25 June - 4 July 1973

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TOXICOLOGICAL EVALUATION OF CERTAIN FOOD ADDITIVES WITH A REVIEW OF GENERAL PRINCIPLES AND OF SPECIFICATIONS

Seventeenth Report of the Joint FAO/WHO Expert Committee on Food Additives

A Joint FAO/WHO Expert Committee on Food Additives met in Geneva from 25 June to 4 July 1973. The meeting was opened by Dr B. Dieterich, Director, Division of Environmental Health, WHO, on behalf of the Directors-General of the Food and Agriculture Organization of the United Nations and of the World Health Organization. Dr Dieterich, in his opening address, briefly reviewed the origins and work of the Committee as well as WHO programmes in related areas, particularly those on environmental health monitoring and criteria.

1. INTRODUCTION

As a result of the recommendations of the Joint FAO/WHO Conference on Food Additives held in September 1955[1], sixteen meetings of the Joint FAO/WHO Expert Committee on Food Additives have been held (see Annex 1). The present meeting was convened on the recommendations made in the sixteenth report of the Committee. Its terms of reference were: (1) to review the principles for evaluating the safety of food additives; (2) to make a toxicological re-evaluation of the food additives listed in Annex 2; (3) to review the specifications of the substances listed in Annex 2; (4) to discuss an approach to the evaluation of flavouring substances.

2. GENERAL CONSIDERATIONS

2.1 Comments on the agenda

2.1.1 Substances added to the agenda

The agenda was originally drafted partly in terms of compound groupings having similar technological functions. In considering individual substances in the different groups, the Committee decided to review the toxicological evaluation of a number of related substances that did not fall within the specific functional classifications, i.e. certain phosphates, calcium chloride, oxystearin, polyvinyl pyrrolidone, propylene glycol and sorbitol. The monographs on the toxicological re-evaluation of lactic, citric, glutamic and tartaric acids also include the re-evaluation of their salts with certain bases accepted for food use. A review of the specifications for the substances listed above will be carried out by a future Committee.

2.1.2 Substances deleted from the agenda

As a number of important studies on saccharin were in progress, the Committee decided not to deal with this substance for the time being.

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[1] FAO Nutrition Meetings Report Series, 1956, No. 11; Wld Hlth Org. techn. Rep. Ser., 1956, No. 107

2.2 Principles governing the use of food additives

The first meeting of the Joint FAO/WHO Expert Committee on Food Additives was held in Rome in December 1956 and dealt with general principles governing the use of food additives. It was recommended that food additives should be used only after authorization by the appropriate authorities and that their legal control should be based on the system of permitted or positive lists. Two factors have to be taken into account in evaluating a substance proposed for use as a food additive, namely technological efficacy and safety in use. The criteria for both must be satisfied before a substance can be formally accepted as a food additive.

The use of a food additive should be technologically justified on the basis of advantage to the community and the consumer, and, as stated in the Committee's sixth report[1], the level of use should not exceed the lowest level that is effective in good manufacturing practice.

The most important factor for the acceptance of a substance as a food additive is the establishment of its safety in use. This principle was clearly stressed in the Committee's first report[2]. It implies that an adequate toxicological evaluation has to be made (see section 2.4).

Specifications for food additives are required as a safeguard against the presence of harmful contaminants and also to ensure that food additives are adequately defined (see section 2.3), since otherwise their technological usefulness and toxicological properties cannot be assessed in a pertinent manner.

In view of public concern about the use of food additives and, in particular, the number of additives that may be permitted for use in any one food, the Committee drew attention to the many safeguards that exist in this domain. Legislation provides safeguards against adulteration and debasement in the quality or nutritive value of food. More specifically, there are safeguards concerning the acceptability of individual food additives and of combinations of food additives.

The Committee has frequently reiterated the general principles laid down in its first report concerning the safe use of food additives. However, it should be pointed out that the absolute safety of the foods themselves cannot be guaranteed since natural components of normal foods may exhibit toxic properties. By contrast, the scrutiny to which food additives are subjected in arriving at a toxicological evaluation makes for their safety in practice. The potential hazard, if any, that the use of a food additive may present to the consumer must be weighed against the benefits that it confers; for example, a food additive may provide protection against the development in food of microbial toxins or mycotoxins.

When several additives are used in a food, the most important factor to be considered is the level of exposure of the consumer to each food additive rather than the total number used. In fact, the use of a number of food additives to achieve a technological effect, each at a lower level than would be needed if it were used alone, may provide an additional measure of safety, as long as there are no undesirable interactions between them. Such interactions may be chemical or biological in nature. The Committee considered possible chemical interactions between food additives that might yield toxic substances, thus precluding the simultaneous use of the additives in the same food. As regards biological interactions, the Committee acknowledged the theoretical possibility of some subtle potentiation of toxicity, but in practice such an effect has rarely been encountered. Compounds that are closely related in chemical structure or function may exhibit additive and, more rarely, synergistic biological effects. In several instances the Committee has allocated ADIs to groups of such compounds, thus providing a safeguard in the event of a number of such food additives being used in a single food.

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[1] See Annex 1, ref. 6
[2] See Annex 1, ref. 1

In the years that have elapsed since the publication of its first report, the Committee has repeatedly stressed the importance of the general principles concerning the safe use of food additives. It emphasized once again that, provided these principles are adhered to, the goal of the safety in use of food additives is attainable in practice.

2.3 Principles governing specifications

General principles governing specifications were summarized in the tenth report[1] in which the Committee also recommended a review of specifications in the light of current information regarding the production, use, composition, and purity of food additives and methods of analysis. The work of the Committee has, however, been continuously expanding to cover not only simple chemicals but also complex substances such as enzyme preparations and natural flavouring materials. For certain food additives derived from plants or animals, it is necessary to establish microbiological criteria. This is the case, for example, with some natural food colours, vegetable gums, gelatin, carrageenan, pectin, modified starches, and enzymes. The Committee felt that there was a need to review the general principles with regard to their applicability to these substances and to consider the need for additional criteria.

2.4 Principles governing toxicological evaluation

Principles of evaluation and procedures for the toxicological testing of food additives were dealt with in the second[2], fifth[3], sixth[4], and tenth[1] reports of the Committee. A WHO Scientific Group on Procedures for Investigating Intentional and Unintentional Food Additives[5] reviewed the advances made in the various relevant disciplines and made recommendations on principles for toxicological evaluation.

Over the years, the Committee has extended its scope to include the evaluation not only of intentional food additives but also of certain antibiotics, trace elements, contaminants, and processing aids. Consequently, there have been some minor changes in its approach to the problem of toxicological evaluation, especially with respect to the allocation of 'acceptable' intakes.

There are two stages in the toxicological evaluation of a substance proposed for use as a food additive. The first is the collection of relevant data, which are usually derived from experimental testing in laboratory animals and, whenever possible, from observations in man. The second is the interpretation and assessment of the data in order to arrive at a decision about the acceptability or otherwise of the substance as a food additive.

2.4.1 Testing procedures

The procedures for testing food additives are summarized in Annex 3, on the basis of previous reports of Joint FAO/WHO Expert Committees. This summary has been provided only because some early reports are no longer available and no attempt has been made to review the procedures critically. It must be stressed that the summary is intended only to indicate general guidelines. The procedures should be modified as necessary, depending on the nature of the substance to be tested, and should take advantage of recent developments in toxicological techniques.

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[1] See Annex 1, ref. 13
[2] See Annex 1, ref. 2
[3] See Annex 1, ref. 5
[4] See Annex 1, ref. 6
[5] Wld Hlth Org. techn. Rep. Ser., 1967, No. 348

2.4.2 Interpretation of findings

The objective in assessing the toxicological data on food additives is to ensure their safety for the consumer on the basis of all the evidence available to the Committee at the time. Future results with present methods or with techniques yet to be developed will necessitate reassessments that may lead to changes in earlier decisions.

The general procedure adopted has been to establish an acceptable daily intake (ADI) for man for each food additive, or for groups of additives. When the toxicological data are derived from animal experiments, their extrapolation to man involves the application of a safety factor to the highest 'no-effect level' obtained in animal studies.

2.4.3 No-effect level

When applied to data from animal experiments, the term 'no-effect level' refers to the level of a substance that can be included in the diet of a group of animals without toxic effects. With certain substances, the highest level that can be incorporated in a diet fails to produce any effect. However, some food additives do exert toxic effects when fed at high levels and for these the maximum no-effect level is used. The maximum no-effect level should be determined in the most appropriate animal species and be based on the most pertinent criteria of toxicity.

At its meetings, the Committee has considered a variety of effects that, in the present state of knowledge, are not deemed to be of toxicological significance, provided they are fully attributable to normal physiological adjustment and are reversible. They include, for example, changes in intestinal flora, laxative effects due to bulk or osmotic load, cæcal enlargement and diminished growth rate caused by high levels of non-digestible substances, and liver hypertrophy and induction of microsomal enzymes due to gross overloading with certain metabolizable substances.

2.4.4 Extrapolation of data to man

In the extrapolation of animal data to man, the application of a safety factor is required for the following reasons: to allow for any differences in sensitivity between the animal species and man; to allow for wide variations in sensitivity among the human population; to allow for the fact that the number of animals tested is small compared with the size of the human population that may be exposed.

It was recognized by the Committee that the expression of the ADI in terms of body weight (BW) does not reflect the relative exposure of animals of different size as accurately as would the metabolic mass, which is equal to $W_b^{0.75}$. However, in practice the method of expressing the dose in terms of mg/kg body weight has proved satisfactory.

A safety factor of 100, the figure recommended by the Committee in its second report[1], has been widely accepted. But it would be unreasonable to apply this figure too rigidly, for example, in the case of substances that are normal constituents of the human diet or are normal intermediary metabolites. When there are adequate data to show that, in the human body, a substance is converted by digestion or metabolism to a normal constituent of the diet, or that a substance is not absorbed from the gastrointestinal tract, these data are used in the evaluation. When toxicological data derived from experiments in man are available, they may be used to provide a lower safety factor since they obviate the need for interspecies extrapolation.

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[1] See Annex 1, ref. 2

On the other hand, there may be reasons to increase the safety factor - for example, when the amount and/or the quality of toxicological information are limited. Furthermore, the nature of the toxic effect produced by an additive at very high levels might demand an increase in the factor in order to ensure safety in use.

2.4.5 Acceptable daily intake for man

The acceptable daily intake (ADI) for man, expressed on a body weight basis, is the amount of a food additive that can be taken daily in the diet, even over a lifetime, without risk.

An ADI is allocated only to substances for which the available data include either the results of adequate short-term and long-term toxicological investigations or satisfactory information on the biochemistry and metabolic fate of the compound, or both.

An ADI may be allocated temporarily, pending the provision of additional data within a stated period of time. This measure implies that the toxicological data are adequate to ensure the safety in use of the additive during the time for which the temporary ADI applies. If the additional data requested do not become available within the stated period, the temporary ADI may be withdrawn at a future meeting of the Committee.

An ADI without an explicit indication of the upper limit of intake ('not limited') may be assigned to substances of very low toxicity, especially those that are food constituents or that may be considered as foods or normal metabolites in man. An additive having a 'not limited' ADI must meet the criteria of good manufacturing practice - for example, it should have proven technological efficacy and be used at the minimum level of efficacy, it should not conceal inferior food quality or adulteration, and it should not create a nutritional imbalance.

There may be circumstances in which the ADI is not applicable. Thus it may be exceeded for special dietary purposes - for example, in the case of modified celluloses, to reduce the energy content of the diet. On the other hand, the ADI for glutamic acid and glutamates does not extend to foods for infants under three months old.

In previous reports a conditional ADI was allocated to a number of substances, often in addition to an unconditional ADI. Variations in the rules for applying conditional ADIs have given rise to some confusion. For this reason, the Committee considers that the allocation of conditional ADIs should be abandoned.

ADIs are intended as guides only and may be exceeded, after consultation with experts, in circumstances in which there may be important advantages in doing so. In the opinion of the Committee, an ADI provides a sufficiently large safety margin to ensure that there need be no undue concern about occasionally exceeding it provided the average intake over longer periods of time does not exceed it.

2.4.6 The allocation of ADIs to additives that are related chemically and toxicologically

As a number of food additives are closely related chemically and toxicologically, the Committee adopted in its seventh and later reports a system of grouping additives for purposes of evaluation - for example, phenolic antioxidants, emulsifiers of the polyglycerol ester series, the polyoxyethylene sorbitan esters, modified celluloses and propylene glycol derivatives. In such cases, the ADI level is expected to cover all specified members of the group that may be included in the diet. A given food additive may be related to two groups, in which case the level in the diet must not exceed the maximum acceptable level for either group. The problem is not so complicated as it may appear at first sight, since many of the substances in a group of additives have technologically related functions and are therefore likely to be used as alternatives to each other.

2.4.7 Exclusion from the ADI of amounts occurring naturally in foods

The ADI includes only the amounts of the substance used as an additive, excluding 'amounts naturally present in food'. There is a single exception, namely the ADI for phosphates, which includes all phosphates, whatever the source.

2.4.8 Other conclusions

Other conclusions on food additives may be summed up in the following terms:

(a) 'Decision postponed' (pending clarification of matters related to toxicological use);

(b) 'No ADI allocated' (in the absence of sufficient information to establish safety, or of adequate specifications);

(c) 'Not to be used' (where there is sufficient information on which to base such a decision).

2.5 Tissue uptake and storage of macromolecular food additives

Information on the tissue storage of macromolecular materials is frequently lacking. In the case of propylene glycol alginate, labelled with ^{14}C either in the alginate or propylene glycol moiety, whole-body autoradiography of the treated mice served to establish lack of absorption of the alginate[1]. Unlike lipid-soluble substances whose storage within the body is non-specific, some macromolecular materials are localized within lysosomes of cells of the reticuloendothelial system. Degraded carrageenan behaves in this way and is retained at the sites of storage, for example in Kupffer cells of rhesus monkeys, for six months or longer after administration has ceased[2]. The consequences of uptake and storage of macromolecular substances in reticuloendothelial cells are not well understood, but there is some indication that alterations of phagocytic function may occur.

In view of the availability of a range of techniques (analytical, histochemical, ultrastructural, and autoradiographic) to supplement light-microscopic observations, further studies to obtain information on the tissue storage of macromolecular food additives should be carried out.

2.6 Allergenicity of food additives

A number of food additives are known to cause allergic manifestations in susceptible individuals. Sometimes these are due to occupational exposure and, while this aspect is outside the terms of reference of the Committee, the evidence obtained in such instances is used in assessing allergenicity. No approval would be given for the use as a food additive of a substance causing serious or widespread hypersensitivity reactions. For food additives with an allergenic potential, the inclusion on food labels of lists of the individual additives used has been suggested as a means of enabling sensitive individuals and their physicians to identify the possible sources of allergic reactions. The practicability and effectiveness of such a measure is uncertain and the matter requires further consideration.

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[1] Sharratt, M. & Dearn, P. (1971) Unpublished report submitted by BIBRA

[2] Abraham, R., Golberg, L. & Coulston, F. (1972) Exp. Mol. Pathol. 17, 77; Warren, S. & Somers, S. C. (1954) J. Amer. Med. Ass. 154, 189

3. PRINCIPLES GOVERNING THE ASSESSMENT OF FLAVOURING SUBSTANCES

In its eleventh report[1], the Committee evaluated some flavouring substances and suggested criteria to be used in ascertaining which groups should be given priority for further study. The present Committee reconsidered the question of the safety of flavouring substances in the light of the approach adopted by the Council of Europe[2]. It also took cognizance of the various efforts being made to establish the average and maximum use and intake levels of flavourings in some countries.

3.1 Toxicological evaluation

In general, the Committee considered that the procedures adopted by the Council of Europe represent a useful and practical approach; the problem of evaluating flavouring substances is one that cannot be solved simply by adopting the processes traditionally used by the Committee to evaluate other types of food additive. While concurring with the concepts put forward by the Council of Europe, the Committee wishes to draw attention to the following issues:

(a) In listing flavouring substances it is difficult to take into account the many indigenous materials, notably herbs, that are peculiar to specific regions. The Council of Europe lists are not comprehensive and are unlikely to incorporate all the flavouring substances actually used.

(b) In the opinion of the Committee, the guide to the testing and toxicological evaluation of flavouring substances provided by the Council of Europe goes too far beyond the enunciation of principles, entering into detailed protocols that are not only unnecessary but also tend to create a rigid set of testing requirements. The Committee cannot emphasize too strongly the need to maintain flexibility at all times in the approach to toxicity testing since each compound presents an individual and unique problem.

3.2 Specifications

For the preparation of chemical specifications, flavouring products may be categorized as follows:

(a) Extracts (preparations) of plant or animal materials;

(b) Essential oils of plant origin;

(c) Individual chemical compounds obtained from natural sources or produced synthetically.

In accordance with general principles for the acceptance of chemicals for food use, it is necessary to identify and define the pure substance in each case. For the categories listed above, however, the following considerations are of importance:

(a) Preparations manufactured by extraction, steam distillation, maceration, or similar means are complex mixtures prepared by safe and unobjectionable processes involving the use of food-grade extraction or carrier solvents. Specifications have already been prepared for the former, and specifications for the latter will be prepared in the near future. The requirement that these preparations should be manufactured by the use of food-grade solvents,

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[1] See Annex 1, ref. 14

[2] Council of Europe, Natural and artificial flavouring substances (unpublished documents)

or by steam distillation or some other innocuous operation, is considered adequate. The source of raw materials as well as limits for possible contaminants must be specified.

(b) The processes used in the manufacture of essential oils may be similar to those described above, but essential oils almost always contain a major constituent that can be identified and defined by chemical or physical means. Trade specifications are available for such products and it should be possible to examine them and develop suitable specifications for their use in food. Limits may be necessary for constituents of possible toxicological significance.

(c) For individual chemical substances, whether natural or synthetic, it should be possible to prepare suitable specifications in the usual form. The principles set out in the eleventh report[1] would apply in such cases.

3.2.1 Methods for the analysis of flavours in food

The Committee recognized the problems involved in developing suitable analytical procedures, particularly for flavouring materials present in the final food ready for consumption. The methods so far developed cannot discriminate between flavouring substances present naturally in food and the same substances added during processing.

4. REVIEW OF SPECIFICATIONS

The Committee reviewed the specifications prepared at previous meetings for antimicrobials, antioxidants, anticaking agents, emulsifiers, gums, modified starches, and certain other food additives. Some specific recommendations and general observations were made. A revision of the nomenclature is required in certain instances, and the chemical composition of many substances needs to be described more precisely.

Since many of the specifications were prepared at different meetings, there are some discrepancies in the definitions or descriptions of the substances. The specifications thus need editing to bring them into line as regards technical terms and general presentation. Furthermore, a certain number of specifications need updating in the light of present chemical, technological, and toxicological knowledge.

There are inconsistencies in the manner in which limits on heavy metal contaminants, arsenic, and fluorine have been applied to closely related additives. While particular manufacturing processes may occasionally demand the application of different limits, there is a need to bring most of the specifications into line in accordance with the principles given in the tenth report[2]. In some instances it should be possible to reduce the limit of impurities and encourage good manufacturing practice.

Attention was also drawn to the possible contamination of fatty acid derivatives with chlorinated dioxin ('chick oedema factor'). More information is needed on the incidence of such contamination and methods for its detection. There is also a likelihood that

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[1] See Annex 1, ref. 14
[2] See Annex 1, ref. 13

polyoxyethylene-containing esters may be contaminated by ethylene glycol, diethylene glycol, and ethylene chlorhydrine during manufacture. More information is required on the extent and level of such contamination.

The new and better assay methods that have become available in recent years must be taken into consideration and incorporated into specifications or general methods where appropriate. The general methods of analysis were fully reviewed. The inclusion of thin-layer and gas-liquid chromatographic procedures and of more advanced methods for the determination of arsenic (colorimetric procedure) and various heavy metals (atomic absorption procedures) was specifically recommended. Some of the obsolete and inaccurate methods for identification and purity tests need to be replaced. The listing of test solutions should be brought up to date.

Some assay methods for certain food additives call for reference standard samples. The monographs should indicate the sources of supply of such substances, and measures should be taken to ensure that they are made available.

The Committee recommended that all specifications should be revised in the light of the above review and published in a single compendium for easy reference. This should also include details of relevant analytical methods, tests, solutions, and reagents.

5. COMMENTS ON FOOD ADDITIVES ON THE AGENDA

The Committee re-evaluated, in the light of new data, a number of food additives that had undergone toxicological evaluation at previous meetings and for which chemical specifications had been prepared. For many of these substances no change in the acceptance was required and no comment will be made on them. For others, the level of the ADI was either increased or, in a few cases, decreased, for the reasons set out below. The results of the evaluations are summarized in Annex 4 and details of the substances are given in the monographs[1].

5.1 Anticaking agents

Calcium, potassium, and sodium ferrocyanide

The metabolic data available for humans suggest that ferrocyanides are reabsorbed by the renal tubules. A full examination in man of the pharmacokinetic behaviour and biotransformation of orally ingested ferrocyanides is required. On the basis of the no-effect level in the rat, the ADI for man was estimated at 0-0.025 mg/kg body weight; a large safety factor was applied pending the results of the further work mentioned above.

Others

Other anticaking agents considered were: salts of myristic, palmitic, and stearic acids (ammonium, calcium, magnesium, potassium), silicon dioxide and certain silicates (aluminium, calcium, sodium aluminosilicate) to which a 'not limited' acceptance was given, and magnesium silicate (talc and magnesium trisilicate) to which a temporary 'not limited' acceptance was given pending further studies.

[1] See p. 2

5.2 Antimicrobials

Benzoic acid

The ADI for this substance, estimated on the basis of the no-effect level in the rat, remains unchanged at 0-5 mg/kg body weight, the conditional ADI being deleted.

Diethylpyrocarbonate

Urethane (ethyl carbamate) is known to be formed in beverages treated with diethylpyrocarbonate. Urethane is a wide-spectrum carcinogen, producing tumours in many organs and in all species tested, and acts across the placental barrier. Since there was uncertainty about the significance of the presence of urethane at low levels, the previous acceptance of diethylpyrocarbonate was revoked.

Hexamethylenetetramine

The toxicological effects of this substance appear to be due to the liberation of formaldehyde and formic acid, the metabolism of which are known. Long-term studies in rodents show a no-effect level of 500 mg/kg. However, there was a report of fetotoxicity in the dog when the substance was administered at a level of 31 mg/kg; at 15 mg/kg there was no effect. The ADI was accordingly lowered to 0-0.15 mg/kg. The Committee noted the *in vitro* demonstration of the chemical interaction of hexamethylenetetramine with nitrite to form nitrosamine. Although the experimental conditions were considerably more severe than those occurring in food processing, it was thought inadvisable to use hexamethylenetetramine when there is a possibility that nitrite might also be present in the food.

Lactic acid and its sodium, potassium, ammonium, and calcium salts; propionic acid and its sodium, potassium, and calcium salts

Since lactic and propionic acids are normal constituents of food and normal intermediary metabolites in man, it was considered unnecessary to set ADI limits for them. There is, however, some evidence that the neonate has difficulties in utilizing the D(-)isomer of lactic acid; it was considered, therefore, that neither this nor the racemate should be used in foods for infants less than three months old. Metabolic studies on the utilization of D-(-)-lactic acid in infants are needed.

Methyl, ethyl and propyl p-hydroxybenzoates

On the basis of adequate long-term studies in rats, the ADI in man was increased to 0-10 mg/kg body weight (as the sum of ethyl, methyl, and propyl esters).

Nitrate and nitrite, potassium, and sodium salts

The toxic effects of nitrates are due to their transformation to nitrites, in food or water or in the gastrointestinal tract in young infants, whose capacity for secreting acid in the stomach is lower than that of adults. High levels of nitrates occur naturally in a number of vegetables and these may create problems in infants less than three months of age. The Committee discussed the possibility of nitrosamine formation resulting from the use of nitrates and nitrites as food additives and emphasized the need for further work on this problem. Cognizance was taken of recent studies showing that ascorbic acid can prevent the nitrosation of oxytetracycline to diemethyl-nitrosamine *in vitro*[1] and the possible prevention of carcinogenesis by nitrosamines formed *in vivo*. It was acknowledged that in the absence of suitable alternatives, nitrates and nitrites are required to control toxin-forming microorganisms such as *Clostridium botulinum*. For nitrates, the previously

[1] Mirvish, S. S., Wallcave, L., Eagen, M. & Shubik, P. (1972) *Science*, 177, 65

estimated ADI (0-5 mg/kg) was retained and the conditional ADI deleted. For nitrates the previous ADI was lowered to 0.2 mg/kg body weight.

Sodium diacetate

The previous conditional ADI was deleted.

Sorbic acid and its calcium and potassium salts

The ADI for man was set at 0-25 mg/kg body weight, a figure that includes the figures previously given for the conditional ADI.

Sulfur dioxide, sodium and potassium metabisulfite, sodium sulfite and sodium hydrogen sulfite

Recent long-term and 3-generation studies on rats, using metabisulfite in a diet with added thiamine, provided a higher no-effect level than that indicated by the earlier studies in which metabisulfite was administered in drinking water. The ADI could thus be increased.

Others

Other antimicrobials considered were: acetic acid and its potassium and sodium salts, hydrogen peroxide, copper and cupric sulfate, and formic acid.

L-(+)-tartaric acid, sodium, potassium, and mixed sodium-potassium salts

The long-term study in rats showed no adverse effects at the highest level tested, and the substances have been used medicinally for long periods. The evaluation was based on the experimental data, the metabolic inertness of tartrates and the fact that they are normal constituents of food. The ADI for man was estimated at 0-30 mg/kg body weight (calculated as L-(+)-tartaric acid).

In the miscellaneous group, the Committee also considered calcium acetate, calcium chloride, calcium sulfate, calcium gluconate, food-grade mineral oil, oxystearin, and sorbitol.

5.3 Antioxidants

Ascorbic acid and sodium ascorbate

Ascorbic acid exhibits no toxicity after the administration of relatively large doses in animals or man. The estimated ADI for man was accordingly raised to 0-15 mg/kg body weight over and above the amount of ascorbic acid naturally present in foods.

Ascorbyl palmitate and stearate

On the basis of adequate long-term studies in the rat, the ADI for man was estimated at 0-1.25 mg/kg body weight (as the palmitate or stearate, or the sum of both).

Butylated hydroxyanisole, butylated hydroxytoluene, and propyl, octyl, and dodecyl gallate

Recent evidence indicates possible effects on reproduction in the rat, when butylated hydroxyanisole, alone or with propyl gallate, is mixed with lard in the diet. The ADIs of the compounds listed above were therefore made temporary, pending further studies on reproduction using butylated hydroxyanisole alone or in mixtures.

Ethylenediamine tetraacetate, calcium disodium, and sodium salts

On the basis of the no-effect level in the rat, the ADI for man was estimated at 0-2.5 mg/kg body weight, which includes the previous unconditional and conditional ADI figures.

Guaiac resin

The Committee decided to use the alternative name of guaiac resin instead of guaiac gum for this substance, in order to distinguish it more easily from the polysaccharide gums, which have a different chemical structure and a different use. A specification for the substance was prepared and will be published in due course. On the basis of the no-effect level in the rat, the ADI for man was estimated at 0-2.5 mg/kg body weight.

Isoascorbic acid and sodium salt

Adequate short and long-term studies have been carried out in the rat. The biochemical studies indicate that isoascorbic acid is readily metabolized and does not affect the urinary excretion of ascorbic acid. On the basis of the no-effect level in the rat, the ADI for man was estimated at 0-5 mg/kg body weight.

Isopropyl citrate mixture and monoisopropyl citrate

On the basis of the no-effect level in the rat, the ADI for man was estimated at 0-14 mg/kg body weight.

Phosphoric acid, polyphosphates, and their sodium, potassium, calcium and magnesium salts, calcium and magnesium phosphates tribasic

Ingested phosphates from natural sources must be considered together with those from food additive sources, this being a departure from the usual practice in estimating ADIs. The evaluation was based on the normal range of dietary intake and the no-effect level in the rat, to which a safety factor was applied. The acceptable total dietary phosphorus intake for man was estimated at 0-70 mg/kg body weight. This figure applies to the sum of added phosphate and food phosphate and to diets that are nutritionally adequate with respect to calcium. However, in the case of a high intake, proportionally higher amounts of phosphate would be acceptable and vice versa.

While reviewing the specifications for phosphates and polyphosphates, the Committee's attention was drawn to the necessity for tests for cyclic phosphate, which may occur in polyphosphates and create certain health hazards. It was decided to prescribe a limit for cyclic phosphates in the specifications for polyphosphates.

Thiopropionic acid and dilauryl ester

The previous conditional ADI was deleted. Because studies on the distearyl ester were insufficient for evaluation purposes, the ADI does not include this compound.

α-tocopherol and mixed tocopherol concentrate

On the basis of clinical experience with this vitamin, the ADI for man was estimated at 0-2 mg/kg body weight, which includes the previous unconditional and conditional figures.

Others

Other antioxidants considered include citric acid and its salts and nordihydroguaiaretic acid.

5.4 Emulsifiers and stabilizers

Alginic acid and its ammonium, calcium, sodium, and potassium salts

Alginic acid and its four salts were considered together. Additional data were available to show that the alginates *per se* are poorly absorbed. The former conditional ADI - 0-50 mg/kg body weight - was replaced by an ADI of 0-25 mg/kg body weight.

Ammonium salts of phosphatidic acids

Tentative specifications prepared at the thirteenth meeting of the Committee were changed into final specifications. An ADI was established.

Diacetyl tartaric acid and fatty acid esters of glycerol

For these substances, the previous unconditional and conditional ADIs were converted to an ADI of 0-50 mg/kg on the basis of the results of biochemical and metabolic studies and feeding tests in animals.

Esters of glycerol and thermally oxidized soybean fatty acid

The Committee was informed that the so-called esters of glycerol and thermally oxidized soybean fatty acid were in fact simple mixtures of thermally oxidized soybean oil and mono- and diglycerides and not, in fact, true esters. It was not considered necessary to have a specification for such mixtures, since the Committee had prepared separate specifications for the two substances involved. It was accordingly decided to withdraw the specification prepared at the fifteenth meeting of the Committee. The tentative specification for thermally oxidized soybean oil had been prepared at the same meeting.

In the absence of adequate information relating these compounds to compounds that had been studied toxicologically, the Committee was unable to establish an ADI.

Fatty acid esters of glycerol with acetic, citric, lactic and tartaric acids

These substances were treated as a group and given a collective 'not limited' ADI except for tartaric acid, the ADI of which should not exceed 30 mg/kg body weight.

Lecithin

In view of biochemical and nutritional experience with lecithin, the Committee changed the ADI to 'not limited'.

Mono- and diglycerides

The Committee considered that mono- and diglycerides differed little from food so that their use need not be limited.

Polyglycerol esters of fatty acids

The Committee examined metabolic data showing that the fatty acid moiety of this group of compounds is metabolized by the body in the same way as normal fatty acids and the polyol portion is rapidly excreted via the kidney. In the light of these data, together with the findings from extensive studies on animals and some data from man, the former conditional ADI was converted to an ADI of 0-25 mg/kg body weight.

Polyglycerol esters of interesterified ricinoleic acid

On the basis of no-effect levels determined in long-term animal-feeding studies, the ADI was established at 0-7.5 mg/kg body weight.

Polyoxyethylene (20) sorbitan esters of lauric, stearic, palmitic, and oleic acids and triesters of stearic acid

The Committee reviewed this series of compounds and decided to retain the former unconditional ADI as the ADI.

Polyoxyethylene (8) stearate and polyoxyethylene (40) stearate

The Committee decided to group these substances on the ground that they were broken down and treated by the body in a similar manner. An ADI was therefore established for the two compounds used in combination.

Propylene glycol esters of fatty acids

There is evidence that the propylene glycol esters of fatty acids are hydrolysed to propylene glycol and fatty acids. The Committee's evaluation was based on the content of propylene glycol, for which an ADI had been established.

Sorbitan esters of palmitic and stearic acids and triesters of stearic acid

The Committee considered that the partial esters of sorbitan have been thoroughly investigated in both short-term and long-term experiments, as well as in man. The data was considered as convering sorbitan mono- and tristearates and sorbitan monopalmitate, and an ADI was assigned to the whole group.

Stearyl citrate

The data for this compound were carefully re-evaluated and the previous ADI increased.

Others

Other emulsifiers considered include: cholic and desoxycholic acid; hydroxylated lecithin; mixed tartaric, acetic, and fatty acid esters of glycerol; stearoyl lactylic acid and salts; sucrose esters of fatty acids; and sucroglycerides.

5.5 Thickening agents

5.5.1 Celluloses

The Committee allocated a group ADI of 0-25 mg/kg body weight to hydroxypropyl cellulose, hydroxypropyl methyl cellulose, methyl cellulose, methyl ethyl cellulose, and sodium carboxymethyl cellulose. A 'not limited' acceptance was given to microcrystalline cellulose.

Pectins

The specification for pectin prepared by the Committee at its thirteenth meeting was reviewed. A revised tentative specification covering both the nonamidated and amidated pectins was prepared in the light of additional information now available. The specification will be published in due course. A separate ADI was established for amidated pectin.

5.5.2 Gums

Carrageenan and furcellaran

When the Committee first considered carrageenan, it was unaware either of the numerous varieties coming under this general heading or of the possible toxicological implications of difference in sources, methods of isolation and preparation, molecular forms, and molecular-weight fractions. That these factors cannot be ignored is suggested by reports of the ulcerogenic and other effects of carrageenans in various animal species. The precise nature of the carrageenan tested was, however, not always clearly stated. One unequivocal conclusion that may be drawn from the animal work is that a particular preparation of degraded carrageenan (used as a therapeutic agent and not as a food additive) is ulcerogenic under certain circumstances. There is no definite evidence that any of the various forms of undegraded carrageenan are capable of eliciting pathological changes in experimental animals or man. At present, there is little information on the proportions of degraded carrageenans occurring in the various food additive forms. Also, there is a paucity of data on the extent

to which carrageenans are degraded during the processing of acid foods containing them as additives. It is imperative to establish the exact significance of such degradation from a toxicological standpoint.

In the present state of knowledge it is therefore concluded that forms of carrageenan of low molecular weight should be avoided in food. These considerations also affect specification and analysis, questions that are dealt with in the monograph on carrageenan[1]. Similarly, the evaluation of food-grade carrageenan given in this report takes into account the new biological data available. The specifications will need suitable amendments in the light of additional information, e.g. on viscosity and on the dispersity of molecular weights.

Others

The Committee considered also agar, arabic gum, and guar gum, for each of which a 'not limited' acceptance was given. For carob bean gum, karaya gum, and tragacanth gum, no evaluation was possible with the data provided.

5.5.3 Modified starches

On the basis of the recommendations contained in the thirteenth report[2], and in view of the fact that both general and individual specifications for different enzyme preparations intended for food processing have since been prepared, the Committee decided that only these enzyme preparations should be used for the manufacture of enzyme-treated starches. It was also decided to prescribe the same limits of impurities for enzyme-treated starches as for chemically treated starches.

The Committee finalized tentative specifications for hydroxypropyl distarch phosphate that had been prepared at the thirteenth meeting. The opportunity was taken of reviewing the specifications for other chemically treated starches and carrying out some minor amendments in the light of more detailed information now available. A test procedure for the determination of residual manganese in bleached starch was provided. With the exception of hydroxypropyl distarch phosphate and starch sodium succinate, all the modified starches had already been given a 'not limited' ADI.

5.6 Miscellaneous

A miscellaneous group of compounds unrelated functionally or structurally was considered by the Committee.

L-glutamic acid and its monosodium, potassium, ammonium, and calcium salts

The Committee considered glutamic acid together with its sodium, potassium, ammonium, and calcium salts. Although findings concerning the susceptibility of neonates to glutamates were considered controversial, the Committee concluded it would be prudent not to apply the ADI to infants under 12 weeks of age. Data suggesting that ingestion by mothers of high levels of glutamates had harmful effects on offspring (exposed *in utero* or when suckling) were considered less convincing, and the former ADI of 0-120 mg/kg body weight, calculated as glutamic acid, was retained.

Polyvinyl pyrrolidone

It was noted that, in the past, the Committee had expressed certain reservations about the dependability of the available data on the body storage of polyvinyl pyrrolidone, particularly in the intestinal lymph nodes. Because of more general concern about the effects of stored macromolecules, the Committee decided to discontinue the previous conditional ADI and not to set an ADI for this substance.

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[1] See p.2
[2]

1,2-propylene glycol

The Committee took note of the metabolic behaviour of the compound as well as toxicological data, and an ADI of 0-125 mg/kg body weight was set.

6. FUTURE WORK

1. The Codex Alimentarius Commission, the principal organ of the Joint FAO/WHO Food Standards Programme, has the function of drawing up international food standards to protect the health of the consumer and facilitate international trade in food. To provide the necessary scientific basis for the Codex Food Standards, the Committee should continue to evaluate those food additives on which it is proposed to include provisions in the standards.

2. In 1970, recognizing the potential hazards of food additives to the consumer, the Twenty-third World Health Assembly adopted a resolution[1] requesting Member States to transmit to WHO any decision to limit or prohibit the use of a food additive. WHO was requested to transmit this information to other Member States and where desirable, to arrange for the toxicity of the food additive to be evaluated by the Joint FAO/WHO Expert Committee on Food Additives.

In addition, it is considered advisable that, before finalizing the agenda of any future meetings of the Committee, FAO and WHO should request the members of the WHO Expert Advisory Panel on Food Additives and the FAO panel of experts to collect new toxicity and chemical data on food additives and to solicit suggestions for the Committee's discussions.

3. Future meetings of the Committee should continue to give consideration to the technological efficacy of food additives, including their mechanism of action. This will help Member governments and the appropriate Codex Committees to define good manufacturing practices and facilitate decisions on the acceptance of additives for food use.

4. The general principles governing the establishment of specifications should be reviewed in the light of the current work and future programme of the Committee. In addition, the review of all the specifications should be completed as soon as possible, and suitably revised or edited specifications, together with the required methods of analysis, should be issued in a single compendium.

5. The methods and procedures for the toxicity testing of food additives should be comprehensively reviewed and brought into line with advances in toxicology and cognate disciplines.

6. Further information is needed on the chemical fate of food additives in foods and their interaction with food components, with particular reference to the formation of toxic substances that would need to be taken into account in toxicological evaluations.

7. Recent studies demonstrate that the capacity to absorb and store certain macromolecular materials within the body extends to some substances used as food additives. It is recommended that similar studies should be conducted on the full range of additives added to food in macromolecular form.

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[1] World Health Organization (1973) Handbook of resolutions and decisions of the World Health Assembly and the Executive Board, Vol. 1, 1948-1972, Geneva, p.156 (WHA23.50)

7. RECOMMENDATIONS

1. Meetings of the Expert Committee should be held regularly by FAO and WHO in order to deal with the topics enumerated in section 6.

2. WHO should systematize the means at its disposal for the identification of food additives that new data have shown to be of dubious safety.

3. In considering the toxicity of food additives, consideration has been given to a number of natural constituents of food that have deleterious effects. In consultation with FAO, WHO should convene a meeting of experts to assess the health hazards presented by these substances.

ACKNOWLEDGEMENT

The Committee records its appreciation of the helpful information provided by Dr P. Shubik, Director of the Eppley Institute for Research in Cancer, College of Medicine, University of Nebraska, Omaha, Nebraska, USA.

ANNEX 1

REPORTS AND OTHER DOCUMENTS RESULTING
FROM PREVIOUS MEETINGS OF THE JOINT FAO/WHO
EXPERT COMMITTEE ON FOOD ADDITIVES

1. General Principles Governing the Use of Food Additives. First Report. FAO Nutrition Meetings Report Series, 1956, No. 11; Wld Hlth Org. techn. Rep. Ser., 1956, No. 129

2. Procedures for the Testing of Intentional Food Additives to Establish their Safety for Use. Second Report. FAO Nutrition Meetings Report Series, 1958, No. 17; Wld Hlth Org. techn. Rep. Ser., 1958, No. 144

3. Specifications for Identity and Purity of Food Additives (Antimicrobial Preservatives and Antioxidants). Third Report. These specifications were subsequently revised and published as Specifications for Identity and Purity of Food Additives, vol. I. Antimicrobial Preservatives and Antioxidants, Rome, Food and Agriculture Organization of the United Nations, 1962

4. Specifications for Identity and Purity of Food Additives (Food Colours). Fourth Report. These specifications were subsequently revised and published as Specifications for Identity and Purity of Food Additives, vol. II. Food Colours, Rome, Food and Agriculture Organization of the United Nations, 1963

5. Evaluation of the Carcinogenic Hazards of Food Additives. Fifth Report. FAO Nutrition Meetings Report Series, 1961, No. 29; Wld Hlth Org. techn. Rep. Ser., 1961, No. 220

6. Evaluation of the Toxicity of a Number of Antimicrobials and Antioxidants. Sixth Report. FAO Nutrition Meetings Report Series, 1962, No. 31; Wld Hlth Org. techn. Rep. Ser., 1962, No. 228

7. Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation : Emulsifiers, Stabilizers, Bleaching and Maturing Agents. Seventh Report. FAO Nutrition Meetings Report Series, 1964, No. 35; Wld Hlth Org. techn. Rep. Ser., 1964, No. 281

8. Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation : Food Colours and Some Antimicrobials and Antioxidants. Eighth Report. FAO Nutrition Meetings Report Series, 1965, No. 38; Wld Hlth Org. techn. Rep. Ser., 1965, No. 309

*9. Specifications for Identity and Purity and Toxicological Evaluation of some Antimicrobials and Antioxidants. FAO Nutrition Meetings Report Series, 1965, No. 38A; WHO/Food Add/24.65

*10. Specifications for Identity and Purity and Toxicological Evaluation of some Food Colours. FAO Nutrition Meetings Report Series, 1966, No. 38B; WHO/Food Add/66.25

11. Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation : Some Antimicrobials, Antioxidants, Emulsifiers, Stabilizers, Flour-treatment Agents, Acids and Bases. Ninth Report. FAO Nutrition Meetings Report Series, 1966, No. 40; Wld Hlth Org. techn. Rep. Ser., 1966, No. 339

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*12. Toxicological Evaluation of Some Antimicrobials, Antioxidants, Emulsifiers, Stabilizers, Flour-treatment Agents, Acids and Bases. FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29

13. Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation : Some Emulsifiers and Stabilizers and Certain Other Substances. Tenth Report. FAO Nutrition Meetings Report Series, 1967, No. 43; Wld Hlth Org. techn. Rep. Ser., 1967, No. 373

14. Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation : Some Flavouring Substances and Non-Nutritive Sweetening Agents. Eleventh Report. FAO Nutrition Meetings Report Series, 1968, No. 44; Wld Hlth Org. techn. Rep. Ser., 1968, No. 383

*15. Toxicological Evaluation of Some Flavouring Substances and Non-Nutritive Sweetening Agents. FAO Nutrition Meetings Report Series, 1968, No. 44A; WHO/Food Add/68.33

*16. Specifications and Criteria for Identity and Purity of Some Flavouring Substances and Non-Nutritive Sweetening Agents. FAO Nutrition Meetings Report Series, 1969, No. 44B; WHO/Food Add/69.31

17. Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation : Some Antibiotics. Twelfth Report. FAO Nutrition Meetings Report Series, 1969, No. 45; Wld Hlth Org. techn. Rep. Ser., 1969, No. 430

*18. Specifications for the Identity and Purity of Some Antibiotics. FAO Nutrition Meetings Report Series, 1969, No. 45A; WHO/Food Add/69.34

19. Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation : Some Food Colours, Emulsifiers, Stabilizers, Anticaking Agents, and Certain Other Substances. Thirteenth Report. FAO Nutrition Meetings Report Series, 1970, No. 46; Wld Hlth Org. techn. Rep. Ser., 1970, No. 445

*20. Toxicological Evaluation of Some Food Colours, Emulsifiers, Stabilizers, Anticaking Agents and Certain Other Substances. FAO Nutrition Meetings Report Series, No. 46A; WHO/Food Add/70.36

*21. Specifications for the Identity and Purity of Some Food Colours, Emulsifiers and Stabilizers, Anticaking Agents and Certain Other Substances. FAO Nutrition Meetings Report Series, No. 46B; WHO/Food Add/70.37

22. Evaluation of Food Additives. Specifications for the Identity and Purity of Food Additives and their Toxicological Evaluation : Some Extraction Solvents and Certain Other Substances; and a Review of the Technological Efficacy of Some Antimicrobial Agents. Fourteenth Report. FAO Nutrition Meetings Report Series, 1971, No. 48; Wld Hlth Org. techn. Rep. Ser., 1971, No. 462

*23. Toxicological Evaluation of Some Extraction Solvents and Certain Other Substances. FAO Nutrition Meetings Report Series, 1971, No. 48A; WHO/Food Add/71.39

*24. Specifications for the Identity and Purity of Some Extraction Solvents and Certain Other Substances. FAO Nutrition Meetings Report Series, 1971, No. 48B; WHO/Food Add/71.40

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*25. A Review of the Technological Efficacy of Some Antimicrobial Agents. FAO Nutrition Meetings Report Series, 1971, No. 48C; WHO/Food Add/71.41

26. Evaluation of Food Additives. Some Enzymes, Modified Starches, and Certain Other Substances : Toxicological Evaluations and Specifications and a Review of the Technological Efficacy of Some Antioxidants. Fifteenth Report. FAO Nutrition Meetings Report Series, 1972, No. 50; Wld Hlth Org. techn. Rep. Ser., 1972, No. 488

27. Toxicological Evaluation of Some Enzymes, Modified Starches and Certain Other Substances. FAO Nutrition Meetings Report Series, 1972, No. 50A; WHO Food Additives Series, 1972, No. 1

28. Specifications for the Identity and Purity of Some Enzymes and Certain Other Substances. FAO Nutrition Meetings Report Series, 1972, No. 50B; WHO Food Additives Series, 1972, No. 2

29. A Review of the Technological Efficacy of Some Antioxidants. FAO Nutrition Meetings Report Series, 1972, No. 50C; WHO Food Additives Series, 1972, No. 3

30. Evaluation of Certain Food Additives and the Contaminants Mercury, Lead and Cadmium. Sixteenth Report. FAO Nutrition Meetings Report Series, 1972, No. 51; Wld Hlth Org. techn. Rep. Ser., 1972, No. 505

31. Evaluation of Mercury, Lead, Cadmium and the Food Additives Amaranth, Diethylpyrocarbonate and Octyl Gallate. FAO Nutrition Meetings Report Series, 1972, No. 51A; WHO Food Additives Series, 1972, No. 4

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* These documents can be obtained on request from: Food Additives, World Health Organization, 1211 Geneva 27, Switzerland, or: Food Policy and Food Science Service, Food and Agriculture Organization of the United Nations, 00100 Rome, Italy.

ANNEX 2

LIST OF SUBSTANCES ON THE AGENDA

1. Antimicrobials (including hexamethylenetetramine)
2. Antioxidants (including ascorbic acid)
3. Anticaking agents
4. Emulsifiers (including esters of glycerol and thermally oxidized soybean fatty acids and thermally oxidized soybean oil)
5. Gums (including carrageenan)
6. Modified starches
7. Miscellaneous
   - (a) Mineral oils
   - (b) Monosodium glutamate
   - (c) Saccharin

ANNEX 3

PROCEDURES FOR TESTING FOOD ADDITIVES

As no single pattern of tests could adequately cover a range of substances so diverse in structure and function as food additives, it would be unreasonable to impose a rigidly uniform set of experimental procedures. It cannot be emphasized too strongly that the conduct of experiments designed to test the safe use of food additives is the responsibility of the specialized scientist. For these reasons, it is possible only to formulate general guidelines on testing procedures.

Acute toxicity studies

The following statements from the second report of the Joint FAO/WHO Expert Committee on Food Additives[1] remain valid:

> The phrase 'acute toxicity test' implies the study of the effects produced by the test material when administered in a single dose. The acute toxicity tests should give sufficient information to enable comparisons of the toxicity of related materials to be made and to provide the necessary information for the planning of further studies. Acute toxicity tests may indicate variation among species and yield some information on the signs of intoxication and pathological effects.
>
> It is advisable to employ at least three species, one of which should be a non-rodent. Both sexes should be used in at least one species. When doses greater than 5 g per kg of body weight produce no deaths in the test animals an accurate determination of the lethal dose is unnecessary. With lethal doses under 5 g/kg, the LD_{50} in one species should be determined by an appropriate method. For other species it is desirable to determine the approximate lethal dose, where this is less than 5 g/kg, in order to indicate whether there is an important difference in species susceptibility.
>
> The test material should be administered orally and parenterally. Where possible it should be administered as a solution in water, edible oil or other suitable solvent; if this is not possible an inert suspending agent may be used. In all cases control data should be available on any vehicle employed.
>
> The animals should be observed for a period of 2 to 4 weeks, depending on their condition. Observation should include the onset, nature and duration of toxic signs, as well as mortality. It is important that autopsies be performed on some animals that die and on some of the survivors. Microscopic examination of tissues should be carried out if the macroscopic study indicates that it is needed.

Studies on acute toxicity in several animal species should make it possible to gain an idea of the apparent mode of action of the chemical, e.g. whether it acts as an anti-cholinesterase, central nervous system depressant or convulsant, a metabolic stimulant, or a liver- or kidney-damaging agent. Comparison with well known chemicals may be useful when a new chemical falls into the same general category.

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[1] See Annex 1, ref. 2, p. 9

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Biochemical studies

A number of types of study are included under this heading. Significant biochemical aspects include mode, rate and degree of absorption, levels of storage in organs and tissues, metabolic transformation, and mode and rate of elimination. Modifications of substances during metabolism may significantly affect their toxicity. Knowledge whether or not a food additive is rapidly metabolized into innocuous degradation products, rapidly excreted, or accumulated in certain organs or tissues may be of great value in assessing potential hazards.

It is highly important that information about the metabolism and distribution of a substance undergoing testing should be obtained at an early stage since it may then be possible to make similar investigations in man. The information from such investigations will make it possible to choose, for further experimentation, the animal species corresponding most closely to man in the absorption and metabolism of the substance and thus to obtain data on animal toxicity that will have enhanced predictive value.

In biochemical studies, the possibility of reactions of the additive with food constituents should be taken into account. Such reactions are of two types. First, the nutritional value of the food may be affected; this possibility may be studied by chemical or biological assay methods. Second, new and possibly toxic substances may be formed; these must be investigated by the usual toxicological procedures. Cooking, storage, or the application of other technological procedures may also alter the test substance. It may be necessary to undertake a toxicological investigation of treated food materials; here a margin of safety may be introduced by conducting the tests with food that has been deliberately overtreated to a measured extent.

Some additives may be converted into substances already present naturally in food in much greater amounts. If the biochemical evidence shows that the sole effect of the additive is to make a small contribution to existing metabolic loads from food components, there is no need for detailed toxicological studies, a case in point being that of the lactic and fatty acid esters of glycerol, which are completely hydrolysed in the intestinal lumen with the formation of substances already present in the diet in much greater quantities.

If a series of chemical analogues can be shown to give rise to the same main metabolic product and to other compounds that are already present in the organism in greater quantities, or that can be readily and safely metabolized, it may be sufficient to carry out toxicological studies on a suitable representative of the series.

The effects of the additive on important enzyme systems in blood or tissues should be studied. For example, an increase in the level of certain marker enzymes in the serum, such as transaminases and other intracellular enzymes, may be indicative of tissue damage. Another aspect to be investigated is the induction of microsomal enzyme systems, especially in the liver. The relevance to man of these biochemical changes needs careful assessment.

Short-term studies

In the second report of the Committee[1], short-term studies are discussed in the following terms:

> The purposes of the short-term test are to examine the biological nature of toxic effects, to assess possible cumulative action, the variation in species sensitivity, the nature of macro- and microscopic changes, and the approximate dose level at which these effects occur. It may yield information

[1] See Annex 1, ref. 2, pp. 10-11

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sufficient to show that the test material is too toxic to warrant further study. It may also provide guidance for the selection of dosage for long-term tests and indicate special studies that may be necessary.

At least two species, including a rodent and a non-rodent, should be used. The number of animals should be enough to allow for a statistical evaluation of the data. In the feeding experiments a sufficient number of levels should be selected to ensure that at least one level has no effect, and that doses are included which produce definite toxic effects, if this is possible. If no effects are observed at dosage levels of 10% in the diet, no useful purpose is served by employing higher levels. It is essential that a control group on the untreated diet be included in the experiment.

Observations should include general appearance, behaviour, growth and mortality. In some cases estimation of food intake, studies of blood and urine chemistry and organ function tests may be indicated. The study of the organs should include macroscopic and microscopic examination and measurement of the relative organ weights in the test and control groups.

These recommendations are still applicable except when the available data show that in the rat (considered in the report as the animal of choice) the metabolism of the chemical being tested is not comparable with its metabolism in man.

It may be feasible or necessary to administer test substances by gavage or by capsule, e.g. in cases of limited solubility, unpleasant taste, or need for accurate dosing. These procedures may produce effects different from those produced by adding a substance to the diet, either by increasing or decreasing the rate of absorption from the gastrointestinal tract or by influencing the metabolic pathway in the bacterial flora of the upper intestine. The validity of these forms of administration will depend on the substance tested.

Long-term toxicity

Where it is considered necessary to carry out long-term toxicity and carcinogenicity studies, the following principles formulated by a WHO Scientific Group on Procedures for Investigating Intentional and Unintentional Food Additives[1] should be followed:

Scientific judgement is necessary in determining the duration of animal studies for the evaluation of an individual food additive. Where adequate biochemical and toxicological data on closely related chemicals are available, the objective becomes the detection of any deviation from the established pattern. This can usually be determined by intensive studies of a few months' duration when these are adequately designed and evaluated. Appropriate studies in humans add significantly to the adequacy of the data.

In the absence of such definitive data, or if there are reasons to suspect carcinogenic potential, longer-term studies must still be relied upon for reassurance. Recent advances in the quality of research animals, and particularly in the control of pathogens, have increased the life-span of some strains of animals. In spite of this, feeding studies adequately designed and evaluated extending up to eighteen months in mice and two years in rats are still considered adequate to ensure a minimum safeguard in evaluating the carcinogenic potential of a chemical additive. In special cases it may be desirable to prolong the observations in these species.

[1] Wld Hlth Org. techn. Rep. Ser., 1967, No. 348, pp. 13-14

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If there are good reasons to doubt the relevance to man of the data obtained in rodent species - for example, if the metabolism of the additive in man is found to be significantly different from that in rodents - it may be desirable to carry out investigations of longer duration in other species. In addition, the investigation of some potential toxic effects, particularly carcinogenicity, requires careful prolonged observation of the offspring. Detailed study of general appearance and behaviour, biochemical effects, metabolism and histopathology should be included and fully reported, both qualitatively and quantitatively.

Special studies

Reproduction, embryotoxicity, and teratogenicity studies

During the preceding tests, or in a pilot experiment, careful attention should be paid to any changes likely to affect reproduction. If there is any evidence to suggest that reproduction may be affected, specific studies should be undertaken.

Reproduction studies must be carried out in a suitable species over at least two generations and may have to be continued over three or more. They should be designed to provide relevant information on fertility, progress of pregnancy, post-partum condition, and progress of mothers and offspring[1].

Reproduction studies may be designed to include investigations on embryotoxicity and teratogenicity. Alternatively it may be more convenient to conduct separate investigations on these aspects. Many suggested procedures for these studies have been published, but no one particular design has yet emerged as being universally acceptable.

Carcinogenicity and mutagenicity

Special consideration should be given to testing for the possible carcinogenicity and mutagenic potential of substances to be used in food. The Committee noted that these matters had been dealt with by a WHO Scientific Group on the Principles for the Testing and Evaluation of Drugs for Carcinogenicity[2], a WHO Scientific Group on the Evaluation and Testing of Drugs for Mutagenicity[3], and the Canadiam Department of Health and Welfare[4]. It was also noted that a Scientific Group on the Assessment of the Carcinogenicity and Mutagenicity of Chemicals will be convened by WHO in 1973.

Certain aspects need particularly careful evaluation. If, for example, a promoting effect is detected in the course of a carcinogenicity test it cannot be ignored. However, routine testing for promoting effects should not be used until more is known about its mechanism and how broadly it can be applied. Likewise, even when cancer is produced by chemicals administered by other than the oral route, their evaluation for food additive use requires experiments using oral administration.

Tissue culture methods for the study of neoplastic transformation, using chemical carcinogens, have not yet been fully developed, and it would be premature to introduce these methods into toxicity studies of food additives.

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1 Wld Hlth Org. techn. Rep. Ser., 1967, No. 364
2 Wld Hlth Org. techn. Rep. Ser., 1969, No. 462
3 Wld Hlth Org. techn. Rep. Ser., 1971, No. 482
4 Canada, Department of Health and Welfare (1973) The testing of chemicals for carcinogenicity, mutagenicity, and teratogenicity, Ottawa

Annex 3

A procedure at present under investigation in routine carcinogenesis studies includes the use of two parent and offspring generations to take into account the transplacental transport of carcinogens and their transfer to milk. No recommendation can yet be made as to the suitability of this method for the toxicological investigation of food additives.

Observations in man

Observations in man are of prime importance because of the differences between one species and another in reactions to toxic substances and the subsequent uncertainty when extrapolating data from animal experiments to human beings.

Studies in man may be carried out by the careful observation of individuals who have ingested the test compound. Additional data may be obtained by studying individuals exposed to additives occupationally or through accidental ingestion. Finally, studies may also be carried out in populations consuming a given additive at high levels because of ethnic proclivities or for therapeutic purposes.

ANNEX 4

ACCEPTABLE DAILY INTAKES*

Substances	Acceptable daily intake for man (mg/kg body weight)
Anticaking agents	
calcium, potassium, sodium ferrocyanide	0-0.025[1]
salts of myristic, palmitic, and stearic acids (aluminum, ammonium, calcium, magnesium, potassium, sodium)	Not limited
silicon dioxide and certain silicates (aluminium, calcium, sodium alumino silicate)	Not limited
magnesium silicate (talc and magnesium trisilicate)	Not limited[1]
Antimicrobials	
acetic acid and its potassium and sodium salts	Not limited
benzoic acid and its potassium and sodium salts[10]	0-5
p-hydroxybenzoate, butyl	No ADI allocated[2]
p-hydroxybenzoate, ethyl[3]	0-10
p-hydroxybenzoate, methyl[3]	0-10
p-hydroxybenzoate, propyl[3]	0-10
diethyl pyrocarbonate[4]	Not to be used
formic acid	0-3
cupric sulfate	No ADI[5]
hexamethylenetetramine	0-0.15
hydrogen peroxide	No ADI[6]
lactic acid and its ammonium, calcium, potassium, and sodium salts	Not limited[7]
nitrate, potassium and sodium salts[8]	0-5
nitrite, potassium and sodium salts[9]	0-0.2[1]
propionic acid and its calcium, potassium, and sodium salts	Not limited
sodium diacetate	0-15
sorbic acid and its calcium, potassium and sodium salts[11]	0-25
sulfur dioxide and sulfites[12] (sodium and potassium metabisulfites, sodium sulfite, sodium hydrogen sulfite)	0-0.7

* For footnotes, see p.36

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Substances	Acceptable daily intake for man (mg/kg body weight)
Antioxidants	
ascorbic acid and its sodium salt	0-15[13]
ascorbyl palmitate and stearate[14]	0-1.25
butylated hydroxyanisole	0-0.5[15]
butylated hydroxytoluene	0-0.5[15]
citric acid and its calcium, potassium, and sodium salts	Not limited
ethylenediaminetetraacetate, calcium disodium and sodium salts[16]	0-2.5
gallates, dodecyl, octyl, propyl[17]	0-0.2
guaiac resin	0-2.5
isoascorbic acid and its sodium salt	0-5
isopropyl citrate mixture and monoisopropyl citrate[18]	0-14
nordihydroguaiaretic acid	No ADI allocated[2]
phosphoric acid and its salts	0-70[19]
tartaric acid (and its potassium, sodium, and sodium potassium salts)[20]	0-30
thiodipropionic acid and dilauryl ester[21]	0-3
α-tocopherol and mixed tocopherols concentrate[22]	0-2
Emulsifiers	
acetic acid and fatty acid esters of glycerol[23]	Not limited
alginic acid and its ammonium, calcium, potassium, and sodium salts[24]	0-25
ammonium salts of phosphatidic acid	0-15[1]
cholic and desoxycholic acid and their salts	0-1.25
citric acid and fatty acid esters of glycerol[23]	Not limited
diacetyl tartaric acid and fatty acid esters of glycerol	0-50
esters of glycerol and thermally oxidized soybean fatty acid	Decision postponed[25]
hydroxylated lecithin	No ADI allocated[2]

Annex 4

Substances	Acceptable daily intake for man (mg/kg body weight)
Emulsifiers (contd)	
lactic acid and fatty acid esters of glycerol[23]	Not limited
lecithin	Not limited
mixed tartaric and acetic acid and fatty acid esters of glycerol[23]	Not limited
mono- and diglycerides	Not limited
polyglycerol esters of fatty acids	0-25[26]
polyglycerol esters of interesterified ricinoleic acid	0-7.5
polyoxyethylene (20) sorbitan esters of lauric, stearic, palmitic and oleic acids and triesters of stearic acid[27]	0-25
polyoxyethylene (8) and polyoxyethylene (40) stearate[28]	0-25
propylene glycol alginate[34]	0-25
propylene glycol esters of fatty acids[29]	0-125
sorbitan esters of palmitic and stearic acids and triesters of stearic acid[30]	0-25
stearoyl lactylic acid, calcium and sodium salts	0-20
stearyl citrate	0-50
sucrose esters of fatty acids and sucroglycerides	0-2.5[1]
Thickening agents	
A. Celluloses:	
hydroxypropyl cellulose[31]	0-25
hydroxypropyl methyl cellulose[31]	0-25
methyl cellulose[31]	0-25
methylethyl cellulose[31]	0-25
microcrystalline cellulose	Not limited
pectin (non-amidated)	Not limited
pectin (amidated)	0-25[1]
sodium carboxymethyl cellulose[31]	0-25

Annex 4

Substances	Acceptable daily intake for man (mg/kg body weight)
Thickening agents (contd)	
B. Gums:	
agar	Not limited
arabic gum	Not limited
carob bean gum	No ADI allocated[2]
carrageenan and furcellaran	0-75
guar gum	Not limited
karaya gum	No ADI limited[2]
tragacanth gum	No ADI allocated[2]
C. Modified starches and dextrins:	
acetylated distarch adipate	Not limited
acetylated distarch glycerol	Not limited
acetylated distarch phosphate	Not limited
acid-treated starches	Not limited
alkali-treated starches	Not limited
amylose and amylopectin	Not limited
bleached starches	Not limited
distarch glycerol	Not limited
distarch phosphate	Not limited
enzyme-treated starches	Not limited
hydroxypropyl distarch glycerol	Not limited
hydroxypropyl distarch phosphate	No ADI allocated[2]
hydroxypropyl starch	Not limited
mono-starch phosphate	Not limited
oxidized starches	Not limited[1]
phosphated distarch phosphate	Not limited
starch acetate	Not limited
starch sodium succinate	No ADI allocated[2]
white and yellow dextrins	Not limited

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Substances	Acceptable daily intake for man (mg/kg body weight)
Miscellaneous food additives	
calcium acetate, chloride and sulfate	Not limited
food-grade mineral oil	Not limited
L-glutamic acid, its ammonium, calcium, monosodium, and potassium salts[32]	0-120
oxystearin	0-25
phosphates (see phosphoric acid, p. 32)	
polyvinyl pyrrolidone	No ADI allocated[33]
1,2-propylene glycol	0-125
sorbitol	Not limited

Annex 4

FOOTNOTES

1 Temporary acceptance.

2 Evaluation not possible with data provided.

3 As sum of ethyl, methyl, and propyl esters of p-hydroxybenzoic acid.

4 The treatment level allocated in previous reports is withdrawn.

5 Maximum acceptable daily load = 0.5 mg/kg body weight expressed as Cu.

6 This substance is to be used only if better methods of milk preservation are not available.

7 Neither D(-)-lactic acid nor (DL)-lactic acid should be used in infant foods.

8 As sodium nitrate.

9 As sodium nitrite.

10 As sum of benzoic acid and sodium and potassium benzoate (expressed as benzoic acid).

11 As sum of sorbic acid and calcium, potassium, and sodium sorbates (expressed as sorbic acid).

12 As SO_2.

13 This figure is in addition to the ascorbic acid present naturally in foods.

14 As ascorbyl stearate or ascorbyl palmitate, or the sum of both.

15 As BHA, BHT, or the sum of both. Temporary acceptance.

16 As $CaNa_2EDTA$.

17 As sum of dodecyl, octyl, and propyl gallate. Temporary acceptance.

18 As monoisopropyl citrate.

19 Total dietary phosphorus load for man. Attention should be paid to the reverse relationship with calcium intake.

20 As L-(+)-tartaric acid.

21 As thiodipropionic acid.

22 As α-tocopherol.

23 As sum of total glycerol esters of fatty acids and acetic, citric, lactic, and tartaric acids, provided that the total food additive intake of tartaric acid does not exceed 30 mg/kg.

24 As alginic acid.

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25 Previously allocated temporary ADI is withdrawn. Decision postponed pending adequate specifications.

26 As polyglycerol esters of palmitic acid.

27 As total polyoxyethylene (20) sorbitan esters.

28 As total of polyoxyethylene (8) and (40) stearates.

29 As propylene glycol.

30 As total sorbitan esters.

31 As the sum of total modified celluloses. The ADI may be exceeded for dietetic purposes, i.e. when use is primarily intended to take advantage of the non-caloric properties of these additives.

32 As glutamic acid, additional to glutamic acid intake from all non-additive dietary sources. ADI not applicable to infants under 12 weeks of age.

33 Evaluation not possible with data provided. Previously allocated conditional ADI is withdrawn.

34 The contribution from propylene alginate to total dietary propylene glycol intake from all sources should be included in the ADI for propylene glycol.

ANNEX 5

FURTHER TOXICOLOGICAL STUDIES AND INFORMATION REQUIRED OR DESIRABLE

1. Anticaking agents

Calcium, potassium, sodium ferrocyanide: Metabolic studies in man[1]. If these reveal any untoward effects, a long-term study in one species will be required.

Magnesium silicate: Studies to elucidate the reported kidney damage in dogs by magnesium silicate. Long-term studies on talc demonstrated to be free from asbestos-like particles. A satisfactory method for estimating asbestos-like particles in talc and magnesium silicate[2].

2. Antimicrobials

p-hydroxybenzoate, ethyl: Further biochemical studies in man and animals.

p-hydroxybenzoate, methyl: Additional studies in man.

Lactic acid and its salts: Metabolic studies on the utilization of D(-) and DL-lactic acids in infants.

Nitrites, potassium and sodium salts: Full evaluation when results of investigations in progress become available.

Sulfur dioxide and sulfites: Additional mutagenic studies in a mammalian system. Studies over longer periods in man.

3. Antioxidants

Butylated hydroxyanisole: Studies of the effect on reproduction of mixtures of BHA, BHT, and propyl gallate, and of BHA alone[2].

Butylated hydroxytoluene: Studies of the effect on reproduction of mixtures of BHT, BHA, and propyl gallate[2].

Gallates, dodecyl, octyl, propyl: Studies of the effect on reproduction of mixtures of BHA, BHT, and propyl gallate[2].

Thiodipropionic acid and dilauryl ester: Biochemical studies and observations on human subjects. Specifications for the distearyl ester.

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[1] Required by June 1974
[2] Required by June 1976

Annex 5

4. Emulsifiers

Ammonium salts of phosphatidic acids: Submission of the results of the long-term study in the rat and metabolic studies in several species[1]. Studies on the metabolic fate of these compounds in man.

Polyglycerol esters of fatty acids: Properly conducted biochemical studies on other members of this group that do not conform to the specifications already established, particularly those containing short chain fatty acids.

Stearoyl lactylic acid, calcium and sodium salts: Studies confirming that this compound is metabolized by man in the same way as by other species.

Stearyl citrate: Short-term studies in two species (one a non-rodent mammalian species); some of these studies should use combinations with hard fats. Emphasis should be placed on the evaluation of kidney function.

Sucrose esters of fatty acids and sucroglycerides: Metabolic studies on representative individual sucrose esters. Two-year studies on another sucrose ester in a non-rodent mammalian species[2].

5. Thickening agents

Arabic gum: Studies of the metabolic fate in man as well as more adequate studies in animals. Studies on the allergenic reactions reported following ingestion in man.

Guar gum: Results on the short-term study which is in progress to check the reliability of the older studies.

Oxidized starches: Results of histopathological studies[1].

Pectin (amidated): Provision of full specifications[1].

6. Miscellaneous food additives

Food-grade mineral oil: Further work on the elucidation of the significance of stored mineral oil in the body.

L-glutamic acid, its ammonium, calcium, monosodium, and potassium salts: Oral non-adverse effect levels of glutamates in neonatal animals. Age correlations between neonatal experimental animals and the human infant. (Required if use is to be extended to infant foods)

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[1] Required by June 1974
[2] Required by June 1976

Joint FAO/WHO Food Standards Programme

CODEX ALIMENTARIUS COMMISSION

CAC/FAL 1-1973

LIST OF ADDITIVES EVALUATED FOR THEIR SAFETY-IN-USE IN FOOD

First Series

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
WORLD HEALTH ORGANIZATION

Joint FAO/WHO Food Standards Programme
CODEX ALIMENTARIUS COMMISSION
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First Series

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
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INTRODUCTION

The Joint FAO/WHO Codex Alimentarius Commission

The Joint FAO/WHO Codex Alimentarius Commission (the Commission) was established to implement the Joint FAO/WHO Food Standards Programme. Membership of the Commission comprises those Member Nations and Associate Members of FAO and/or WHO which have notified the Organizations of their wish to be considered as Members. By 1 April 1973 99 countries had become Members of the Commission. Other countries which participate in the work of the Commission or of its subsidiary bodies in an observer capacity are expected to become Members in the near future.

The purpose of the Joint FAO/WHO Food Standards Programme is to elaborate international standards for foods aimed at protecting the health of the consumer, ensuring fair practices in the food trade, and by their acceptance by governments resulting in the harmonization of national food legislations, thereby facilitating international trade. In addition to compositional criteria and labelling, these food standards incorporate provisions in respect of food hygiene, food additives, pesticide residues, other contaminants, and methods of analysis and sampling.

The Joint FAO/WHO Codex Committee on Food Additives

The Codex Committee on Food Additives is an intergovernmental subsidiary body which advises the Commission on all matters relating to food additives. It meets every one to one and a half years and submits reports ("ALINORM" reports) to the Codex Alimentarius Commission. The Codex Committee on Food Additives has the responsibility, among others:

(a) to endorse or establish permitted levels of use for individual food additives and maximum permitted levels for contaminants in specific food items;
(b) to prepare lists of food additives and contaminants for toxicological evaluation by the Joint FAO/WHO Expert Committee on Food Additives;
(c) to consider specifications of identity and purity for food additives; and
(d) methods of analysis for their determination in food.

The Codex Committee on Food Additives relies upon data supplied by Member governments and the recommendations and evaluations of the Joint FAO/WHO Expert Committee on Food Additives. In addition, it examines such matters as technological justification for use, good manufacturing practices, and levels already permitted in national food legislation.

The Joint FAO/WHO Expert Committee on Food Additives

The Joint FAO/WHO Expert Committee on Food Additives is composed of experts who serve in a personal capacity. The Expert Committee evaluates food additives on the basis of all available data and, where appropriate, establishes "acceptable daily intakes" and chemical specifications for the additives. The conclusions of the experts are published in reports and monographs by WHO as the "World Health Organization Techn. Rep. Ser." and the "WHO/Food Add. series", respectively, and by FAO as the "FAO Nutrition Meetings Report Series". The views and recommendations of the FAO/WHO Expert Committee on Food Additives are taken by the Codex Committee on Food Additives as the basis for reaching decisions concerning the safety or otherwise of substances intended to be added to food.

Notes on Food Additive Lists A, B and C

For practical purposes food additives have been grouped in three lists, i. e. A, B and C. List A has been sub-divided into two parts (List A(1) and List A(2)). List A(1) contains those additives which have been fully evaluated by the Joint FAO/WHO Expert Committee on Food Additives and

have either been given an "acceptable daily intake" or have not been limited toxicologically (see meaning of "Not limited", page 4). List A(1) also includes those additives, the "acceptable daily intake" of which is "conditional". List A(2) includes those additives the evaluation of which is not complete but which have been accepted for use in food on a provisional basis. The inclusion of a substance in either of these lists does not mean a technological justification of its use in specific foods, although only those substances have been included in List A the technological usefulness of which, under certain circumstances, has been recognized by the Codex Committee on Food Additives. List B (reference CX/FA 73/2) has been published separately as a working document of the Codex Committee on Food Additives and has been circulated to governments and interested International Organizations for comment. It contains those additives, the evaluation of which by the Joint FAO/WHO Expert Committee on Food Additives is pending, and, in some instances, interest in which, from a technological point of view, has not yet been established by the Codex Committee on Food Additives. It is envisaged that additives will be added to or deleted from this list on the basis of decisions of the Codex Committee on Food Additives and recommendations of the Joint FAO/WHO Expert Committee on Food Additives. List C has been sub-divided into two parts, i. e. List C(1) and List C(2). List C(1) contains those substances which, in the opinion of the Joint FAO/WHO Expert Committee on Food Additives, based on experimental data, are unsafe for use in food. List C(2) includes those additives, the use of which is restricted for toxicological reasons.

The purpose of Lists A(1) and A(2) is to provide information to those concerned with regulating the use of chemical substances in the preparation of food. It is not intended that these lists be regarded as complete or final or that substances not appearing in List A(1) or List A(2) be regarded as unsafe or suspect from a point of view of health unless they appear in List C(1). Rather, Lists A(1) and A(2) are subject to continuous change; further substances may be included as they are cleared toxicologically by the Joint FAO/WHO Expert Committee on Food Additives and found to be of technological usefulness by the Codex Committee on Food Additives. Substances may also be removed from these lists in the light of new toxicological evidence. While these lists of food additives are made available to governments and interested bodies for information only, they contain substances which, in the view of the Codex Alimentarius Commission, are suitable for use in food within the limits of the "acceptable daily intake" and in the light of the "General Principles for the Use of Food Additives". As regards limits in food for the individual additives in List A, these are included in Codex commodity standards (see list of approved uses on page 51). It is the aim of the Commission to eventually draw up a Codex List of food additives which will be sufficiently complete to provide the basis for international acceptance of lists of food additives.

Similarly, List C is not complete and is subject to revision (addition or deletion) by the Codex Committee on Food Additives in the light of the findings of the Joint FAO/WHO Expert Committee on Food Additives. The absence, therefore, of a substance from this list does not imply that it is safe for use in food unless that substance appears on either List A(1) or A(2). Lists C(1) and C(2) are intended to inform governments as to which food additives they should not permit to be used in food or the use of which additives they should restrict.

Food additives in Lists A and B have been grouped on the basis of their technological functions, bearing in mind that a large number of additives are multi-functional. For example, citric acid, which is a chelating agent and a relatively strong acid, could be grouped with "acidifiers", "antioxidant synergists" or "flavours". For this reason, it has been found convenient to list a large number of substances together under the heading "Acids, Bases and Salts", a group which represent a chemical rather than functional sub-division. As further additives are included in Lists A(1) and A(2), it may be necessary to revise the present system of the grouping of food additives.

List of Additives and their Uses Approved by the Codex Alimentarius Commission

Codex commodity committees have proposed a number of provisions for food additives in Codex Commodity Standards to the Codex Alimentarius Commission. Many of these have been endorsed by the Codex Committee on Food Additives and adopted by the Commission at Step 8 of the Procedure for the Elaboration of Codex Standards. Those food additive provisions which have been included

in Recommended International Codex Standards, are listed at the back of this document (see page 83). It is to be noted that the list of food additive provisions (usually expressed as mg or g per kg of a specified food or "limited by good manufacturing practice") is not final, but represents the situation as at 1 April 1973. As further commodity standards will be adopted by the Commission containing food additive provisions there will be a need to bring the list of approved uses up-to-date.

Specifications of Identity and Purity for Food Additives

Specifications of identity and purity have been drawn up by the Joint FAO/WHO Expert Committee on Food Additives for nearly all the additives appearing in these lists. These specifications are being further considered by the Codex Committee on Food Additives with a view to their eventual adoption by the Codex Alimentarius Commission as recommended international Codex specifications.

Technological Efficacy of Food Additives

Recently, the Joint FAO/WHO Expert Committee on Food Additives has reviewed the technological efficacy of certain food additives. These reviews have been published in monographs by WHO in the "WHO Food Additives Series and by FAO in the "FAO Nutrition Meetings Report Series". Reference to these monographs has been made, where applicable, in this document. These monographs should not be regarded as recommendations for use, "tolerances", legal restrictions or clearances, but as a review of data available in literature.

Definition of Terms Used in this Document

The definitions given below are intended to clarify the various terms used in the Codex Alimentarius. Governments may wish to adopt different definitions in their national legislations. The definition of "contaminant" has been included in this publication for the sake of completeness.

Food Additive: "For the purposes of the Codex Alimentarius, "food Additive" means any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may be reasonably expected to result (directly or indirectly) in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods. The term does not include "contaminants" 1/ or substances added to food for maintaining or improving nutritional qualities".

Contaminant: "For the purposes of the Codex Alimentarius, "contaminant" 1/ means any substance not intentionally added to food, which is present in such food as a result of the production (including operations carried out in crop husbandry, animal husbandry and veterinary medicine), manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food or as a result of environmental contamination. The term does not include insect fragments, rodent hairs and other extraneous matter".

Natural Flavours and Flavouring Substances are preparations and single substances respectively, acceptable for human consumption, obtained exclusively by physical processes from vegetable, sometimes animal, raw materials, either in their natural state or processed for human consumption.

Nature-Identical Flavouring Substances are substances chemically isolated from aromatic raw materials or obtained synthetically; they are chemically identical to substances present in natural products intended for human consumption, either processed or not.

Artificial Flavouring Substances are those substances which have not yet been identified in natural products intended for human consumption, either processed or not.

1/ Pesticide residues are contaminants for the purposes of the Codex Alimentarius but are separately defined (See Appendix VII, ALINORM 72/35).

Note on the General Principles for the Use of Food Additives

The General Principles given on page 5 have been adopted by the Ninth Session of the Codex Alimentarius Commission. They are intended as information for governments and for use by Codex commodity committees when proposing the addition of chemical substances to food.

Use of References

All references quoted in the respective columns should be consulted to get a complete picture of the toxicological or chemical evaluation of the additive by the Joint FAO/WHO Expert Committee on Food Additives. Where there is more than one reference, the one underlined contains significant details of toxicological evaluation or contains the actual specification of identity and purity of the additive. Numbers in brackets refer to publications of the Joint FAO/WHO Expert Committee on Food Additives, a list of which is given on page 86 of this document. Copies of the reports and monographs quoted can be obtained from the Distribution and Sales Section of FAO, 00100 Rome, Italy, and the Distribution and Sales Service of WHO, 1211 Geneva 27, Switzerland.

Key to Abbreviations

ADI	"Acceptable Daily Intake" expressed as mg/kg body-weight.
C	"Conditional", the conditions applying to the ADI having been stated in the particular publications quoted.
UC	"Unconditional" (where an ADI is not qualified, it represents an "unconditional" ADI).
T	"Temporary" or "Tentative" (temporary or tentative ADIs are subject to reconsideration by the Joint FAO/WHO Expert Committee on Food Additives in the light of certain further work required. Until such time the evaluation of the substance concerned is not regarded as complete).
Not limited	This statement refers to the "Acceptable Daily Intake" and means that, in the opinion of the Joint FAO/WHO Expert Committee on Food Additives, the total daily intake of the substance arising from its use or uses in accordance with good manufacturing practice, does not represent a hazard to health. For this reason, the establishment of an ADI is not deemed necessary.
FAS	Food Additive Specifications established by the Joint FAO/WHO Expert Committee on Food Additives and issued by the Secretariat of the Codex Alimentarius Commission to Codex Contact Points in 1967. 1/
GMP	Good Manufacturing Practice.
Limited by GMP	This statement refers to the limitation of a food additive in specified foods. It means that, for example, the additive in question is self-limiting in food for technological, organoleptic or other reasons and that, therefore, the additive need not be subject to maximum limits. However, it means that the food additive must be used according to good manufacturing practice, and in accordance with the General Principles for the Use of Food Additives.

1/ Available from the Joint FAO/WHO Food Standards Programme, FAO, 00100 Rome, Italy.

GENERAL PRINCIPLES FOR THE USE OF FOOD ADDITIVES

1. All food additives, whether actually in use or being proposed for use, should have been or should be subjected to appropriate toxicological testing and evaluation. This evaluation should take into account, among other things, any cumulative, synergistic or potentiating effects of their use.

2. Only those food additives should be endorsed, which so far as can be judged on the evidence presently available, present no hazard to the health of the consumer at the levels of use proposed.

3. All food additives should be kept under continuous observation and should be re-evaluated, whenever necessary, in the light of changing conditions of use and new scientific information.

4. Food additives should at all times conform with an approved specification, e.g. the Specifications of Identity and Purity recommended by the Codex Alimentarius Commission.

5. The use of food additives is justified only where they serve one or more of the purposes set out from (a) to (d) and only where these purposes cannot be achieved by other means which are economically and technologically practicable and do not present a hazard to the health of the consumer:

(a) to preserve the nutritional quality of the food; an intentional reduction in the nutritional quality of a food would be justified in the circumstances dealt with in sub-paragraph (b) and also in other circumstances where the food does not constitute a significant item in a normal diet;

(b) to provide necessary ingredients or constituents for foods manufactured for groups of consumers having special dietary needs;

(c) to enhance the keeping quality or stability of a food or to improve its organoleptic properties, provided that this does not so change the nature, substance or quality of the food as to deceive the consumer;

(d) to provide aids in the manufacture, processing, preparation, treatment, packing, transport or storage of food, provided that the additive is not used to disguise the effects of the use of faulty raw materials or of undesirable (including unhygienic) practices or techniques during the course of any of these activities.

6. Approval or temporary approval for the inclusion of a food additive in an advisory list or in a food standard should:

(a) as far as possible be limited to specific foods for specific purposes and under specific conditions;

(b) be at the lowest level of use necessary to achieve the desired effect;

(c) as far as possible take into account any Acceptable Daily Intake, or equivalent assessment, established for the food additive and the probable daily intake of it from all sources. Where the food additive is to be used in foods eaten by special groups of consumers, account should be taken of the probable daily intake of the food additive by consumers in those groups.

LIST A(1) OF FOOD ADDITIVES

List A(1) contains additives which have been cleared toxicologically for use in food by the Joint FAO/WHO Expert Committee on Food Additives
(See Notes on Food Additive Lists A, B and C in the Introduction)

	ADI (mg/kg body-weight)	Toxicological Evaluation 1/	Specification 2/
9.27 Methyl salicylate	0-0.5	(12) p 63	(13) p 39
9.28 gamma-Nonalactone	0-1.25	(12) p 67	(13) p 40
9.29 Nonanal	0-0.1 (C) 3/	(12) p 69	(13) p 41
9.30 Octanal	0-0.1 (C) 3/	(12) p 71	(13) p 42
9.31 Piperonal	0-2.5	(12) p 73	(13) p 44
9.32 gamma-Undecalactone	0-1.25	(12) p 76	(13) p 45
9.33 Vanillin	0-10	(12) p 78	(13) p 46

1/ See also (11) p 8.
2/ See also (11) p 7, (13) p 1 and the reference to toxicological evaluation.
3/ Further work is required before an unconditional ADI can be established. (See Reference (12)).

10. FLAVOUR ENHANCERS

	ADI (mg/kg body-weight)	Toxicological Evaluation	Specification
10.1 L-Glutamic acid and ammonium, calcium, monosodium and potassium salts (Not to be added to the diet of infants under 12 weeks of age)	0-120 (Additional to intake from all dietary sources; does not apply to infants under 12 weeks of age)	(16) p 20 (19) p 15 (20) p 42 (29)	(21) p 23
10.2 Ethyl maltol (See 9.15, List A(1))	0-2	(19) p 23 (20) p 37	(21) p 15

crease will be passed on to the consumer whether or not he can understand or concur with the basis for apportioning the costs.

So who will decide what test should be used, and how will he be able to choose? Responsibility has devolved upon a single federal agency, the Bureau of Biologics of the Food and Drug Administration. This agency not only exercises licensing control over interstate sale of diagnostic kits for detecting hepatitis but also licenses blood banks only if they adopt the testing procedure required by the Bureau. The Bureau has no control over pricing policies per se and yet is currently in the position of unavoidably supporting a monopoly because Austria is the only test in its class that has met the Bureau's standards. This unusual situation may explain why, in addition to pursuing a more aggressive policy of preventing post-transfusion hepatitis, the Bureau has expended exceptional effort in soliciting the views of those who may question the merit of universal implementation of the test by blood banks. Continued dialogue should prevent over-reaction while the medical-scientific community regains leadership defaulted through legal precedents arising from uneven levels of effort in various localities.[4]

Additional data are needed, but ethical and practical problems will make new studies progressively more difficult to carry out. It is troublesome ethically to withhold a test that, though obviously imperfect, appears to be a relative improvement. The diminished efficiency of detecting residual hepatitis after appropriate donor selection and the application of moderately sensitive tests for hepatitis B antigen further discourages additional study. The residual may also be distorted by containing an abnormally high proportion of cases that, unlike hepatitis B, are more often mild and of uncertain clinical consequence and might be overlooked in the absence of intensive study. Additional data will have to depend on analysis of specimens collected before sensitive tests for hepatitis B antigen become routine. By then the blood-bank technician's natural preference for an agglutination test may be met by methods using red blood cells coated with hepatitis B antibody.[5] The greater simplicity of the agglutination test and the multiplicity of potential manufacturers should make it less expensive than radioimmunoassay but no less difficult to evaluate regarding efficacy.

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SAFETY OF HYDROLYSATES IN PARENTERAL NUTRITION

ELSEWHERE in this issue of the *Journal*, an article by Olney, Ho and Rhee questions the safety of protein hydrolysates for use as the nitrogen source in total parenteral nutrition of the human infant. This study reports the production of a central-nervous-system lesion in the infant mouse after subcutaneous injection of protein hydrolysate solutions containing glutamate, aspartate and cysteic acid. That these "acidic" amino acids alone or in combination produce central-nervous-system lesions in the suckling mouse is a matter of record.

The neurotoxic effects of these amino acids in species other than the mouse are debatable. Descriptions of glutamate-induced neurotoxic effects in the rat vary, with some investigators reporting a null effect that may be strain related. No neurotoxic effects of glutamate have been described in the newborn guinea pig or dog. Although Olney and his colleagues have reported that the arcuate nucleus of the infant subhuman primate is damaged by high doses of glutamate (administered as monosodium glutamate, MSG),[1] other laboratories have failed to confirm their report of a massive lesion in the arcuate region.[2,3] In addition, the two reports by Olney et al. of an arcuate lesion in the primate differ drastically in description.[1,4] From a massive lesion of the arcuate nucleus whose histopathologic description was considered to resemble that found in the infant mouse,[1] the lesion is now described as a microlesion involving 50 to 90 cells seen only in epon sections.[4] Although a difference in the route of glutamate administration was involved in these studies,[1,4] the published blood levels of glutamate do not differ enough to explain the drastic descriptive change in the nature of the lesion.

It is evident that the question of glutamate-induced neuronal damage in the young of species other than the mouse has been and continues to be a controversial issue. One index that can be measured objectively is the rise in plasma glutamate and aspartate levels after administration of glutamate, aspartate or protein hydrolysates. Induction of neuronal damage in infant animals requires substantial elevation in plasma levels of the specific amino acids involved. For example, Perez and Olney[5] report a 400-fold increase in plasma glutamate levels after the subcutaneous administration of glutamate (2 mg per gram of body weight) to the infant mouse. The data in Table 1 demonstrate a 10-fold increase in both plasma glutamate and aspartate concentrations within five minutes of the subcutaneous injection of young mice with 50 μl per gram of body weight of a 5 per cent casein hydrolysate – 5 per cent dextrose solution (Travenol Laboratories). On the other hand, Stegink and Baker have demonstrated

Table 1. Plasma Levels of Glutamate and Aspartate in Young Mice[a] Injected Subcutaneously with a 5 per Cent Casein Hydrolysate–5 per Cent Glucose Solution at 0.05 Ml per Gram of Body Weight.[b]

Time	Glutamate	Aspartate
min	*moles/100 ml*	
0	7.8[c]	1.9
5	74.8	24.1
10	42.9	11.5
15	30.3	10.0
20	28.1	11.8
30	23.5	6.7
60	14.5	5.0
90	11.5	4.0
120	8.5	1.5

[a]20-day-old Swiss Webster albino mice; average weight 15 g.
[b]Equivalent to 0.2 mg of glutamate + aspartate/g of body weight.
[c]Each data point obtained from pooled blood samples of 4 animals.

conclusively that human infants given protein hydrolysates as the nitrogen source during parenteral nutrition maintain normal plasma concentrations of glutamate and aspartate.[6] It is highly unlikely that elevations of blood glutamate and aspartate were obscured in their study. Plasma amino acid patterns (blood obtained from the external jugular) paralleled the amino acid composition of the particular hydrolysate infused.

Generally, pediatric patients receiving total parenteral nutrition are infused with solutions containing 2 or 2.5 per cent protein hydrolysate and 25 per cent glucose at flow rates of 120 to 150 ml per kilogram of body weight per 24 hours. Infants infused with a casein-hydrolysate-based preparation receive the equivalent of 0.01 mg per gram of body weight per hour of glutamate plus aspartate. On the basis of studies of glutamate metabolism in the three-day-old pig, this level is readily metabolized.[7]

Olney and his co-workers have again demonstrated the neurotoxicity of certain amino acids administered to the suckling mouse. This dose-dependent effect is associated with marked changes in plasma concentrations of glutamate and aspartate. To impugn the safety of protein hydrolysate preparations for use as nitrogen sources in total parenteral nutrition for the human infant on the basis of these observations is unwarranted. It is difficult to imagine glutamate-induced neuronal damage without substantial elevation of plasma glutamate and aspartate levels. However, one cannot but agree with Olney and his co-workers that ". . . it is important to give continuing attention to possible means of improving the composition of [the] parenteral solutions. . . ."

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RISKS VS. BENEFITS OF ANTIPSYCHOTIC DRUGS

It is a truism that a drug potent enough to do good may also do harm. It has also been too frequently true that new drugs have been pressed into service with unrestrained enthusiasm until the evidence for toxic effects have become overwhelming. The antibiotics are classic examples of both phenomena. More recently, it has begun to appear that the antipsychotic drugs (phenothiazines, thioxanthines and butyrophenones) may represent further examples. A recent report prepared by an American College of Neuropsychopharmacology–Food and Drug Administration Task Force for this journal[1] suggested that neurologic side effects of these drugs require thoughtful consideration of the risks associated with their chronic use. Simultaneously, Crane reviewed the current status of clinical psychopharmacology,[2] and very appropriately pointed out many of the sociologic influences that have made it difficult for psychiatry to use medications in a completely rational manner. These include demands upon physicians to "do something" tangible for chronically malfunctioning psychiatric patients, who are often considered burdens to their families and an expense to the public, and who may seem inscrutable or incurable. Psychiatric institutions are under pressure to reduce numbers of hospital beds or reduce the length of hospitalization, and do so largely by administrative fiat, hoping to reduce costs and to return patients "to the community," as if that represented progress in the treatment of illness. Such pressures have led to the sometimes senseless ritualization of drug therapy, the continuation of medications for persons who are unimproved or even asymptomatic for many months or even years. They have also led to the assumption that drugs are indispensible to maintaining chronic psychotic patients out of the hospital, and that potential benefits outweigh the risks of serious toxicity, which are assumed to be small.

The facts, however, as well as one can know them, seem to be that chronically administered antipsychotic drugs are neither indispensable nor without serious risks. Antipsychotic drugs are clearly the single most effective aspect of the management of acute or active psychosis, particularly schizophrenic illnesses and mania, and the withholding of these medications with-

it should, whereas the others did not.

AFTERNOON SESSION

The Use of Monosodium Glutamate In Sea Food Products

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Author's abstract

Attention is called to the many types of protein hydrolysates on the market and to their great variability in content of monosodium glutamate, salt, ammonia, inert matter and moisture. Each product must stand on its individual merit and no conclusion can be drawn as to the suitability for seasoning foods of hydrolysates as a class. Generally, pure monosodium glutamate is preferred as a flavor improver for seafoods. It is a pure, standardized product and gives reproducible results. Usually, concentrations of monosodium glutamate of from 0.15 to 0.5% are best for clam and fish chowders, fish cakes, soups and purees. In pastes and sandwich spreads as much as one percent can be used. Canned oysters are improved by MSG but the flavor of shrimps is not enhanced in the opinion of some members of our taste panel. Anchovy and lobster spreads are markedly improved in flavor. Heat and processing change the flavor of MSG somewhat but do not usually produce off- or undesirable flavors.

Most clam chowders found on the U. S. market contained MSG. The latter does not appear to mask or cover up the odor or flavor of stale or partially decomposd seafoods. Monosodium glutamate showed no preservative value in seafoods.

The Japanese used monosodium glutamate and protein hydrolysates freely in their canned and dried Army rations such as canned fish and gravy and many meat-vegetable or soybean combinations which contained little meat.

When we started working with these hydrolyzed protein products a few years ago, looking for a way to enhance the flavor of some of the marine foods in New England and the East Coast, we got many samples of monosodium glutamate and the different hydrolysates in various stages of impurity and tried them out.

What is monosodium glutamate? Let us talk first about what are often passed off as glutamates and hydrolysates. It seems to me that

is a rather important aspect of the subject, and I would like to present it for what it is worth—even though it may mean stepping on the toes of some manufacturers. But if these other products spoken of do have something, let us know what they have. The products we obtained included liquids, pastes, and powders; some were black, some white, and all colors in between were represented. They gave us quite different results, and I should like to emphasize what was said—that these products act differently, depending on whether they are in the presence of a little salt, a lot of salt or no salt, and also whether they are in a product like pickled herring with acid added to them. Acid also changes the flavor complex considerably.

These hydrolyzed proteins are really remarkable products. You have heard the discussion of what they do from Dr. Crocker and other speakers. You have heard their properties described in terms of Dr. Crocker's famous formula of salty, bitter, sweet, and sour. We did try these various flavoring agents in such products as salt fish and codfish cakes. We tried them on clams and clam chowder, on canned lobster and lobster paste, on crabs and crab cakes, and on gefüllte fish, a product that is canned to some extent in this country and liked by many people not of the Jewish faith. We tried them on some fish pastes, oyster stew, marinated herring, and anchovies. Some of these were already in a semi-fermented or decomposed condition and some, like our cheeses, are not exactly up to snuff as regards absence of decomposition, but the Food and Drug Administration does not offer much objection.

There is a degree of hydrolysis in some of these fish products already, due to bacterial action, and in some respects the flavor resembles these hydrolyzed proteins. In fact, that is what it is.

What concentrations did we use in these products? If you taste a product at about 1% salt, we don't think you can taste under .1% of monosodium glutamate. I suspect .05% is true in water or products which don't have much flavor. From there you can go to 3 percent. One of our speakers said that .5% gave you a maximum taste threshold and, if you use more, your curve does not go up. In other words, you don't get twice the flavor from adding 1% that you do from .5%.

We found that heat does affect monosodium glutamate and these hydrolysates, but not as much as it does the hydrolysates themselves. A canning process of 240-250° F. definitely does something to the flavor. In some cases it improves it; in some cases it makes it inedible. The only way to know is to try it out. I know of no way to predict what products monosodium glutamate is going to improve and which it is not. The field it covers and the products it does improve are certainly remarkable. I would not believe that the same product that could improve marinated herring with a 1 to 2% acid would also improve lob-

ster paste or crab cake and codfish cakes, but it does. You have to use it with caution. You have to try it out, and I am sure there is a huge field there and we have hardly dented the surface.

For example, we put a little monosodium glutamate in canned oysters. They are not much to talk about and have never been too popular. If you put a little monosodium glutamate in them, it really adds something. It is the same with clam chowder. I don't know about the products of other industries, but it is being rather widely used in the marine industry. It is rather new of course and the method used is more or less hit or miss. Interest in this subject on the part of industry generally and others as well is shown by the number of people here and the many notables sitting in this audience. Its range of usefulness is great. The use of soybean sauce and Worcestershire sauce in so many of the English products—puddings, gravy products, etc.—suggests the diversity of products in which monosodium glutamate can be used. Whereas the English have perhaps used some glutamate in their fish products and pastes, it has not been done to any great extent as yet.

Some of the hydrolysates carry a very strong color, since some of them have caramel added. In marine products, that is generally unfortunate, because we want to keep the product as light as possible. I think for marine products we want to use pure monosodium glutamate. Neither do we want an excessive amount of salt, ammonia salts, or sodium chloride. There is no question but that monosodium glutamate carries its own salt, but it goes up to a certain point on the scale of salinity and then you don't seem to notice it. Most people like sea foods fairly salty. They think that everything that comes from the sea ought to be salty. Of course, a crab or shrimp is not any saltier than a fresh water fish. Again, when we add a lot of salt, we have to be careful about adding glutamate since we don't wish to overdo the saltiness. Excessive salt covers up the glutamate flavor. We have found that in a good many cases glutamate as a flavor factor seems to work much better in very lightly salted than in the more highly salted products. For example, salt codfish flakes are used in quite a few products. Glutamate does not work well in them, unless the salt content has been reduced somewhat.

I have just noted some of the satisfactory concentrations that we have found in some of these products: clam chowder—.1 to .3%—it does not make much difference whether added whole or mixed. We got so much variation with these hydrolysates that I would rather not talk about them. I have no objection to using them, but you can't tell what you are getting. Fish chowder—.1 to .3%; lobster paste or crab paste and fish paste—.5 to 2%. You can use quite a little in these pastes; it brings out the flavor nicely. In lobster and crab meat, we use the

"tomalley" which consists of gelatinized protein in the body of the lobster and any eggs that are present. The eggs give a bright orange color to the paste. These are packed in Canada. Canada used to let England make up these pastes, but now they are packing them and so are we. Crab paste is a very fine spread indeed, especially with crab cakes and the gelatinized or heat-coagulated proteins that are in the body after you pick out the white meat. In smoked herring paste you can also use a pretty good amount of monosodium glutamate: 0.5 to 1.0%, usually around 0.75%. Until some years ago whiting was not utilized at all. The natives of New England would not eat it. But in St. Louis, where they like fish and chips, literally carloads of this whiting were consumed. It has now gotten back to Cape Cod. It makes a pretty good canned product and a nice paste. One-half percent of monosodium glutamate greatly improved whiting. Smoked oyster paste: 0.3 to 1.3%. Canned oysters: .1 to 2%. Canned fish cake: .2 to 3%. Canned crab meat: glutamate is not very satisfactory, but in canned products such as deviled crabs and crab pastes, it makes a wonderful improvement. Shrimp paste: a little but not too much gives zest to the characteristic flavor of the shrimp.

That brings me to a point that is new to most of us. I was in the Army working on foods in the Pacific during the war. 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 soybean 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 had 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.

This product is very useful in marine products if added carefully. We don't want to kill the characteristic flavor of the sea foods. We must not destroy that flavor. We must add only enough monosodium glutamate to bring out that flavor, not so much that we cover it up.

What should you call it—is it flavor, seasoning, or salt? I think it is a seasoning, because it is a sort of an overall taste flavor and feel. It appeals to all of those senses. Therefore, I think it is different from salt, sugar or syrups of various kinds which we use in culinary work. I do think we ought to standardize the product. If we sell hydrolysates, let us sell them as such. The work we did was hit or miss. One thing

to remember—the original saltiness of your product makes a great difference in how you use this glutamate.

Somebody brought up the subject of soy sauce manufacture. I had a little personal experience making soy sauce by the enzyme method. It might be called a hydrolysate problem. There was a Chinese working in the Department of Agriculture about 1920, when I was getting out of school, and we worked together on making a soy sauce. He brought over what he called a greatly improved method. The method was to take about 50 percent soybeans (two varieties were used since some have a bad flavor and some good) and about 50% barley. These were soaked for two or three days and then sprinkled with a powdered mold, which he called "moyashi." It is very rich in enzyme, and soybeans and barley blended nicely with it. Then we put them in a room on shelves at 100 percent humidity. The mold grows through the beans and cereal, and by switching them around you have a culture through the beans and barley. Then you threw them into a barrel of salt, 10% brine, and stirred them up for a few weeks. The longer you store it, the better the soy sauce becomes. That is still the Oriental method. Practically the whole substance of the bean and barley goes into solution. If you siphon out this stuff after a year or so, you find very little sediment. The Chinese know the difference between good and bad soy sauce. Unfortunately the bad is often foisted off on us.

L-GLUTAMATE DEHYDROGENASE IN RAT AND HUMAN FETAL LIVER SLICES

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Preliminary experiments showed that hepatic glutamate dehydrogenase (EC 1.4.1.2. L-glutamate : NAD oxidoreductase) appears almost simultaneously with the development of the liver in the earliest human fetuses studied. Glutamate dehydrogenase activity was readily evident at 12 weeks of development and had doubled by 16 weeks. Glutamate dehydrogenase was present on the 17th day of development in the rat liver and increased to a maximum some two weeks after birth, remaining at that level until at least eight weeks of age. The present paper shows that the activity of glutamic dehydrogenase is increased greatly when liver slices are incubated *in vitro* in the presence of glutamate or certain other substances.

METHODS

Sprague-Dawley female rats, 17-19 days pregnant, were obtained from the Charles River Breeding Laboratories in Wilmington, Mass. Human fetal liver tissue was obtained at therapeutic abortions. The livers were sliced into 50 mg portions and placed in 25 ml Erlenmeyer flasks containing 10 ml balanced salt solution (Earle, 1943) with or without 20 mg/ml of glutamate or malate. A portion (0.25 gm) of the liver was homogenized immediately in 2.0 ml of ice-cold 0.1 M Tris-HCl buffer, pH 7.6,

and frozen for subsequent assays to determine enzyme activity at zero time.

Slices were incubated from 2 to 8 hours at 37° C in a Dubnoff shaker. A mixture of 95% O_2 - 5% CO_2 was bubbled slowly through the solution for the duration of the incubation. At appropriate times liver slices were removed, rinsed, blotted dry, weighed, and homogenized in Tris buffer. The homogenate was rapidly frozen in dry ice-acetone and stored for subsequent assays of enzyme activity.

Previous experiments had shown that the combination of homogenizing, freezing and thawing released essentially all of the glutamate dehydrogenase activity. After thawing, the homogenate was centrifuged in a Servall refrigerated centrifuge at 20,000 x g for 20 minutes. The glutamic dehydrogenase activity of the resulting clear supernatant solution was measured in a Beckman DB spectrophotometer with a recording potentiometer by the increase in optical density at 340 mu during the first minute of reaction. Each cuvette contained, in total volume of 1 ml, 0.01 ml of supernatant, 4 umoles of NAD, 20 umoles potassium glutamate, and 59 umoles 0.1 M Tris buffer, pH 7.6. The conditions for the assays of other dehydrogenases were identical save for the replacement of glutamate by the proper substrate in the reaction vessel. Glutamate dehydrogenase was subjected to disc electrophoresis on 4% polyacrylamide gel using the technique of Van Bogaert et al (1967).

RESULTS

Incubation of slices of 20-day fetal rat liver in Earle's balanced salt solution resulted in no marked loss of activity of glutamate, lactate, malate or isocitrate dehydrogenase during a four-hour incubation (Fig. 1). The activity of these dehydrogenases in slices of adult liver was maintained for

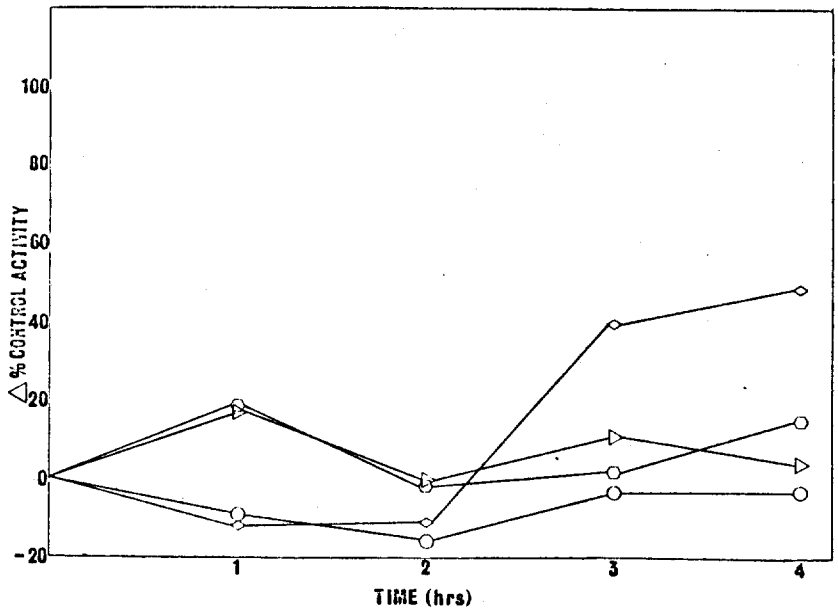

Fig. 1. Changes in the specific activities of glutamate dehydrogenase (◇——◇), malate dehydrogenase (⬡——⬡), lactic dehydrogenase (▷——▷), and isocitrate dehydrogenase (○——○) expressed as the percentage of activity at zero time. Fetal (21 day) rat livers were excised and incubated in 10 ml Earle's balanced salt solution at 37° C with 95% O_2-5% CO_2. The results represent the average of two fetal preparations.

at least eight hours of incubation. The activity of glutamate dehydrogenase, but not of the other dehydrogenases, increased when either fetal or adult liver slices were incubated. An even greater increase in the specific activity of the glutamate dehydrogenase was noted when fetal liver slices were incubated in the presence of potassium glutamate, 20 mg/ml (Fig. 2). The decrease in the specific activity of GDH after four hours of incubation suggests that the fetal GDH is less stable than the GDH of adult liver which

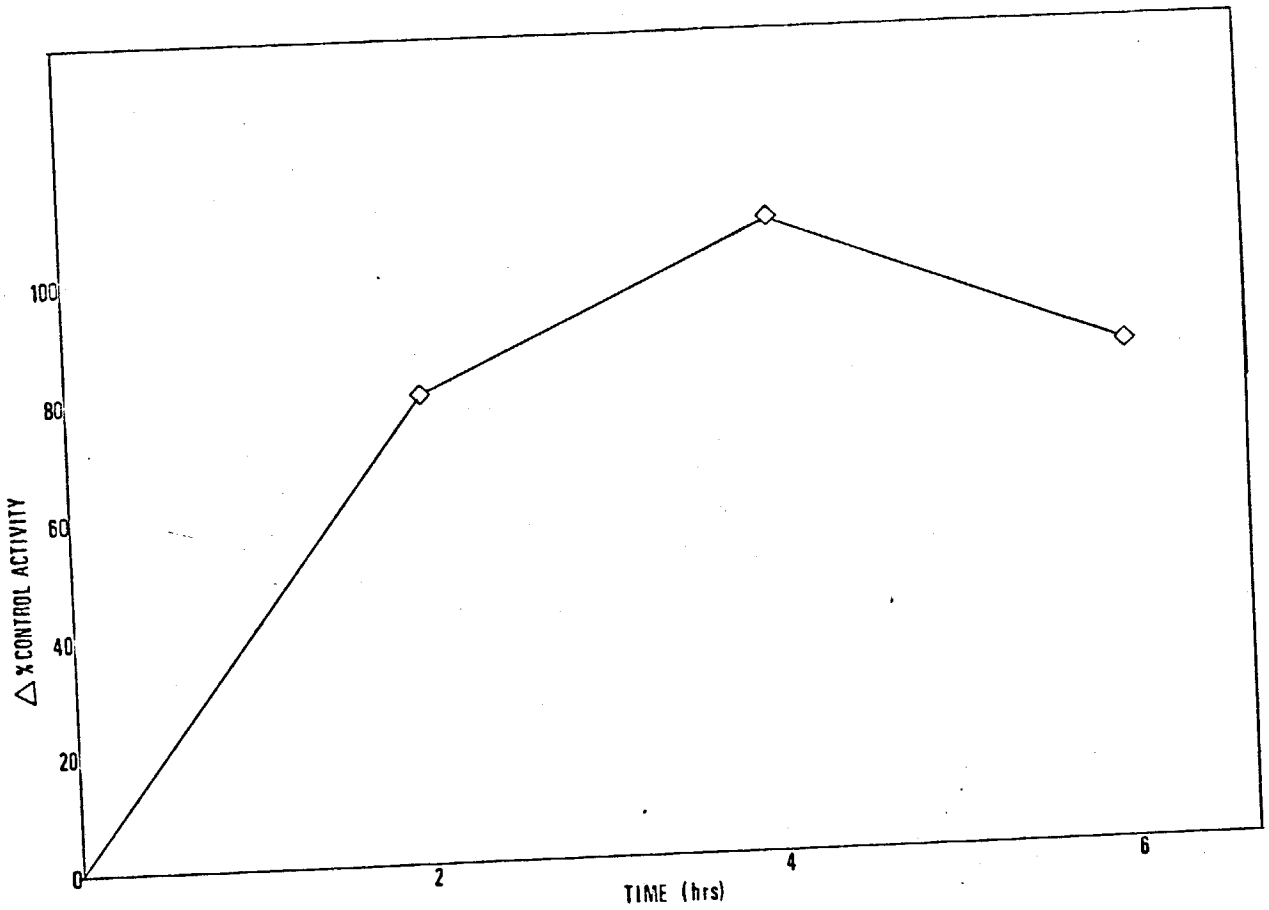

Fig. 2. Increase in specific activity of glutamate dehydrogenase of fetal rat liver during incubation of liver slices in Earle's solution containing 20 mg/ml potassium glutamate. Each point represents the average of two 20 day rat fetal liver preparations.

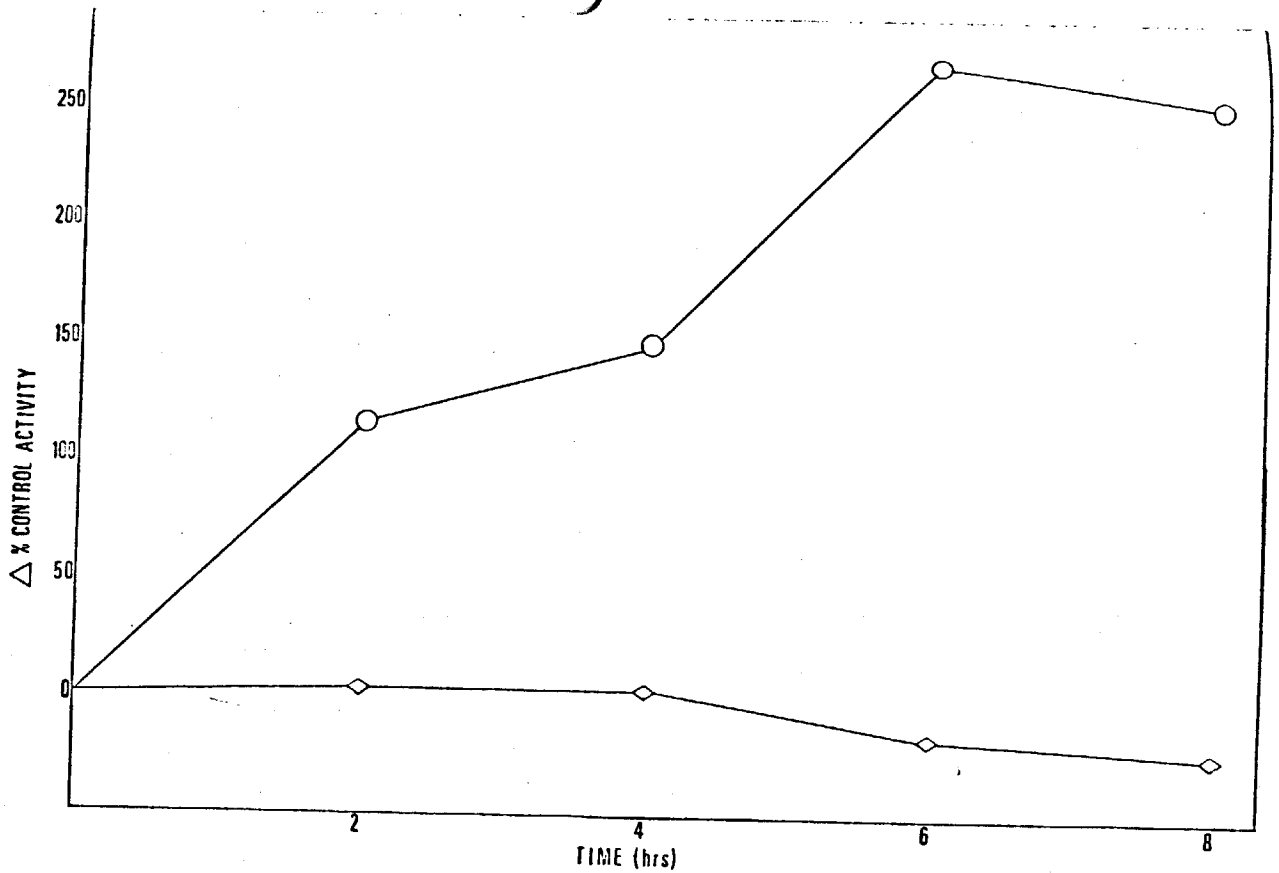

Fig. 3. Specific activities of glutamate dehydrogenase (○——○) and malate dehydrogenase (◇——◇) during incubation of slices of fetal (20 day) rat liver in the presence of 20 mg/ml sodium malate in Earle's solution.

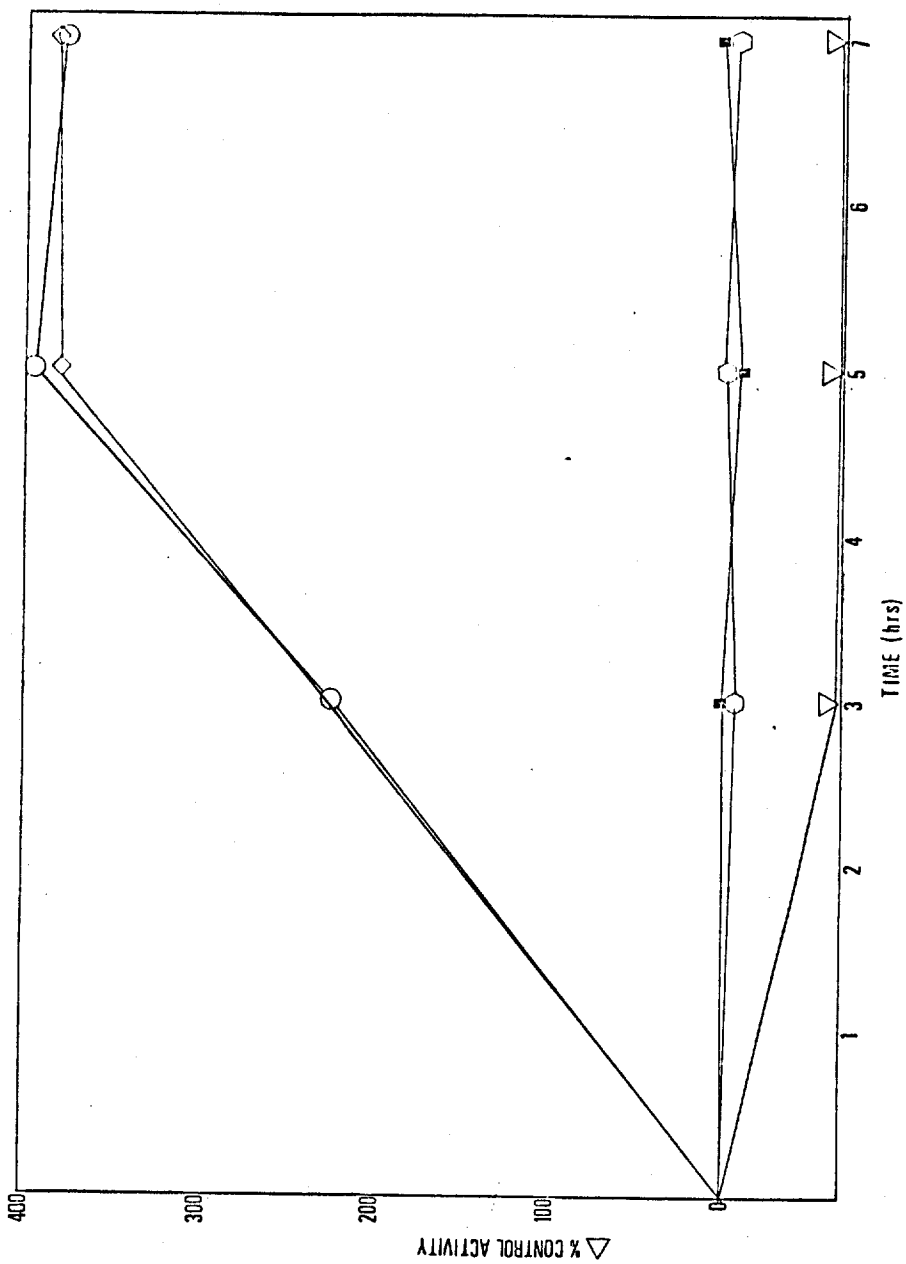

Fig. 4. Effect of the addition of sodium malate, 20 mg/ml, to the incubation medium upon glutamic dehydrogenase and malate dehydrogenase of human fetal liver slices. Glutamate dehydrogenase activity with (○——○) and without (▽——▽) added malate and glutamate

dehydrogenase activity with malate plus 20 ug actinomycin D per ml (◇——◇). Malate dehydrogenase with (⬡——⬡) and without (□——□) added malate. The fetus was a 16-week female measuring 12 cm crown-rump length.

maintained a high activity even after ten hours of incubation.

The activities of isocitrate, lactate and malate dehydrogenases were not increased when liver slices were incubated in a medium containing their respective substrates. However, malate was even more effective than glutamate in increasing the activity of glutamate dehydrogenase in fetal liver slices during prolonged incubation (Fig. 3). In the presence of 20 mg malate/ml of Earle's salt solution the GDH activity of rat fetal liver slices increased to 275% of the control values at six hours, whereas the malate dehydrogenase activity had decreased to 75% of the control value for MDH. A series of further experiments confirmed the finding that malate is even more effective than glutamate in increasing the activity of glutamate dehydrogenase in rat fetal liver slices.

Similar experiments with slices of human fetal liver showed that glutamate dehydrogenase activity was increased by the addition of glutamate or malate to the Earle's salt solution (Fig. 4). The GDH activity of the slices incubated in malate increased fourfold over control values in five hours. A comparable increase occurred when 20 mg of actinomycin D/ml of Earle's salt solution was present along with the malate.

The electrophoretic patterns of the glutamic dehydrogenase of fresh human fetal liver and from liver slices incubated four hours in the presence of malate were identical (Fig. 5). The greater activity of the GDH after incubation is reflected in the relatively larger size of the bands.

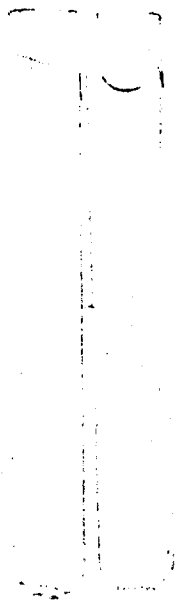

Fig. 5. Zymograms of glutamate dehydrogenase from human fetal livers (right) extracted immediately and (left) extracted from liver slices after four hours of incubation in Earle's solution containing 20 mg malate per ml.

DISCUSSION

That the activity of an enzyme may be regulated by its substrate has been documented for many enzymes. Thus, the injection of tyrosine into adult animals increased the activity of tyrosine-α-ketoglutarate transaminase sevenfold over a five-hour period (Lin and Knox, 1957), whereas the administration of tyrosine to fetal rats *in utero* failed to increase appreciably the low levels of tyrosine-α-ketoglutarate transaminase in fetal liver

(Sereni et al, 1959). The activity of tryptophan pyrrolase in fetal rat liver was also unaffected by injections in utero of L-tryptophan (Nemeth, 1959). The enzymes present in the livers of newborn and adult animals and in adults injected with substrate are chromatographically identical (Nemeth, 1961).

As controls for the present experiments both young and mature intact rats were injected with sodium glutamate or sodium malate but neither altered the activity of glutamate dehydrogenase in the liver. This might indicate simply that neither substance was accumulated by the hepatic cells in the intact animal. The concentrations of the free amino acids are greater in the livers of 22-week human fetuses than in the adult liver (Ryan and Carver, 1966).

The possibility that the increase in activity represents de novo synthesis of protein requiring the de novo synthesis of DNA-dependent RNA appears unlikely, since the addition of actinomycin D (20 ug/ml) did not prevent the increased activity. Other experiments showed that puromycin (100 ug/ml) did not prevent the increased activity of glutamate dehydrogenase in slices of adult rat liver. The possibility that the increase in activity in glutamate dehydrogenase results from the stabilization of the enzyme or from a decreased turnover of the enzyme in the presence of the substrate seems unlikely. In long-term incubations the activity of glutamate dehydrogenase after eight hours of incubation decreased to a greater extent in media containing substrate than in media without substrate. In other experiments the addition of glutamate to extracts of either fetal or adult liver did not protect the enzymatic activity; the fraction of activity remaining in extracts without substrate after 24 hours of storage in the cold was as great as that in extracts to which substrate was added.

Glutamate dehydrogenase is a tetramer with a molecular weight of 10^6 (Olson and Anfinsen, 1952) which undergoes dissociation to monomers of 2.5×10^5 (Frieden, 1958). The addition of estrogens, ATP or 1,10-phenanthroline increases alanine dehydrogenase activity and inhibits glutamate dehydrogenase activity. Conversely, ADP, DPN or TPN increases GDH activity and decreases alanine dehydrogenase activity (Tomkins et al, 1961). To test the possibility that incubation of tissue slices might be affecting the molecular configuration of the enzyme, the alanine dehydrogenase activity of several preparations was investigated along with the glutamic dehydrogenase activity. In all experiments the ratio of alanine dehydrogenase and glutamic dehydrogenase activities remained constant, which suggests that the enzyme was not undergoing a change in association during the incubation.

ACKNOWLEDGEMENT

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EFFECT OF GLUTAMIC ACID MONOHYDROCHLORIDE TO PREGNANT RATS AND THEIR FETUS

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"Effect of glutamic acid monohydrochloride to pregnant rats and their fetus."

Purpose of study

The object of the study was to determine whether or not glutamic acid monohydrochloride might have teratogenic action.

Though glutamic acid monohydrochloride was used as the test substance in this study, its biological activities are considered to be extremely resemble to those of free glutamic acid.

Experimental procedures

Adult male and female rats of Wistar strain were used in this study.
The experiments commenced on 1 March, 1966.
Grouping of animals was as follows :

Group	Treatment	No. of Rats ♂	No. of Rats ♀
1	Water (Control)	5	5
2	Glu·HCl 5mg/2ml	6	10
3	Glu·HCl 25mg/2ml	6	8

Test substance was administered in two consecutive periods; the first was consecutive 15 days and the second consecutive 18 days.
Aqueous solutions of the test substance were injected into stomach of nonanesthetized animals by polyethylene stomach tube. The concentrations of the solutions were either 5mg Glu·HCl/2ml or 25mg Glu·HCl/2ml. Volume of the solution of the test substance administered at one time was a 1/100 ml per gram of body weight. Administration was carried out once a day at around 4 p.m. of each administration day.
Before or during the second administration period male and female animals were housed together in a cage so that female animals might be in various pregnancy stages in the second administration period.

All rats had free access to the distilled water and to quantities of standard laboratory feed, Japan clear mouse, rat and hamster CE-2 formula.

Body weight gain and feed intake were measured everyday.

Sexual cycle of female rats was examined through the periods of test substance administration and 2 weeks immediately before the administration periods of test substance.

About 30th days after the administration period (including gestation period), all parent animals were sucrified by ether and subjected to gross and macroscopic examination, including organ weight measurement. F1 animals except animals for mating were examined as some manner described above at more than one month of age. F2 animals were autopsied at 7 days of age.

Results

The experimental results are summarized as follows from the data attached.

1. Administration of glutamic acid hydrochloride did not influence the body weight gain and the feed intake in both male and female rats and sexual cycle of female rats.

2. Body weight change of pregnant rats was not influenced by administration of glutamic acid hydrochloride.

3. Weight of internal organs of female rats in the glutamic acid hydrochloride treatment groups were all in normal ranges.

4. Rather larger spleen and smaller liver and kidney were observed in the male rats administered 125mg glutamic acid hydrochloride per Kg body weight and rather larger spleen and hypophysis and smaller gland of seminal vesicle were found in the male rats administered 25mg glutamic acid hydrochloride per Kg body weight. These results, however, might not be attributable to administration of glutamic acid hydrochloride.

5. No teratogenic effects were observed in pups and youngs and no abnormalities of internal organs in macroscopic examination were also observed.

Experimental Data

Fig. 1, 2 and 3

Change of weight and feed intake of male rats.

Fig. 4

Effect of Glu·HCl to sexual cycle, weight gain and feed intake of female rats.

Fig. 5

Effect of Glu·HCl to the pregnant rats.

Fig. 6 and 7

Change of weight and feed intake of control and dosed group rat in the duration of pregnancy.

Fig. 8a and 8b

Relation between administration of Glu·HCl and weight of internal organs of female rats.

Fig. 9a, 9b and 9c

Relation between administration of Glu·HCl and weight of internal organs of male rats.

Fig. 10a and 10b

Weight of internal organs of youngs which were produced by the group administrated 25 mg Glu·HCl/kg weight.

Fig. 11a and 11b

Weight of internal organs of the F_2 rat from F_1 whose parent rats were administrated 25 mg Glu·HCl/kg weight.

Fig. 1

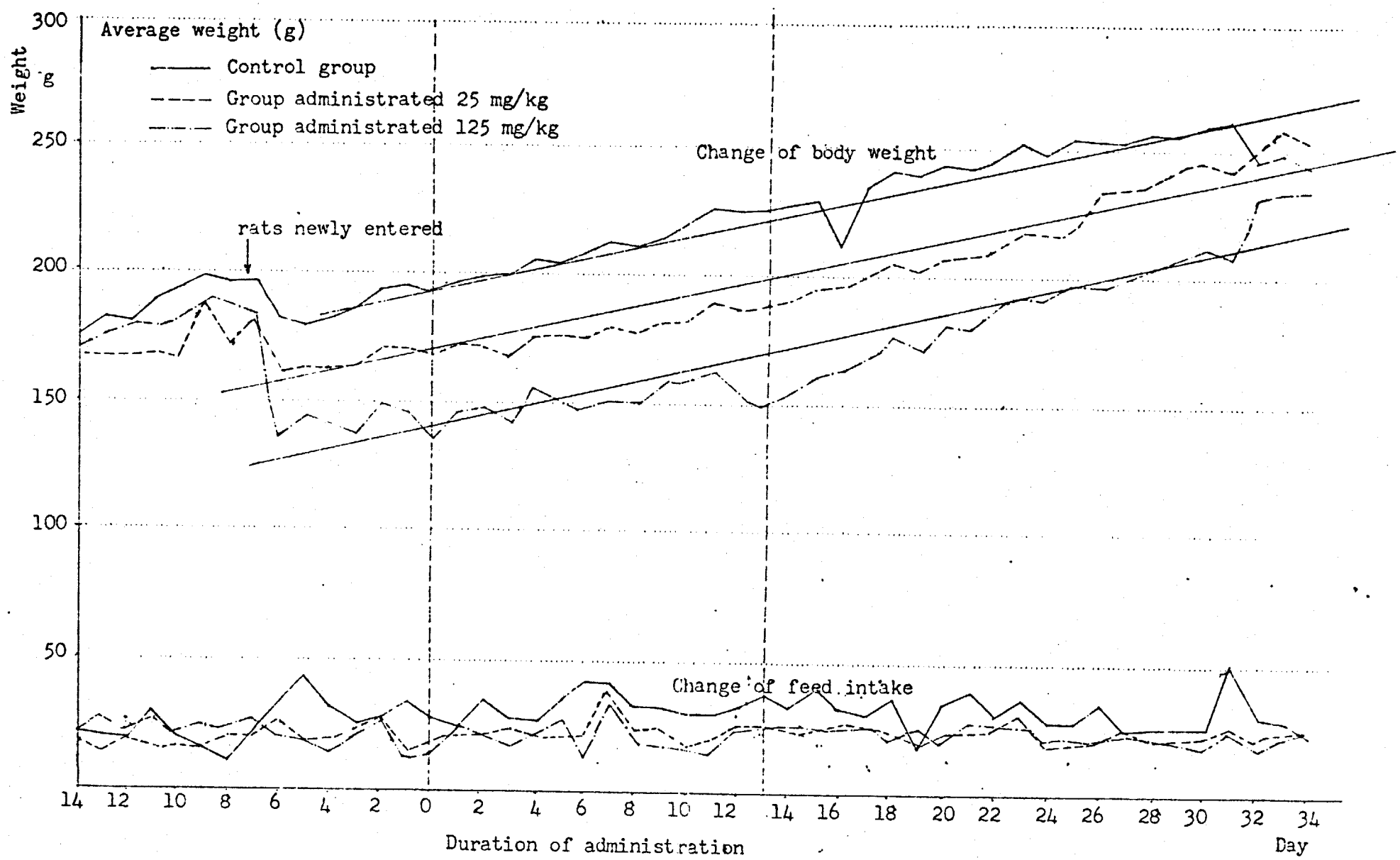

Average weight (g)
Control group
Group administrated 25 mg/kg
Group administrated 125 mg/kg
Weight
g
300
250
200
150
100
50
rats newly entered
Change of body weight
Change of feed intake
14 12 10 8 6 4 2 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Duration of administration
Day

Fig. 2

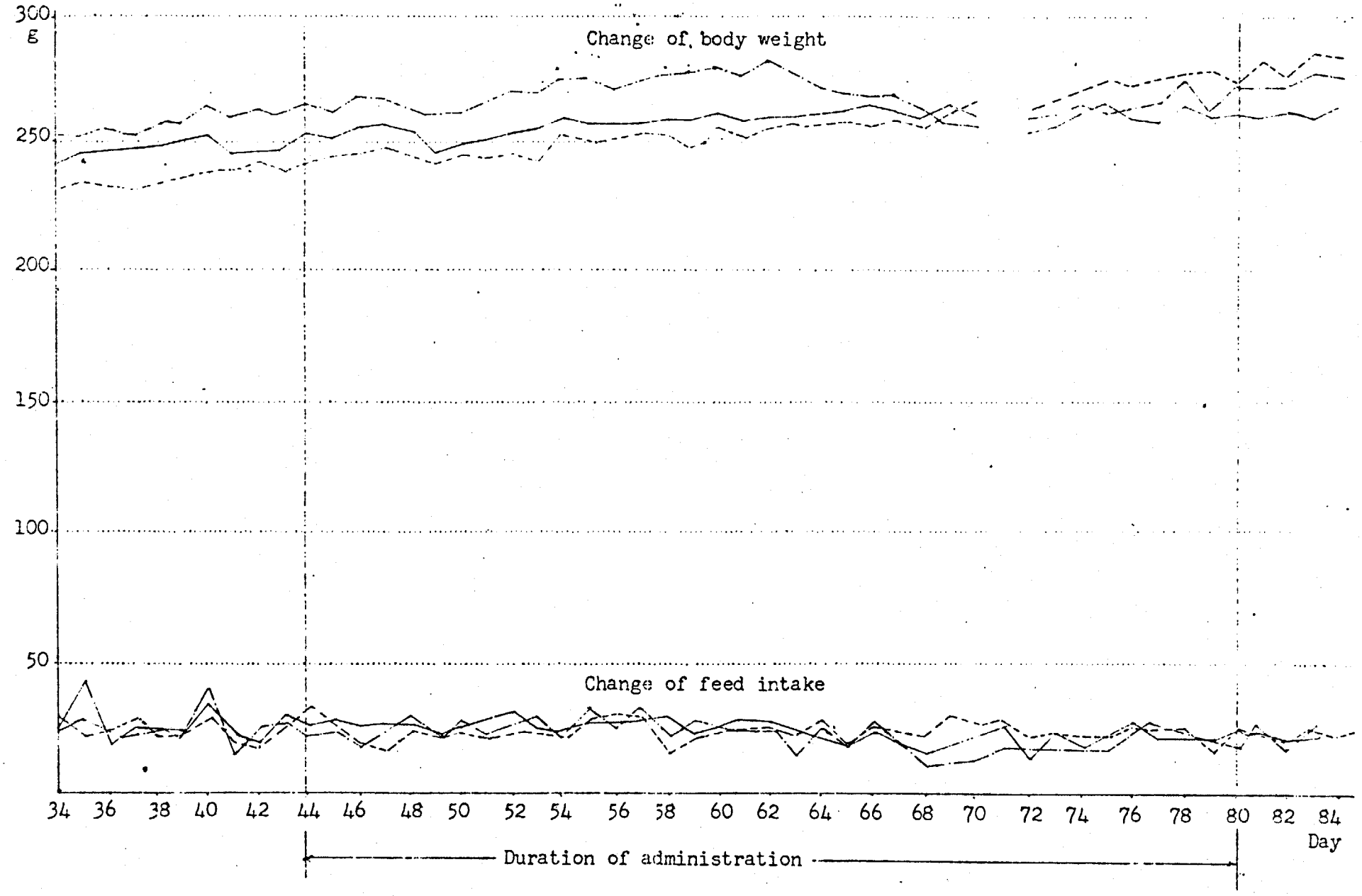

300
g
250
200
150
100
50
Change of body weight
Change of feed intake
34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84
Day
Duration of administration

Fig. 3

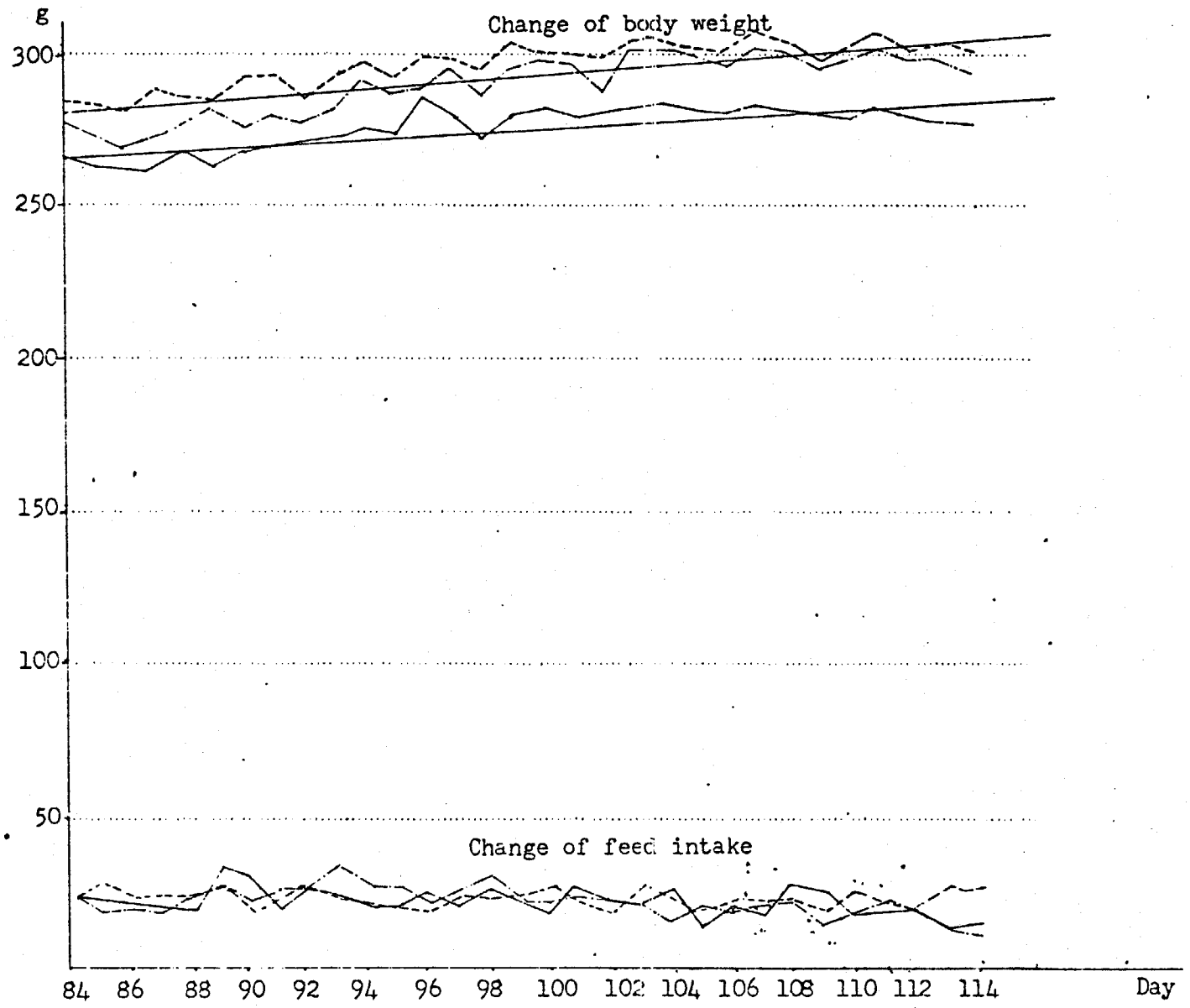

g
Change of body weight
300
250
200
150
100
50
Change of feed intake
84
86
88
90
92
94
96
98
100
102
104
106
108
110
112
114
Day

Fig. 4

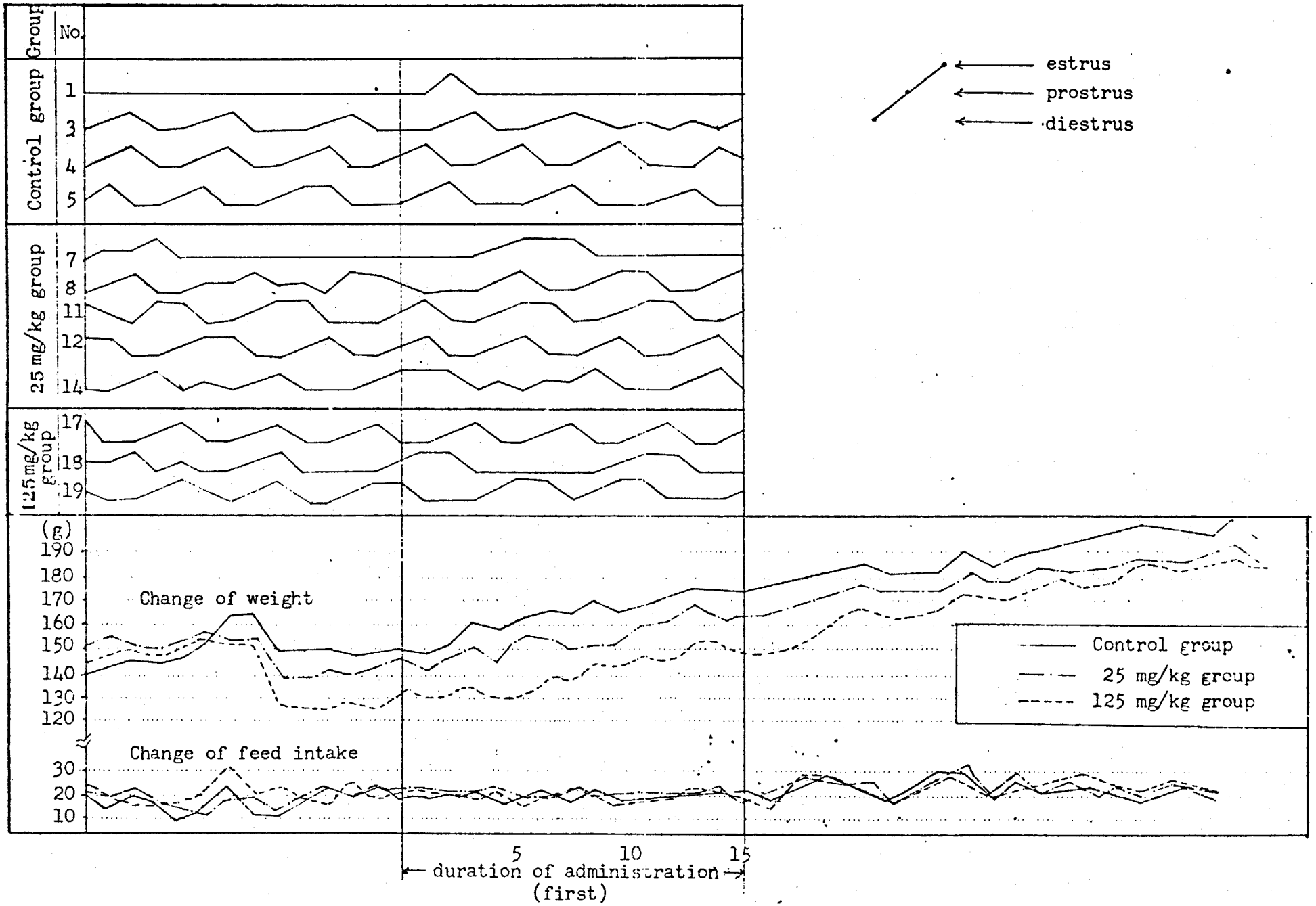
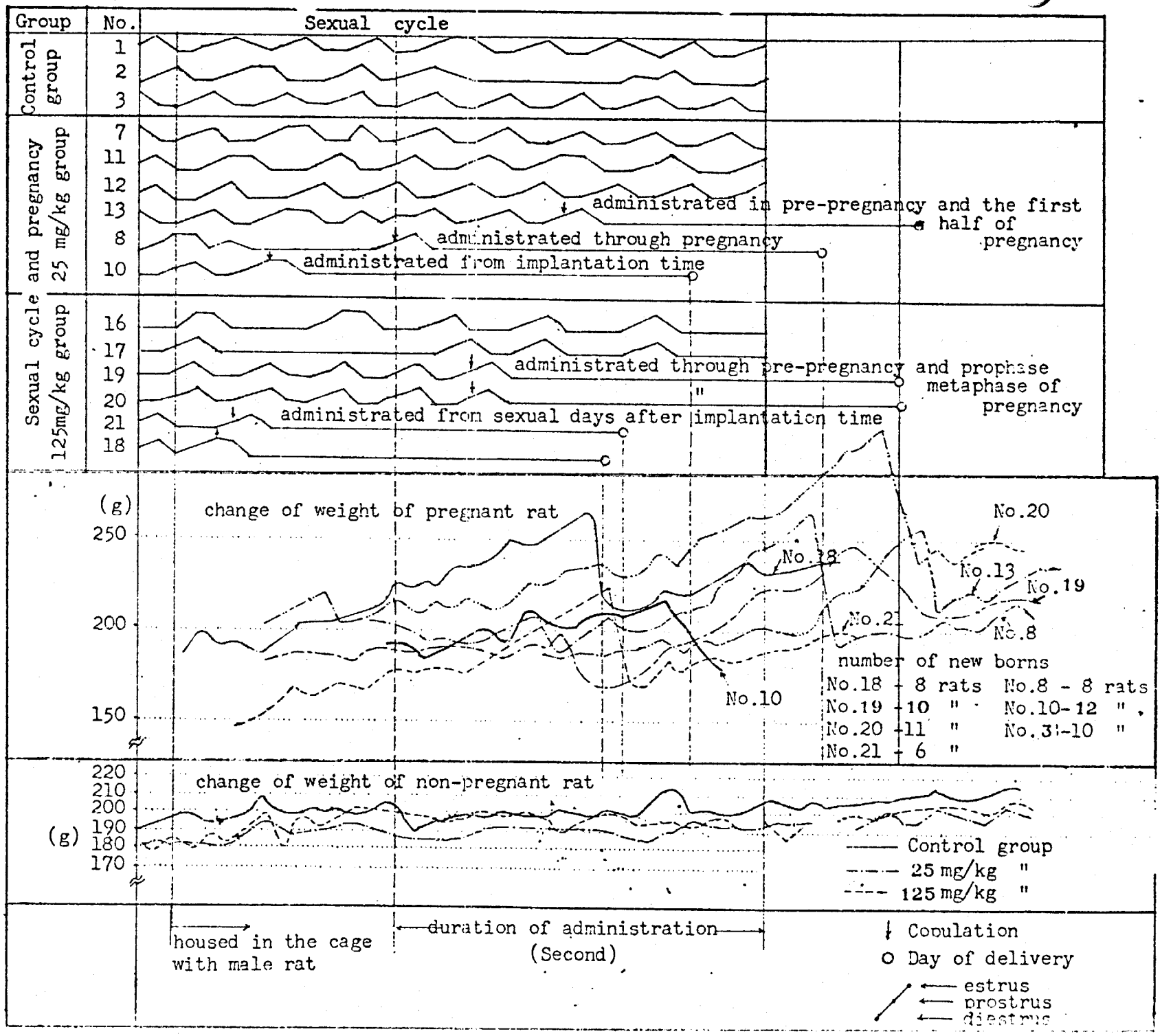

Group
No.
Control group
1
3
4
5
25 mg/kg group
7
8
11
12
14
125 mg/kg group
17
18
19
estrus
prostrus
diestrus
(g)
190
180
170
160
150
140
130
120
Change of weight
Control group
25 mg/kg group
125 mg/kg group
Change of feed intake
30
20
10
5
10
15
duration of administration
(first)

Fig. 5


Group
No.
Sexual cycle
Control group
1
2
3
Sexual cycle and pregnancy 25 mg/kg group
7
11
12
13
8
10
administrated in pre-pregnancy and the first half of pregnancy
administrated through pregnancy
administrated from implantation time
Sexual cycle 125mg/kg group
16
17
19
20
21
18
administrated through pre-pregnancy and prophase metaphase of pregnancy
administrated from sexual days after implantation time
(g)
250
200
150
change of weight of pregnant rat
No.20
No.18
No.13
No.19
No.21
No.8
No.10
number of new borns
No.18 - 8 rats
No.19 - 10 "
No.20 - 11 "
No.21 - 6 "
No.8 - 8 rats
No.10 - 12 "
No.3 - 10 "
220
210
200
190
180
170
(g)
change of weight of non-pregnant rat
Control group
25 mg/kg "
125 mg/kg "
housed in the cage with male rat
duration of administration (Second)
Copulation
Day of delivery
estrus
prostrus
diestrus

(8)

Fig. 6

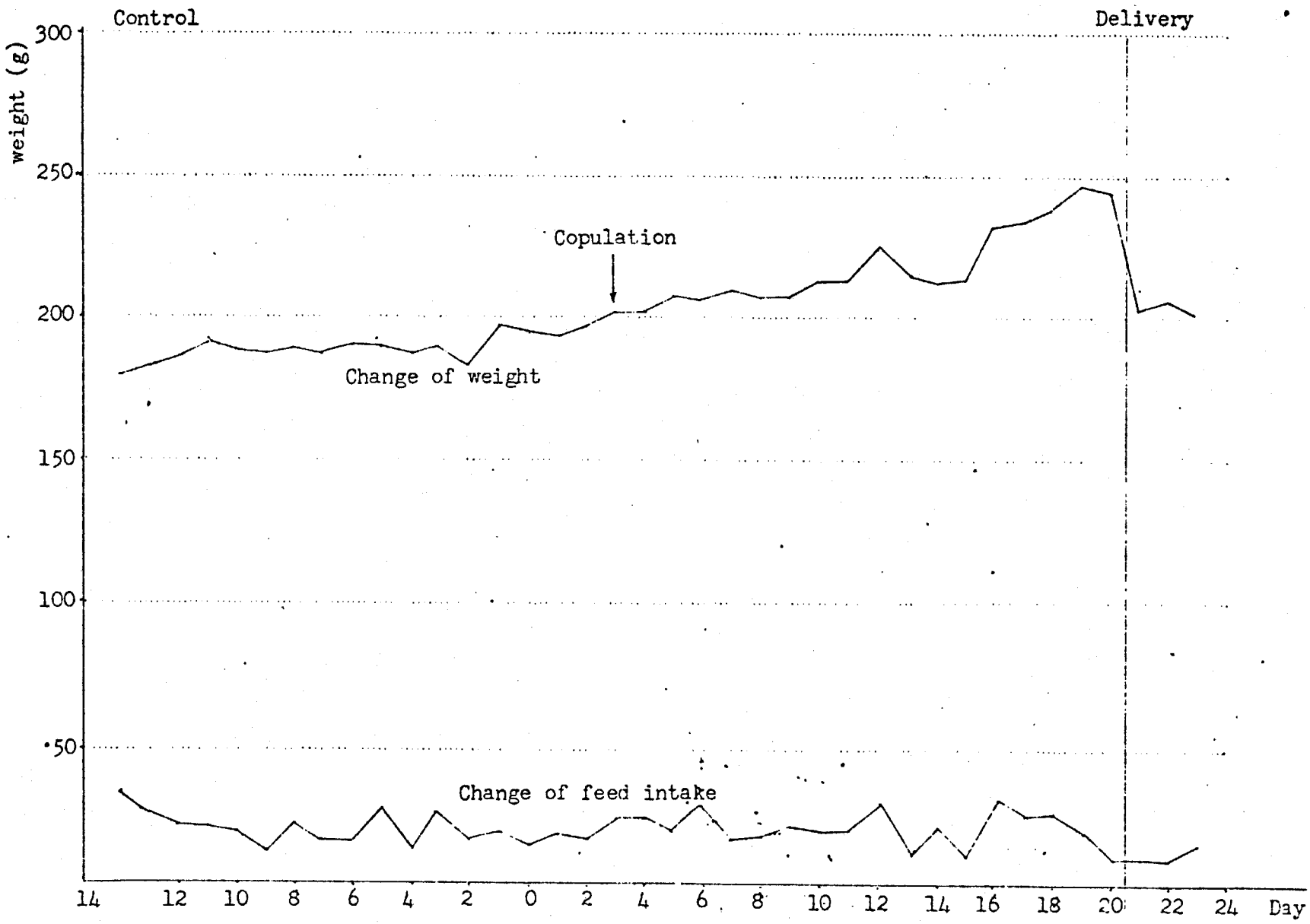

Control
Delivery
weight (g)
300
250
200
150
100
50
Copulation
Change of weight
Change of feed intake
14
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
18
20
22
24
Day

Fig. 7

Administrated 25 mg/kg for 14 days before pregnancy

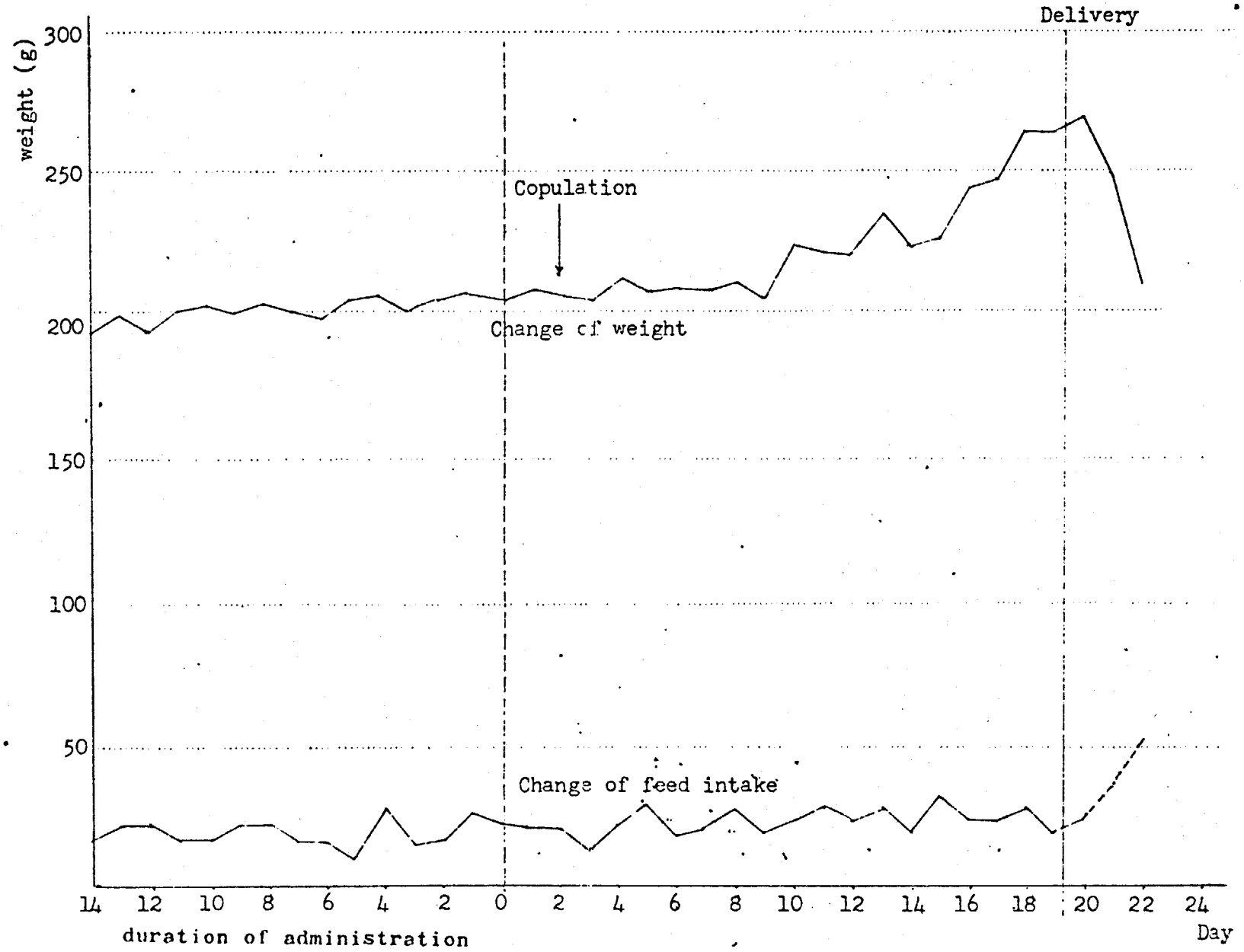


Fig. 8a

Female

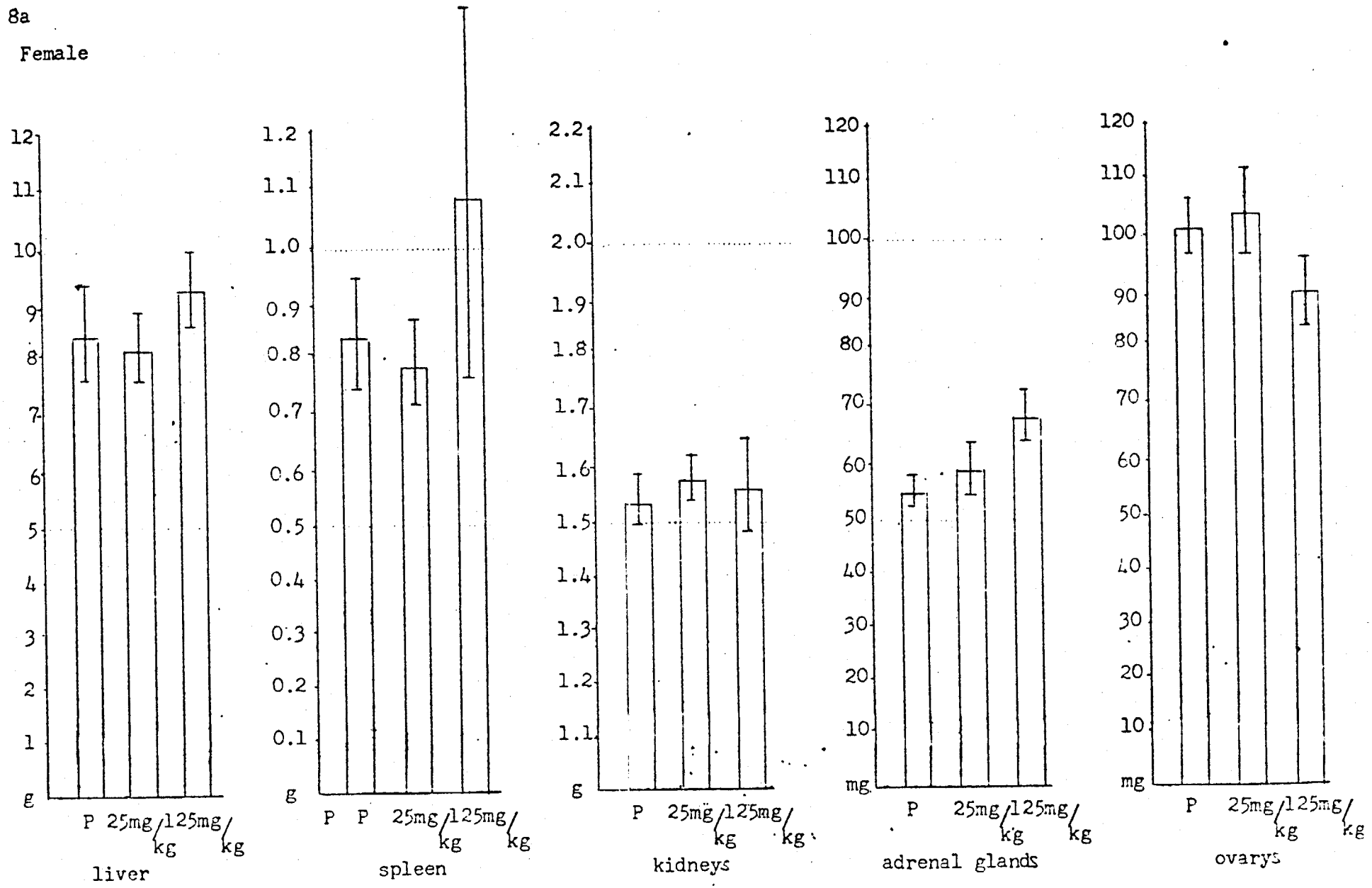


Fig. 8b

Female

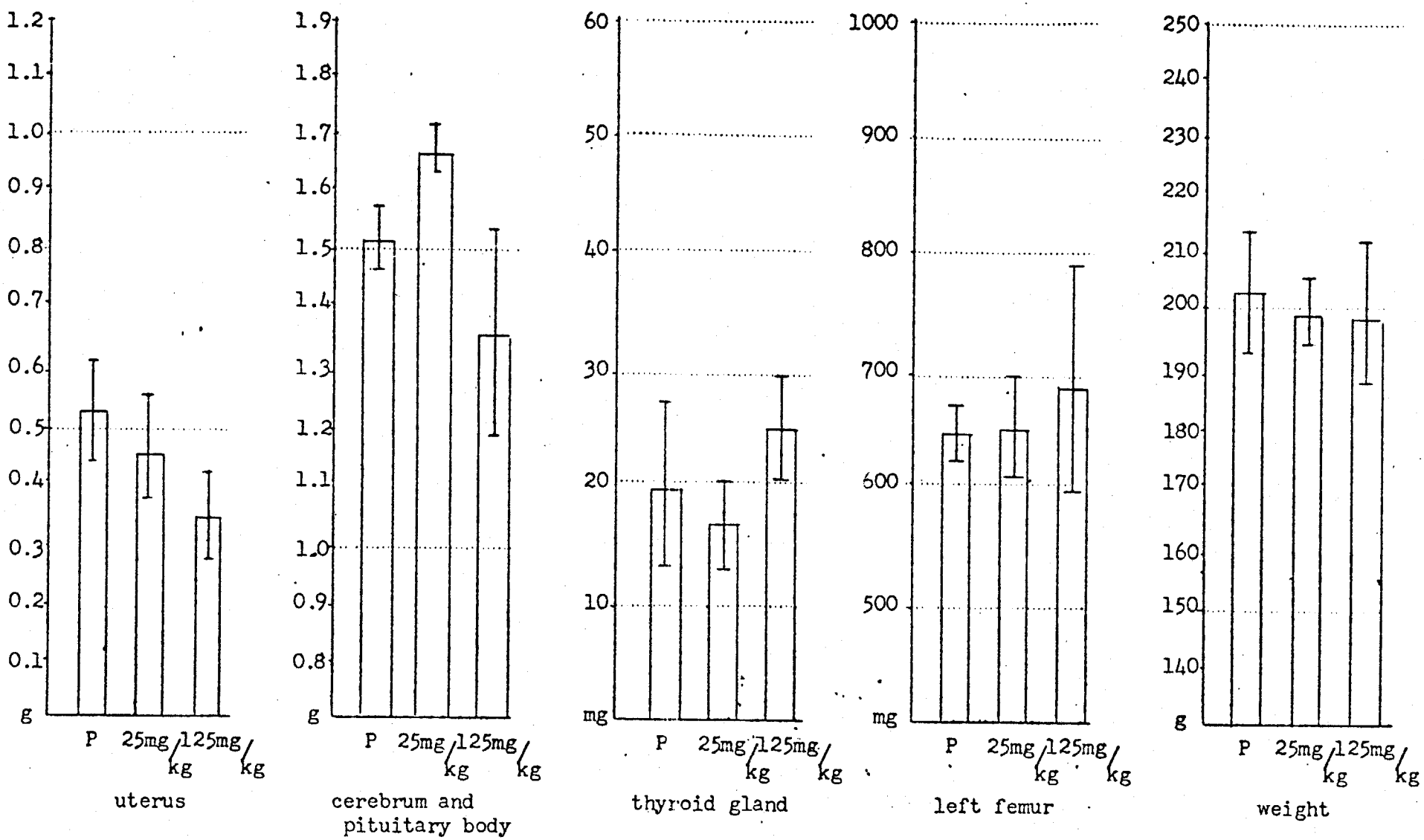


Fig. 9a

Male

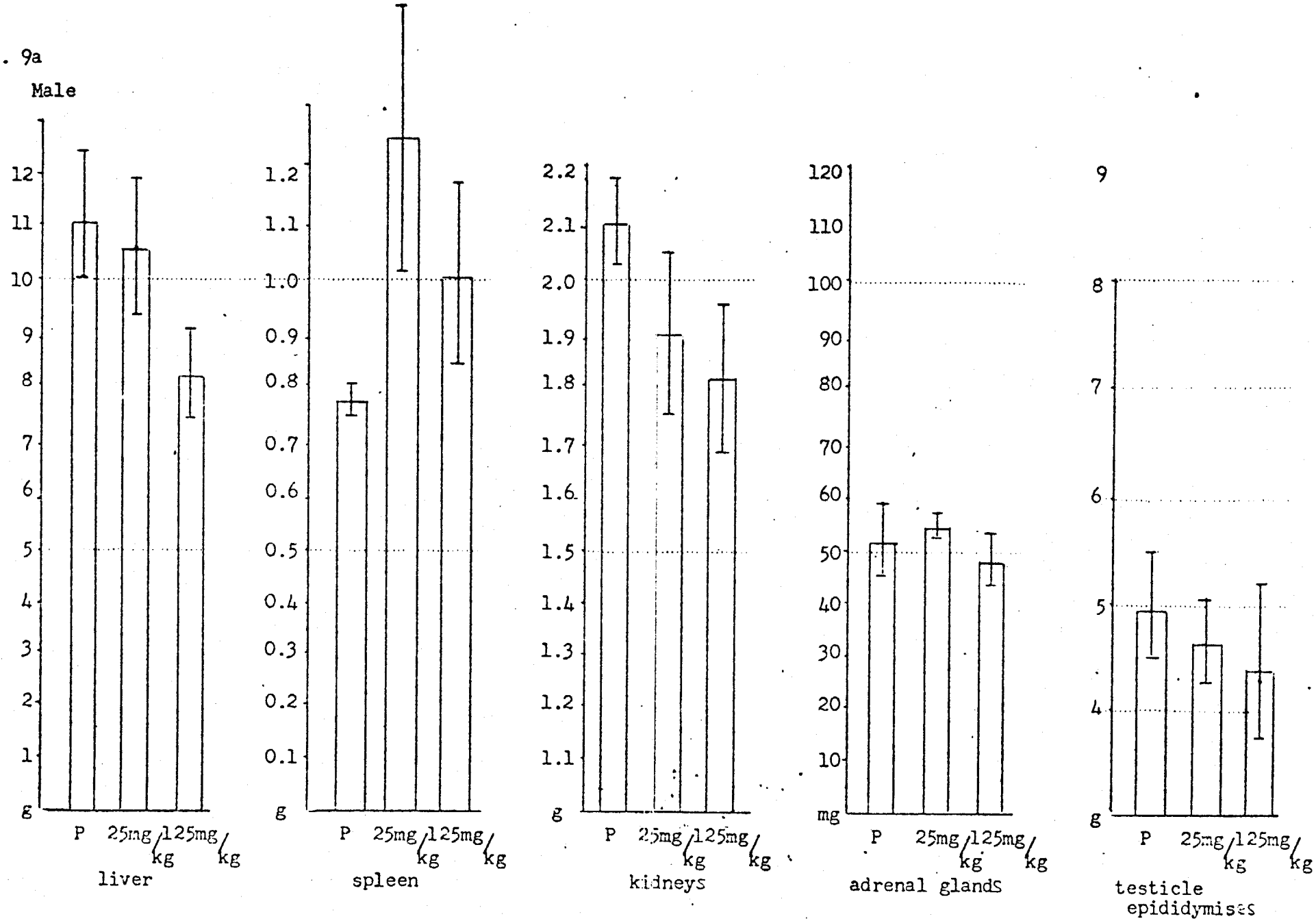

12
11
10
9
8
7
6
5
4
3
2
1
g
P
25mg/kg
125mg/kg
liver
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
g
P
25mg/kg
125mg/kg
spleen
2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
g
P
25mg/kg
125mg/kg
kidneys
120
110
100
90
80
70
60
50
40
30
20
10
mg
P
25mg/kg
125mg/kg
adrenal glands
9
8
7
6
5
4
g
P
25mg/kg
125mg/kg
testicle epididymises

Fig. 9b

Male

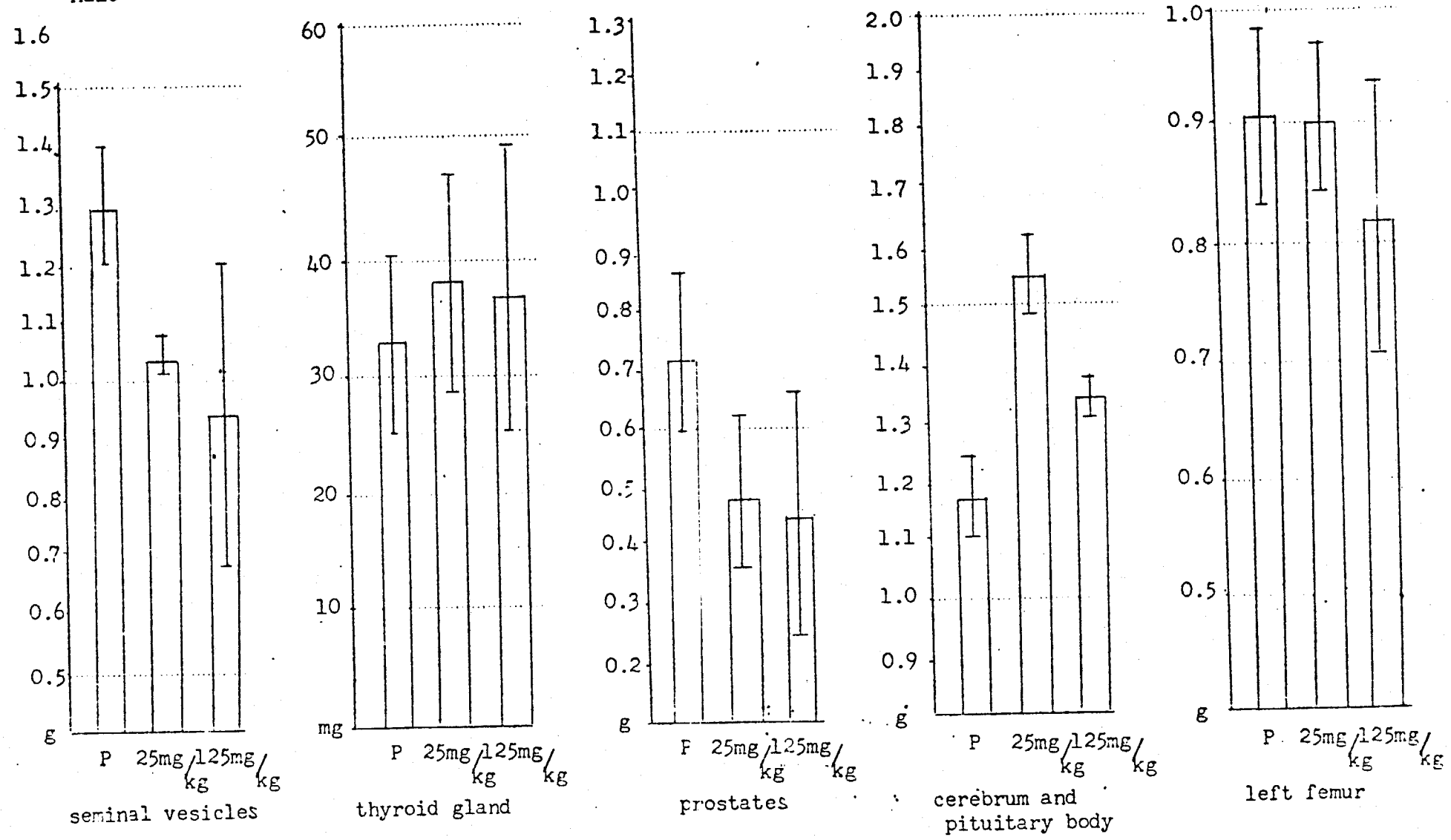

1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
g
P
25mg/kg
125mg/kg
seminal vesicles
60
50
40
30
20
10
mg
P
25mg/kg
125mg/kg
thyroid gland
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
g
P
25mg/kg
125mg/kg
prostates
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
g
P
25mg/kg
125mg/kg
cerebrum and pituitary body
1.0
0.9
0.8
0.7
0.6
0.5
g
P
25mg/kg
125mg/kg
left femur

Fig. 9c

Male

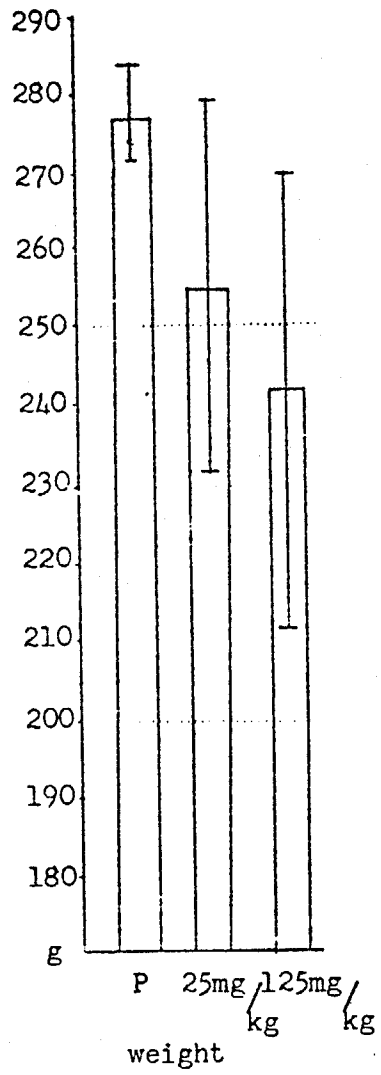

290
280
270
260
250
240
230
220
210
200
190
180
g
P
25mg/kg
125mg/kg
weight

Fig. 10a Female

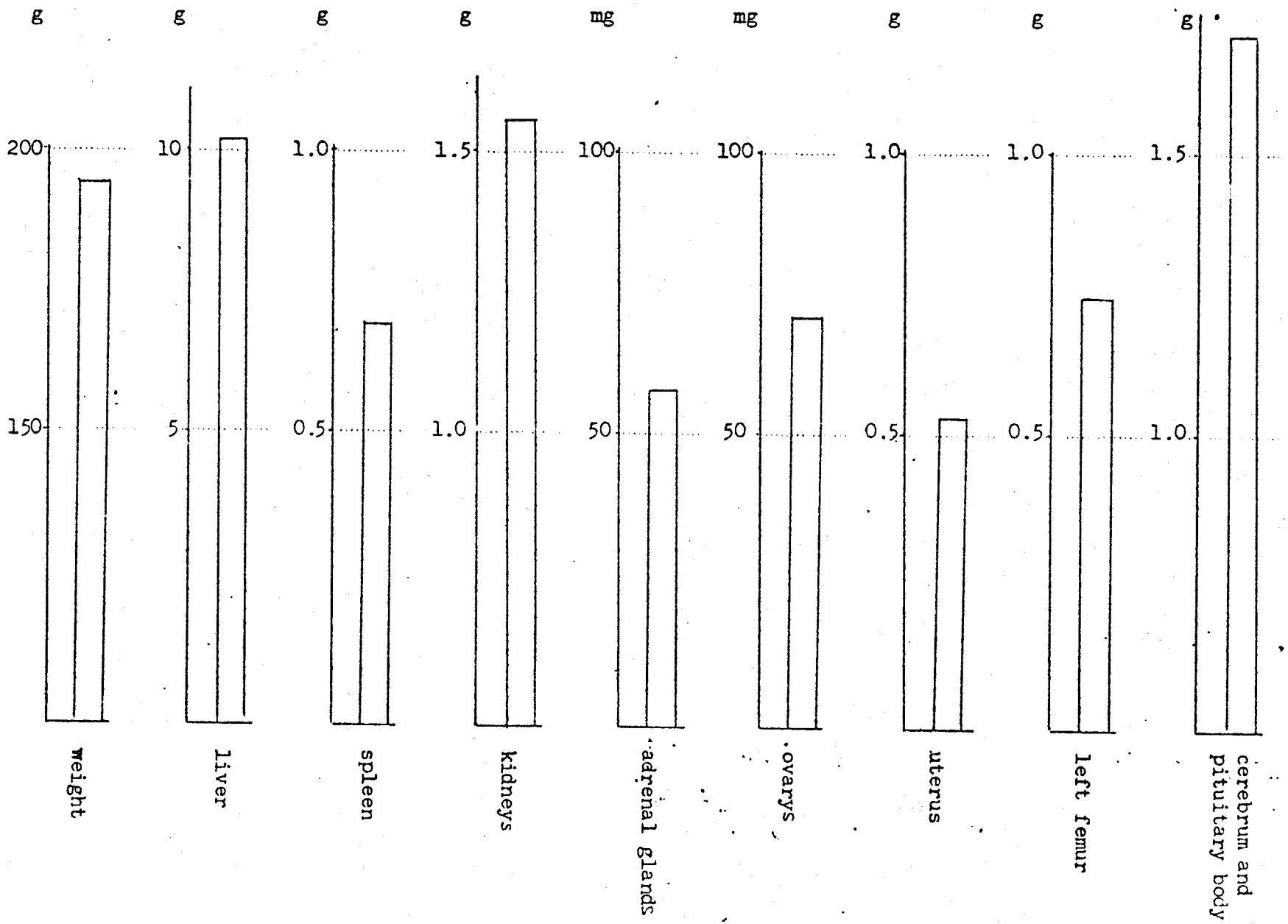


Fig. 10b **Male**

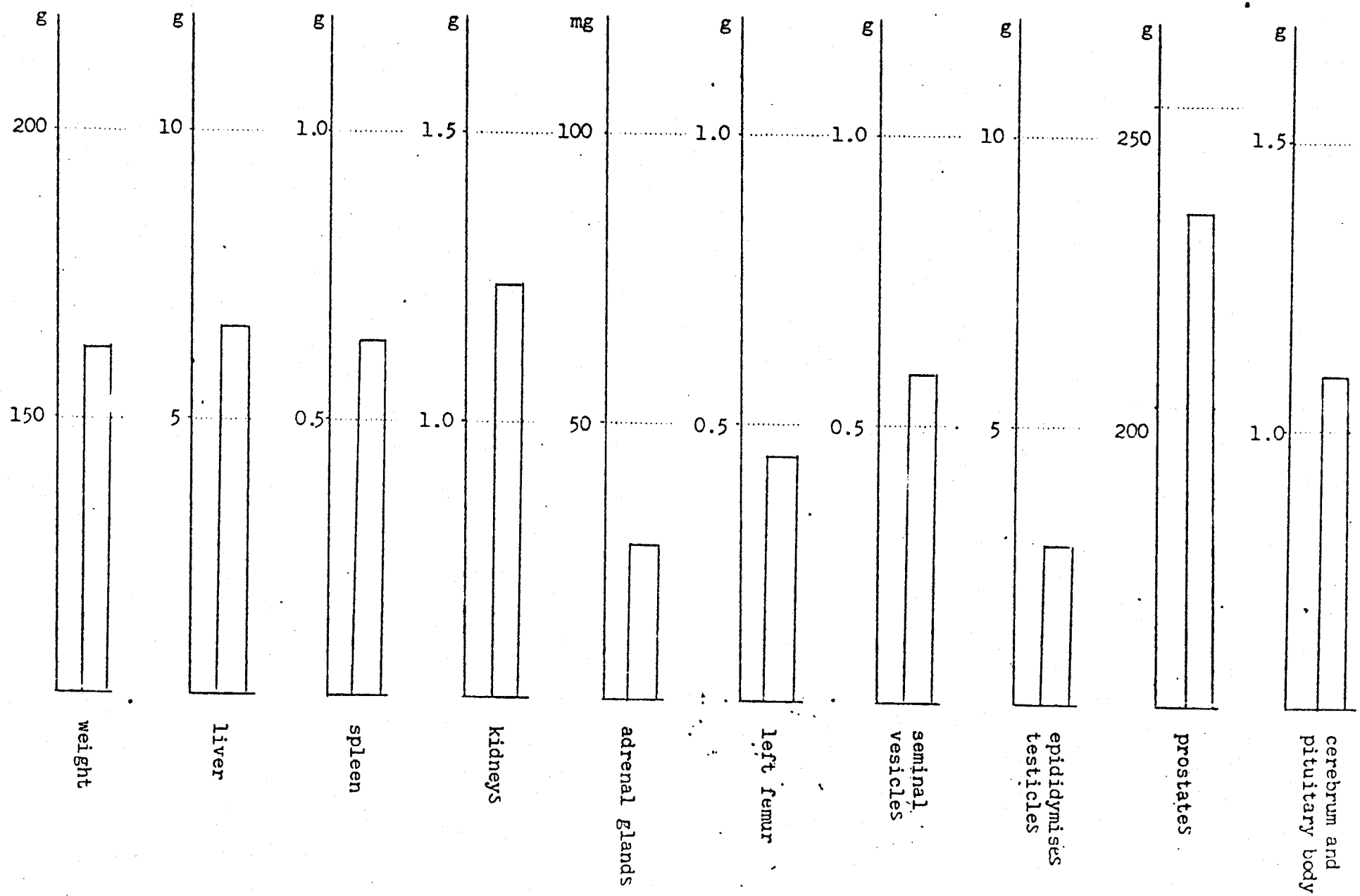

g
200
150
weight
g
10
5
liver
g
1.0
0.5
spleen
g
1.5
1.0
kidneys
mg
100
50
adrenal glands
g
1.0
0.5
left femur
g
1.0
0.5
seminal vesicles
g
10
5
epididymises testicles
g
250
200
prostates
g
1.5
1.0
cerebrum and pituitary body

Fig. 11a **Male**

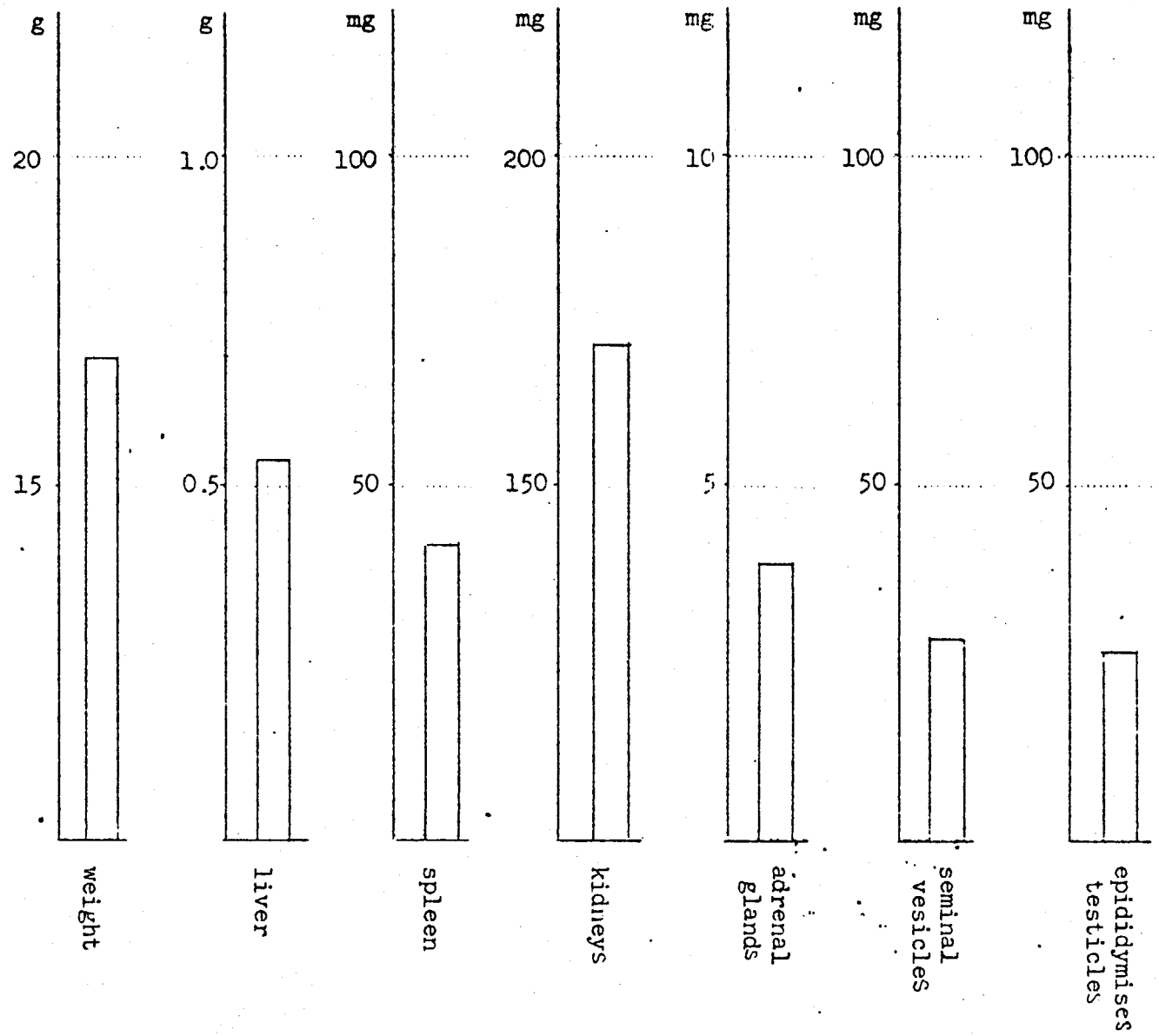
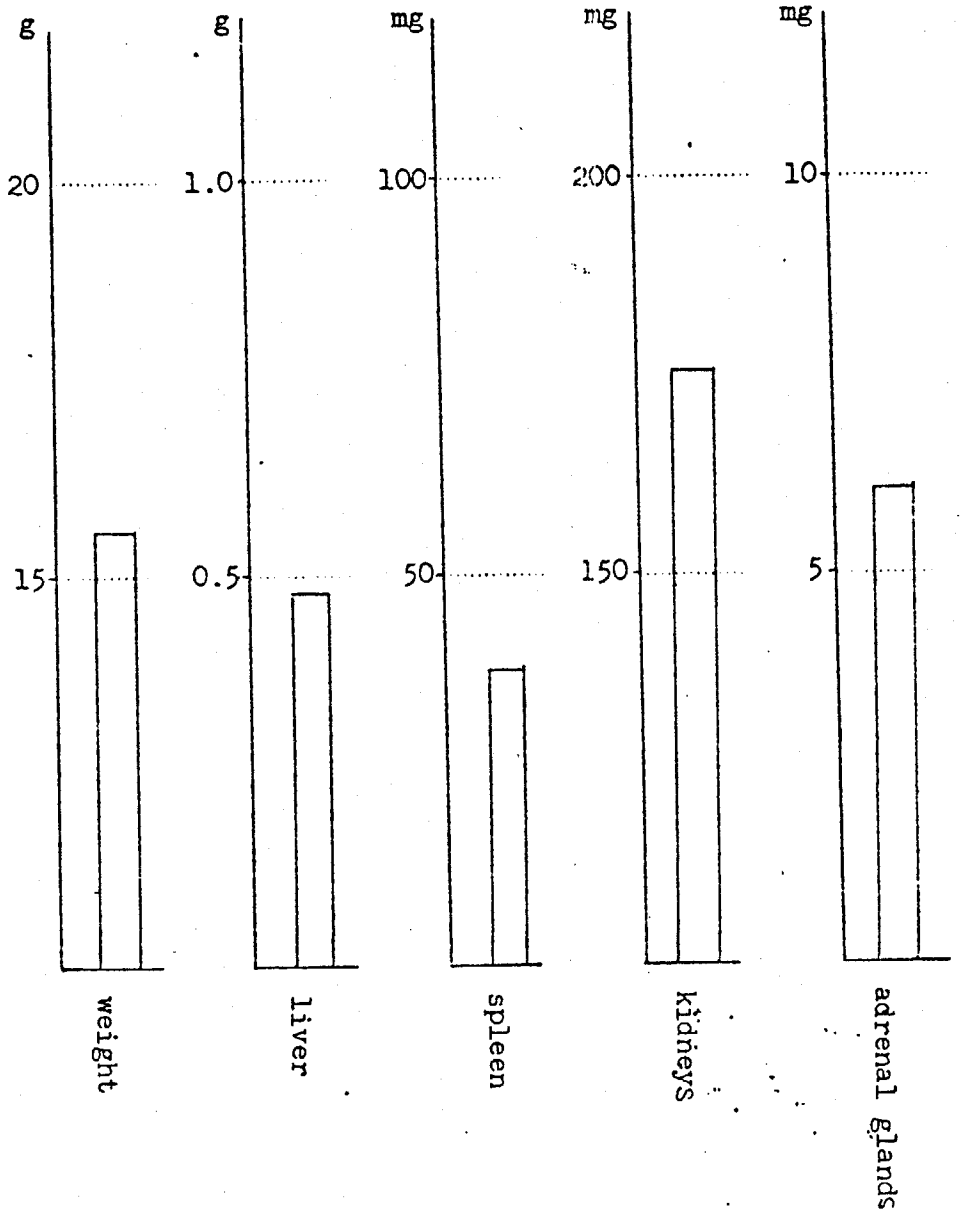

g
20
15
weight
g
1.0
0.5
liver
mg
100
50
spleen
mg
200
150
kidneys
mg
10
5
adrenal glands
mg
100
50
seminal vesicles
mg
100
50
epididymises testicles

Fig. 11b Female



The Taste of Monosodium Glutamate And Other Amino Acid Salts in Dilute Solutions

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Author's abstract

Observations were made on simple systems involving monosodium glutamate with the object of learning a few elementary facts about its usefulness in the absence of highly complex flavoring systems of ordinary foods. Glutamate in distilled water at concentrations from 0.05% to 1.0% is just slightly sweet and slightly salty. No usefulness would be ascribed to glutamate from this experiment. Glutamate with salt produces a taste that is mildly sweet and pleasantly salty, but having an additional effect of an apparent high flavor intensity. This flavor is obviously useful. Increasing concentrations of glutamate require increasing salt concentrations for strongest glutamate taste. At low glutamate concentrations saltiness is diminished.

Glutamate taste is affected by pH. Maximum taste is noted between the ranges pH 6 and 8. Taste diminishes slightly at pH 5 to approximately 80% of original intensity. Greater reductions are apparent at pH 4 and 3.3.

These experimental results obtained by working with simple systems would indicate that in applying monosodium glutamate to foodstuffs, particular attention must be paid to the salt content, the pH of the food and the sweetness. All may require adjustment to achieve the maximum benefits of the glutamate.

Solutions of 20 different amino acids (some L, others DL- form) were tasted in 1% salt solutions at pH 7, in concentration equivalent to .5% monosodium glutamate. Most of the amino acids had very slight taste, usually sweet, although a few were markedly sweet. None had any ability to produce a useful glutamate-like taste. A few of them may have some useful flavor applications when tested under different conditions.

The usefulness of monosodium glutamate for improving the flavor of a variety of foods has been known for a number of years. Other papers on today's program deal with its application in a number of specific uses. The fact that it is valuable in products of noticeably dissimilar taste characteristics has often led people to attribute to it a chameleon-like ability to change its taste to match its taste environment. Whatever the mechanism for its usefulness in this variety of foods, the subject is certainly worthy of considerable study.

During the course of the last few years, I have made a number of observations of glutamate in simple systems with the object to try to learn a few of the elementary facts in the absence of the highly complex flavoring systems of ordinary foods. These observations are offered here, not as an explanation of the glutamate effect, but to promote discussion and to suggest further study along these lines.

The simplest experiment with glutamate is to add it to distilled water in various concentrations ranging from 0.05% up to 1.0% in small increments. The lowest percentage observed gives a taste noticeably different from the distilled water. The taste is one of a pleasant sweetness which increases with the concentration. The slight sweetness is somewhat greater than equal percentages of sucrose at the low levels. The flat taste of distilled water is diminished even by the lowest levels used, and a slight saltiness develops with increasing concentration, so that at 0.5% glutamate there is a saltiness which could be rated approximately equal to an 0.25% sodium chloride solution. The only unusual taste effect is the persistence of a sweetish character which is perceptible in the mouth for periods longer than one-half hour. Observations made on such solutions indicate merely that glutamate is a pleasant tasting substance with considerable persistence. One would scarcely ascribe any useful taste quality to it. Undoubtedly this is the reason that glutamate was known as a chemical compound for about fifty years before any useful character was noted.

However, most foods are eaten with appreciable amounts of salt being present. If we examine a solution containing 1.0% salt and 0.5% glutamate, we will find that the taste is as mildly sweet and pleasantly salty, but there is an additional effect of an apparent high flavor intensity somewhat difficult to describe inasmuch as it is not identical to the high intensity of too sweet sugar solutions or too salty salt solutions. Rather, it seems that all the taste buds are stimulated pleasantly and the stimulation persists for a long time. The closest similar taste I know is that of a salted chicken broth, although the broth is lacking in this high intensity factor. This taste is obviously *very useful* and from here on, I shall refer to it as a normal glutamate taste.

Having noted that salt plays a very important role in the glutamate effect, a series of observations have been made in which the glutamate concentration is varied between 0.05% and 1.0%, while the salt concentration is varied from 0.25% to 1.0%. At the lowest salt level, glutamate concentrations from 0.05% to 0.25% have noticeable normal glutamate tastes. As the concentration of glutamate is increased (still maintaining low salt level) to levels of 0.5% and 1.0%, a distinct sweetness becomes very prominent and the normal glutamate character is overshadowed. When the concentration of salt is increased to 0.5%, the normal glutamate effect is present throughout all concentrations of glutamate observed, but in the highest level (1.0%) additional sweetness is detected. With salt levels at 1.0%, the lower concentrations of glutamate have a normal glutamate taste, and the saltiness of the solution is materially diminished. However, when 1.0% glutamate is used, no reduction of saltiness is noted although the glutamate taste is a normal one. These observations indicate:

1. That salt must be present to produce a useful glutamate taste.
2. That for optimum efficiency and palatability, the concentration of the salt is dependent upon the concentration of the glutamate.

The marked sweetness observed with the low salt—high glutamate solution does not appear to me to have useful application inasmuch as sugar is probably as suitable and less costly for this use. However, further experimentation may find some particular application of this property.

Since the pH of foods varies rather widely, observations were made to learn the effect of pH on the normal glutamate taste. Solutions of glutamic acid equivalent to 0.5% monosodium glutamate were made up in a 1.0% salt solution and adjusted to the following pH's: 3.3, 4, 5, 6, 7 and 8 with sodium hydroxide. The solution at pH 8 had essentially the same taste as the control (pH 7) except that a slight flatness was noted.

At pH 6, again, the taste was essentially identical with the control. However, at pH 5, the normal glutamate taste was noticeably reduced to approximately 80% of the original intensity.

The glutamate taste at pH 4 was even more reduced, although here the acid sourness itself was becoming rather prominent, so as to overshadow the glutamate taste.

Similarly, at pH 3.3, the glutamate taste showed even greater reduction.

These observations indicate that glutamate is more economically used at more neutral pH's.

In all cases, the aftertaste was the same as from neutral solutions. Apparently, the gradual return of the saliva to a neutral condition restored the original taste characteristics. The diminishing of the glutamate taste with increasing acidity suggests that it is affected considerably by the ionization of the second carboxyl group in the molecule.

These experimental results obtained by working with simple systems would indicate that in applying monosodium glutamate to foodstuffs, particular attention must be paid to the salt content, the pH of the food and the sweetness. All may require adjusting for achieving the maximum benefits of the glutamate.

The usefulness of glutamic acid salts as flavoring materials suggests that possibly other amino acids occurring in nature might also have similar properties.

Today, most of the twenty-odd naturally occurring amino acids are available in sufficient purity for conducting small scale tests. A few of them, however, are available only as a DL mixture. In preliminary tests on amino acid solutions no salt was included, and observations were difficult to carry on because of the unnaturalness of the distilled water

taste. Consequently, 1.0% sodium chloride in distilled water was adopted as the standard. In all cases, the pH of the solution was adjusted to 7.0 before tasting. The concentration of each amino acid was equivalent to 0.5% monosodium glutamate.

The DL alanine solution simply had a sweet taste with no other interesting characteristics.

The L (+) arginine had a slightly sweet taste, and it diminished the salty taste somewhat.

Both L (+) aspartic acid and its DL form had a slightly sweet taste, and a very weak glutamate character. However, I did not feel that its glutamate character was sufficiently interesting for commercial use.

The L (—) cystine merely had a slight sweet taste.

Glycine was rather strongly sweet and the saltiness was somewhat reduced.

The L (+) histidine solution was somewhat more salty than the control. It had a slight sweet taste, and also a slight sourness, which is hard to explain in conventional terms since the pH was neutral.

The L (—) hydroxyproline solution was sweet with a rather pronounced bitterness suggesting a burned taste.

Both L (—) leucine and DL isoleucine were slightly sweet and had very slight bitter aftertastes.

The L (+) lysine had a mild sweet taste and a suggestion of glutamate taste although, again, not enough to be of commercial interest.

The DL methionine had a definite sweetness equivalent to about a 2.0% sucrose solution, but it also was slightly bitter.

The DL norleucine was also definitely sweet and exhibited considerable persistence in taste.

The DL norvaline and DL valine were just slightly sweet.

The DL phenylalanine was very sweet, perhaps as sweet as a 5.0% sucrose solution. However, a bitterness was very noticeable so the combination of sweet and bitter was somewhat reminiscent of a chocolate flavor.

The L (—) proline was just slightly sweet.

The DL serine and DL threonine had almost no effect on the flavor of the salt solution.

The DL tryptophane was very sweet, approximately as sweet as a 10.0% sucrose solution. There is an interesting point relating to taste and chemical structure because saccharine has a ring structure somewhat similar to tryptophane.

The L (—) tyrosine had a very slight taste.

From these observations we see that most of the amino acids are pleasant tasting substances which hold no interest as materials for pro-

ducing a glutamate taste. A few of them may have some useful flavor applications when tasted under different conditions.

In commercial food practice today mixtures of amino acids in the form of protein hydrolysates are rather widely used as flavoring materials. With the simple experiments we have performed so far, it has not been evident that the flavor of these hydrolysates is mainly due to their amino acid composition. It appears that the reaction products undoubtedly involving carbohydrates, protein materials and perhaps other trace materials contribute largely to the flavor and aroma of the protein hydrolysates.

Discussion

Dr. Crocker: First of all, I am amazed at the closeness of conclusions of two independent workers. Second, as to the taste versus the effect of glutamic acid. Quite a few of the amino acids have taste of course, but only apparently the L-glutamic acid has glutamic effect. Another point brought up a little earlier in the meeting was with reference to saliva acting on amino acids. I found working with methionine that when you put it in your mouth it has only a sweet taste, but after 15 or 20 minutes it develops a very cheesy flavor and there the saliva has protein-splitting action. If you have only odor to a thing, such as in the case of roast beef, that is not too pleasant, so you put on salt and entertain another set of taste organs—you entertain the organs of taste with salt. It appears that glutamate is successful not because it adds taste, but because it adds feeling. When you can entertain three of your senses in flavor, you are playing a strong cord.

Dr. Gephart: You mentioned that one of the amino acids had a chocolate flavor. At what concentration was this apparent?

Mr. Galvin: Phenylalanine was characteristic of chocolate. It was made up to the equivalent of .5 percent monosodium glutamate in salt water, and the pH was adjusted to 7. It was quite sweet, and had a little bitter connotation thought to be reminiscent of chocolate.

Dr. Anson: It is presumptious to add anything, but I would like to say just a little bit more about the taste of amino acids. Some of them seem to be much more pleasant than others. I compared some protein hydrolysates, and I thought I would be able to predict from the amino acid of the protein what the hydrolysate tasted like, but I was not. I think that illustrates a very practical point. Unless you are a very experienced expert, the ordinary tester is quite incapable of synthesizing tastes or odors. The practical conclusion of that is that you are reduced to a very impractical approach. Unfortunately, that has been the history of flavoring.

Mr. Galvin: We have made experiments in the past which agree with

Dr. Anson's observations on the difficulty of predicting what a complex flavor will taste like from a knowledge of the elementary flavors. Observations made on a number of protein hydrolysates made from wheat, corn, yeast, casein, and one or two others indicated that of all these proteins only the casein hydrolysate tasted something like we thought it should, whereas the others did not.

化学便覧

基礎編 I

日本化学会編

丸善株式会社

クルークレ

	物質名	化学式	分子量	比重	融点 [C]	沸点 [C]	溶解度 水	溶解度 エタノール	溶解度 エーテル	特性
1	D-グルクロン酸	$CHO(CHOH)_4CO_2H$ [+−++]	194.14		シロップ	※	溶	溶		$[\alpha]_D^{20}$ +11.7→+36.3°(水, c=2); シンコニン塩[201°]; ※→ラクトン[175°]
2	D-グルコサゾン	$C_{18}H_{22}O_4N_4$	358.40		205※		不	; 溶		黄; 溶: ピリジン; ※分解
3	D-グルコサミン	CHO / $CHNH_2$ / $(CHOH)_3CH_2OH$ [+−++]	179.18		110※		易	微	不	×MeOH; ※分解; $[\alpha]_D$ +48.6(水, c=1)
4	D-グルコース	$CHO(CHOH)_4CH_2OH$ [+−++]	180.16	1.544 (125)	146	>200※	81.7;	1.9; 21.7		斜方; ×EtOH; ※分解
5	D-グルコースオキシム	$C_6H_{12}O_5(=NOH)$	195.17		137		易	難	不	×MeOH
6	D-グルコースフェニルオサゾン→D-グルコサゾン									
7	D-グルコースフェニルヒドラゾン	$C_6H_{12}O_5(=NNHC_6H_5)$	270.29	α形 160 β形 140−1			易 易	難 微	難 難	菱状晶 針晶
8	D-グルコソン	$CHOCO(CHOH)_3CH_2OH$ [−++]	178.14		シロップ		溶	; 溶		$[\alpha]_D$ −3.3; フェニルヒドラゾン→D-グルコサゾン, 非晶に固化
9	D-グルコン酸	$HO_2C(CHOH)_4CH_2OH$ [+−++]	196.16		125~6	※	易	不		フェニルヒドラジド[200°]; ※→ラクトン[130~5°]
10	グルタコン酸	$HO_2CCH_2CH=CHCO_2H$	130.10		138		易	易	易	針晶; ×Et_2O
11	グルタコン酸ジエチル	$CH_2<CH_2CO_2C_2H_5$ / $CHCO_2C_2H_5$ [trans]	186.21	1.0499 (20)	液	236~8; 125 (12)				n_D^{20} 1.4465°
12	L-グルタミン	$H_2NCO(CH_2)_2CHCO_2H$ / NH_2	146.15		178~86		3.61 (18)	難	不	針晶; ×H_2O
13	L-グルタミン酸	$HO_2CCH_2CH_2CHCO_2H$ / NH_2	147.13	1.538 (20)	206~8 ※		1; 溶	難	不	斜方; ※分解
14	グルタミン酸モノナトリウム	$HO_2CCHCH_2CH_2CO_2Na$ / NH_2	169.12	1.635	195		易	難		針晶; $[\alpha]_D^{20}$ +25.16°
15	グルタル酸	$HO_2C(CH_2)_3CO_2H$	132.12	1.424 (25)	98~9	302~4	83.3;	易	易	単斜
16	グルタル酸ジエチル	$CH_2(CH_2CO_2C_2H_5)_2$	188.23	1.0185 (25)	−24.1	236.5~7	難	易		n_D^{20} 1.4240
17	グルタル酸ニトリル→グルタロニトリル									
18	グルタロニトリル	$CH_2(CH_2CN)_2$	94.12	0.9952 (15)	−29.45	285	溶	溶	不	n_D^{20} 1.4295; $H_2O+H_2SO_4$ (200°)→グルタルイミド
19	クレアチニン	CH_3N-CH_2 / $HN=C-NH>CO$ (+H_2O)	113.12		約291※		1.5 (18)	0.01; 溶 (18)	不	単斜; ×H_2O
20	クレアチン	$CH_3NCH_2CO_2H$ / $HN=CNH_2$ +H_2O	131.14		a) 100※ b) 291※※ (無水)		1.35; 溶	0.008;	不	単斜; ※脱水; ※※分解; アセチル化物[165°];
21	クレイトンエロー→分析試薬 12.2.4									
22	クレオゾール	$C_6H_3(OH)(OCH_3)(CH_3)$ [1,2,4]	138.17	1.098 (20)	5.5	219~21	微	∞	∞	ベンゾイル化物[70~1°]
23	クレソチン酸→6 オキシ-o-トルイル酸									
24	o-クレゾール	$C_6H_4(CH_3)(OH)$	108.14	1.0465 (20)	32	191.1	2.6;	溶	溶	ベンゼンスルホニル化物[39°]; アセチル化物[208°]; フェニルウレタン[145°]

M.W d m.p. b.p.

25 CONFIRMED

QM preference probes now bear out that these 25 foods—of 50 tested—are decidedly improved by the addition of glutamate

CANNED PRODUCTS

1. Asparagus, spear
2. Corn, white, cream
3. Corn, golden, cream
4. Corn, golden, kernel
5. Green beans
6. Peas
7. Spinach
8. Tomato juice
9. Hominy, fried
10. Boned chicken
11. Pork & gravy
12. Salmon
13. Beef & gravy

FRESH PRODUCTS

14. Green beans
15. Hamburger
16. Cod fish, creamed
17. Haddock, fried
18. Beef stew
19. Baked beans & bacon
20. Chicken soup
21. Margarine (army type)
22. Cream of celery soup

DEHYDRATED FOODS

23. Potatoes, mashed
24. Cabbage
25. Onion soup

These New Facts Will Help You Focus

MSG's Power to Perk Up Foods

Panel studies over 18 months tell you "where" and "how much." But—you can't predict benefits merely on the basis of types of foods and flavors

NORMAN F. GIRARDOT and DAVID R. PERYAM

Respectively, Research Laboratories, Pabst Brewing Co., Milwaukee, and Quartermaster Food & Container Institute, Chicago

That monosodium glutamate has a very definite effect on consumer preference for many foods is clearly substantiated by exhaustive studies at the Quartermaster Food & Container Institute, Chicago.

In these investigations, 50 foods or recipes representing various products and flavor types were tested, showing—

1. Twenty-five were definitely improved (at or below 5% level).
2. Three others showed a trend toward improvement (between 5 and 10% level).
3. Eighteen were not changed, and
4. Four were definitely harmed.

It was anticipated that the effectiveness of MSG would depend upon food class or flavor type. This was not consistently true, although there was a tendency for similar foods to give similar results. Meat dishes, fish, and canned vegetables were frequently improved, whereas cereals, milk products, and sweet flavored recipes were not.

These results suggest that, while flavor type may be a useful guide for planning purposes, the final decision as to the use of MSG in a particular food product or recipe intended for general consumption should be made only on the basis of actual tests of that product.

Method Employed

All preference tests involved MSG concentrations at or above threshold of flavor difference as established by the triangle test. Preference testing was by the method of paired comparisons.

The problem that motivated the studies concerned determination of the value of MSG as a flavoring material for use in recipes of the Army's master menu.

Selection of the foods and recipes was based on several considerations. Emphasis was placed on foods considered pertinent to Army feeding and those where available information indicated that improvement might be found.

Different food types—meats, fish, cereals, vegetables, etc.—were included, and attempts were made to choose representatives of broad flavor types, such as bland, sweet, or sour. However, since almost all prepared foods are quite complex in flavor, it was not possible to make clear-cut distinctions on the latter basis.

In most cases, MSG was added as near as possible to the end point in preparation of recipes so that the stated concentrations would represent concentrations in the foods as served.

Primary consideration was to assure a uniform mixture, and in some cases this was best accomplished by adding MSG prior to cooking. With the fried fish items, uniform mixture was not possible, and the product was sprinkled evenly over the fillets before frying. Nine of the canned vegetables, provided through the cooperation of members of the canning industry, had MSG added before final processing. These items were stored at room temperature 4 to 6 months prior to testing.

Two types of testing were employed:

1. Difference testing by a trained panel whose members were selected on the basis of skill in detecting flavor differences caused by MSG, and

2. Consumer preference testing using a group of about 650 employees of the Chicago Quartermaster Depot.

Although random selection within the latter group was attempted, it could not be achieved because the subjects were not equally available.

The study continued over a period of 18 months and involved a total of about 2,150 individual participations in preference tests. It is estimated that only a very few people took part more than ten times. The program was interspersed with preference tests on other foods, and few subjects were ever aware of the variable being checked. Trained panel members, on the other hand, worked with full knowledge.

To assure that only real and controlled flavor differences would be represented in the preference tests, each food was first studied in a series of tests by the trained panel to determine accurately the amount of flavor differences caused by various concentrations of MSG. Employed here was the triangle technique in which the observer received three samples, two alike and one different, and attempted to pick out the odd sample. The two like samples were always untreated controls.

Initially, the concentration of MSG suggested by available information was investigated. If the trained panel was able to detect a significant flavor difference between the treated sample and the control, this level was tested by consumer preference. If no flavor difference was found, the concentration of MSG was progressively increased until there was a significant difference.

Preference was probed by the paired-comparisons method. In each case a sample of the food containing MSG was presented with a control sample of the same food. Each consumer sampled two pairs at a session, either repeating on the same pair of samples or testing the same food at two different concentrations of MSG.

With failure on one level to show a significant preference, concentration of MSG was increased and the food again checked until a preference for the treated sample was established, or until it became evident that further increases would have either no effect or a negative effect.

This procedure was not followed with the nine canned vegetable items provided by industry. For these, the MSG concentrations were determined by the cooperating company's own research department.

The procedure, illustrated in Table 1, shows results on frozen peas. Three concentrations of MSG—0.2, 0.4, and 0.6%—were tested for difference. Since only the highest one resulted in a de-

How Different Foods Respond To Addition of MSG

TABLE I—TESTS Show Effect of MSG on Frozen Peas

MSG Percent by Weight	Triangle Difference			Paired Preference		
	N[1]	Correct Percent	Significant Level	N	Preference for MSG Sample (Percent)	Significance Level
0.20	20	30.0	Insig.	....	.....	.....
0.40	20	35.0	Insig.	....	.....	.....
0.60	20	60.0	1%	40	45.0	Insig.
0.80	....	.....	.....	41	49.0	Insig.

[1] Number of observations.

TABLE II—MOST CANNED VEGETABLES Containing MSG Were Preferred Over Untreated Controls

Product	MSG Percent By Weight	N	Preference for MSG Sample (Percent)	Significance Level (Percent)
Asparagus, whole spear[1]	0.20	80	67.5	1
Corn, white cream style[1]	0.20	80	67.5	1
Green beans[1]	0.30	80	65.3	1
Peas[1]	0.20	80	63.8	1
Spinach	0.20	40	72.0	1
Tomato juice	1.00	82	71.0	1
Corn, golden cream style[1]	0.20	80	62.5	5
Corn, golden whole kernel[1]	0.10	80	61.2	5
Hominy, fried[2]	0.40	40	65.0	5
Lima beans[1]	0.20	80	57.3	Trend
Beans with pork	0.30	80	45.6	Insig.
Sauerkraut	0.30	39	54.0	Insig.
Tomatoes, stewed[1]	0.18	80	50.6	Insig.

[1] Prepared by industry and provided by courtesy of the A. E. Staley Co., Decatur, Ill.
[2] MSG added before cooking.

TABLE III—HOW FRESH, Frozen, and Dehydrated Vegetables Containing MSG Compared With Untreated Controls

Vegetable	MSG Percent By Weight	N	Preference for MSG Sample (Percent)	Significance Level (Percent)
Green beans, fresh	0.20	80	63.8	1
Potatoes, dehydrated, mashed	0.40	79	66.0	1
Cabbage, dehydrated, scalloped[1]	0.20	40	68.0	5
Peas, frozen	0.80	41	49.0	Insig.
Lima beans, frozen	1.00	40	60.0	Insig.
Carrots, dehydrated, creamed	0.10	40	48.0	Insig.
Cauliflower au gratin[1]	0.20	38	55.0	Insig.

[1] MSG added before cooking.

TABLE IV—PREFERENCE Was Shown for Many Meat, Poultry, and Fish Recipes Containing MSG.

Product	MSG Percent By Weight	N	Preference for MSG Sample (Percent)	Significance Level (Percent)
Boned chicken, canned	0.75	40	75.0	1
Hamburger, fresh, fried	0.60	76	65.4	1
Pork and gravy, canned	0.25	80	68.2	1
Cod fish, fresh, creamed	0.40	75	77.0	1
Haddock, fresh, fried[1]	0.40	81	70.0	1
Salmon, canned	0.40	40	70.0	1
Beef and gravy, canned	0.25	80	64.2	5
Beef stew, fresh	0.40	80	61.0	5
Hamburgers, canned	0.50	36	67.0	Trend
White fish, fresh, broiled	0.40	38	66.0	Trend
Chopped ham and eggs, canned	0.75	40	55.0	Insig.
Meat loaf, fresh[1]	0.40	80	52.5	Insig.
Tuna fish, canned	0.60	80	53.8	Insig.

[1] MSG added before cooking.

TABLE V—MANY MISCELLANEOUS Foods and Recipes Were Not Improved by MSG

Product	MSG Percent By Weight	N	Preference for MSG Sample (Percent)	Significance Level (Percent)
Baked beans with bacon[1]	0.50	40	72.0	1
Chicken soup	0.06	40	75.0	1
Oleomargarine (army type)	0.20	42	67.0	5
Cream of celery soup	0.40	40	68.0	5
Onion soup (dehydrated onions)	0.70	40	68.0	5
Army bean soup	0.10	40	58.0	Insig.
Eggs, fresh, scrambled[1]	0.40	79	52.0	Insig.
Cottage cheese	0.05	81	43.2	Insig.
Macaroni with cheese[1]	0.20	77	56.0	Insig.
Cooked rice	0.20	39	38.0	Insig.
Bread, army garrison[1]	0.20	81	46.0	Insig.
Chocolate pudding	0.10	40	40.0	Insig.
Vanilla pudding	0.10	41	56.0	Insig.
Corn meal mush	0.05	40	35.0	5[2]
Orange juice, canned	0.10	41	27.0	1[2]
Milk, dry whole, reconstituted	0.07	40	10.0	1[2]
Oatmeal mush	0.10	39	21.0	1[2]

[1] MSG added before cooking.
[2] MSG sample inferior.

TABLE VI—IMPROVEMENT of Foods by MSG as Related to Types of Flavors

Flavor Type	Improved	Not Improved
Sour	Tomato juice	Orange juice Sauerkraut
Bland	Mashed potatoes Creamed radish Chicken soup Cream of celery soup Oleomargarine Hominy	Vanilla pudding Chocolate pudding Rice Oatmeal mush Cornmeal mush Milk Cottage cheese
Sweet	None	Orange juice Vanilla pudding Chocolate pudding

tectable flavor change, the first check was on preference between the untreated control peas and those with 0.6% MSG. When this comparison resulted in no preference, a second test was conducted with 0.35% MSG. When no preference was established at this high level, work on frozen peas was discontinued.

Compiling the Data

Even though several tests were conducted on each food, only one set of data is given, namely that found to be most favorable to MSG. Triangle-test results were merely preliminary and have been entirely eliminated. Preference tests other than the specific ones shown in Tables II-V gave insignificant results and so were omitted.

However, in referring to the tables, it should be kept in mind that the MSG concentration shown for each food is either at or above that found necessary to bring about a flavor change, and that no concentration above this level gave a more favorable result.

Where not otherwise indicated, MSG concentration represents percent by weight of the food as prepared and ready for serving. For certain foods (indicated by footnotes in Tables) the given concentration represents percent by weight prior to cooking or processing. Within each table the foods have been grouped according to the amount of improvement effected. This corresponds to the statistical significance level as shown in the last column, except in Table V, where four items show a loss in preference.

How They Rated

Canned vegetables (Table II) were most consistently improved. Of the 13 items, only 3 failed to show at least a trend toward improvement. Improvement was not as consistent among the miscellaneous vegetable recipes (Table III), but again a wide variation in effective MSG concentration was found.

There was improvement with most of the meat, poultry, and fish items (Table IV). Here again, only 3 of 13 items failed to show at least a trend.

Results with miscellaneous foods and recipes (Table V) were much less favorable to MSG. Only 5 of the 17 items were improved, and there were 4 foods where detection of a flavor change was accompanied by a loss in preference.

Since these items have been placed together merely for convenience, and since a number of foods are included with which the use of MSG has never been seriously recommended, the low frequency of improvement has no general significance. The table includes 4 soups—and significant improvement was established for 3 of them. It is of interest to note the very low concentrations of MSG which caused a detectable flavor change in some foods, ranging down to 0.05% for corn meal mush and 0.06% for chicken soup.

Table VI shows what happened to a number of foods classified according to the general flavor types of sweet, sour, and bland. The grouping is arbitrary, particularly in regard to bland, where many more items, such as certain vegetables, might also have been included. Some foods which have two dominant flavor characteristics (e.g., orange juice) have been included in two classes. Additional types were not established, since they would have been even less well defined.

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The Hematologic Effects of Monosodium Glutamate in the Rat

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Rats of the Sprague-Dawley strain were fed diets containing large concentrations of monosodium glutamate for extended time periods. The consequences of the regimen were explored in the vascular and reticuloendothelial systems.

A marked increase of basket cells in the peripheral blood was noted after the experimental diet was instituted. After several weeks, large, abnormal lymphoid cells, similar to those characterized in the literature as "neoplastic cells," were observed. A third change associated with this diet was the presence of neutrophils which exhibited an abnormal nuclear pattern resembling the macropolycyte that Cooke described in some cases of granuloyctic leukemia.

Notwithstanding these alterations, it is thought that the principal effect of monosodium glutamate upon the vascular system is not one of leukemogenesis, but rather one of endothelial injury with the transformation of endothelial cells into phagocytes which are unduly fragile and degenerate into basket cells.

INFLUENCE OF α KETO ACIDS ON THE DESAMIDATION OF AMINO ACID AMIDES[1]

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INTRODUCTION

Among the possible mechanisms whereby dehydropeptides may be formed in tissues is that involving the condensation of an amino acid amide with an α keto acid (*1*):

$$RCONH_2 + R'COCOOH \rightarrow RCON{=}C(COOH)R' \quad (1)$$

Such a condensation is used for the mild in-vitro synthesis of this type of compound, and since it involves chemical groups of known biologic occurrence, it does not seem improbable of being at least one of the mechanisms whereby the dehydropeptides are formed in tissues. The dehydropeptides in turn are rapidly split in tissues by dehydropeptidases to products which include ammonia and the corresponding α keto acid (*2-5*):

$$RCON{=}C(COOH)R' \rightarrow RCOOH + NH_2 + R'COCOOH \quad (2)$$

The net results of the successive reactions 1 and 2 are therefore the desamidation of the amino acid amide and the recovery of the original keto acid. The latter thus plays the role of a cosubstrate.

Among the ways in which this hypothesis can be tested is to incubate mixtures of tissue preparations (1) with amino acid amides alone, and (2) with amino acid amides and added α keto acids. If the scheme as presented is correct, the rate of ammonia produced from (2) should be greater than from (1), and the keto acid recovered at the end of the incubation period should be the same in the presence as in the absence of the amides. In this connection, the possibility of transamination reactions involving the transfer of ammonia from the amino acid amide to the α keto acid must also be considered, and the oxidative desamination of the amino acid so formed must be known.

We have employed in this study glutamine and asparagine as the amino acid amides, together with pyruvic acid as the α keto acid. These substrates were chosen because they are widespread in most animal and plant tissues. Implicit in the reactions 1 and 2 involving these compounds are (1) the condensation by some enzymatic process of the keto acid with the acid amide groups at the γ carbon of glutamine and at the β carbon of asparagine, and (2) the subsequent splitting of the dehydropeptides by action of dehydropeptidase. These types of peptide linkages are not susceptible to peptidases which act upon known saturated peptide analogs. In support of the biologic occurrence of the γ peptide linkage, however, is the presence of glutathione in tissues.

We have further extended the testing of the condensation hypothesis of dehydropeptide formation (equation 1) by employing the peptide, pyruvoylglycine, in place of pyruvic acid. If our scheme is correct, the condensation product would be the tripeptide, glutamyl- or aspartyldehydroalanylglycine, in which the dehydroalanyl residue is in the middle of the chain. According to Bergmann and Schleich (*2*), such dehydropeptides, in which the dehydro residue is in the middle of the chain, are not attacked by dehydropeptidases. Therefore, the addition of pyruvoylglycine to the amino acid amides in the presence of tissue extracts, even though the dehydropeptide condensation occurred, should lead to little or no increase in ammonia production. This is, of course, also dependent upon the rate of condensation of the amide with pyruvoylglycine being much greater than the rate of hydrolysis of the pyruvoylglycine. Bergmann and Grafe (*6*) have showed that the enzymatic splitting of pyruvoylglycine by pancreatin is extremely slow.

The experiments whereby these concepts have been tested are described in the present paper.

EXPERIMENTAL PROCEDURE

Tissue extracts were prepared exactly as before (*3, 4*). Each cubic centimeter contained the equivalent of 333 mg. of tissue. The amino acids and amino acid amides were made up to 1.4×10^{-2} M stock solutions. The keto acids were made up to 2.7×10^{-2} M stock solutions, unless otherwise noted. All stock solutions were made up fresh before each run and were brought to pH 7 with dilute sodium hydroxide.

Considerable difficulty was experienced in the final step of the synthesis of pyruvoylglycine, namely, that involved in the preparation of α-α diacetaminopropionic acid by the condensation of acetamide with pyruvic acid (*1*). This step was accomplished by recourse to the method of Böttinger (*7*) and consists in the combination of acetonitrile with pyruvic acid in concentrated sulfuric acid. Since this method is not fully described by Böttinger for this system, a brief description of the procedure employed is given.

Four hundred cubic centimeters of concentrated sulfuric acid was chilled, with stirring, to 10° C., and then 77 gm. of pyruvic acid was added. With good stirring, 100 gm. of acetonitrile was slowly added, care being taken that the temperature did not rise above 50°. The reaction took about 1 hour. The mixture was cooled to 10° and then

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poured into 4 l. of shaved ice. Intense chilling took place, and the reaction product crystallized out. When the ice had melted, the precipitate was filtered off and washed thoroughly with cold water. A single crystallization from alcohol yielded pure diacetaminopropionic acid. The remainder of the synthetic steps toward the preparation of pyruvoylglycine were straightforward, and the final product after recrystallization from ether-petroleum possessed all the properties of pyruvoylglycine, as described by Bergmann and Grafe (*6*), that is, a melting point of 90° nitrogen content of 10.0 percent (theoretical, 9.7 percent), and the faculty of turning a mixture of ammoniacal nitroprusside to a blue color followed by a muddy green color on addition of potassium hydroxide.

The digests were prepared by mixing 1 cc. of the extract with 1 cc. of amino acid or amino acid amide plus either 1 cc. of keto acid or 1 cc. of water. Control digests contained only the tissue extracts plus added water to give the usual volume. The substrate solutions were entirely stable. The ammonia evolved in the solutions was determined by the method described (*3*), and the enzymatic activity was established by noting the excess of ammonia found in the test solutions over that found in the control solutions.

The first experiments were designed to observe the rate of desamidation of glutamine in the presence of pyruvate (table 1). The desamidation rate in extracts of the hepatoma was less than in those of normal liver. In 2 hours under these conditions, glycyldehydroalanine is nearly completely hydrolyzed (*3, 4*).

TABLE 1.—*Time course of evolution of ammonia in digests of rat liver and hepatoma extracts with glutamine and pyruvate* [1]

Digestion period (in hours)	Ammonia N evolved [2]	
	Liver	Primary hepatoma
	Micrograms	*Micrograms*
1	30	4
2	50	10
3	80	16
4	100	24
5	110	31

[1] Mixtures composed of 1 cc. amino acid amide plus either 1 cc. water and 1 cc. keto acid, plus 1 cc. extract equivalent to 333 mg. tissue. Glutamine, 1.4 x 10^{-2}M stock solution. Pyruvate, 2.7 x 10^{-2}M stock solution.
[2] In terms of micrograms. Theoretical maximum amide nitrogen, 196 micrograms, determined by acid (1NHCl) hydrolysis.

The data in table 2 show (1) that the relatively weak desamidation of glutamine and asparagine is considerably increased when pyruvate is added to the mixture, but that pyruvoylglycinate has little or no effect in this regard, (2) that pyruvate has very little effect on the desamidation of glutamate or aspartate, (3) that alanine and alanylglycine yield relatively little ammonia under these conditions, and (4) that the hepatoma is much weaker than liver in reacting to added pyruvate.

TABLE 2.—*Ammonia nitrogen evolved in digest of rat liver and hepatoma extracts with various substrates*

Substrates [1]	Ammonia N evolved [2]	
	Liver [3]	Primary hepatoma
	Micrograms	*Micrograms*
Glutamine	9	10
Glutamine+pyruvate	102	22
Glutamine+pyruvoylglycinate	12	10
Glutamate	3	2
Glutamate+pyruvate	4	4
Glutamate+pyruvoylglycinate	4	3
Asparagine	22	12
Asparagine+pyruvate	135	14
Asparagine+pyruvoylglycinate	36	13
Aspartate	8	2
Aspartate+pyruvate	12	4
Aspartate+pyruvoylglycinate	9	3
Alanine	10	0
Alanylglycine	0	0
Pyruvate	1	0
Pyruvoylglycinate	0	0

[1] [2] Same as in table 1. Incubation period 4 hours at 37° C.
[3] Dialyzed liver extract incubated under the same conditions with glutamine plus pyruvate yielded 90 micrograms of ammonia nitrogen as compared with a value of zero for the same mixture minus pyruvate.

The ammonia noted in rat-liver extracts in the presence of pyruvate is invariably higher than in the absence of pyruvate. Determinations of the amount of pyruvate in the digests of rat-liver extract with glutamine, asparagine, etc., at the end of the incubation period indicated relatively little decrease in the concentration of the pyruvate, as shown in the following tabulation: [2]

Mixture	Pyruvate [1] (in milligrams)
1 cc. extract + 1 cc. pyruvate + 1 cc. water	2.2
1 cc. extract + 1 cc. pyruvate + 1 cc. glutamine	2.2
1 cc. extract + 1 cc. pyruvate + 1 cc. asparagine	2.2

[1] Initial concentration of pyruvate was 3.0 mg.

Studies of the pyruvate effect on the ammonia production from glutamine were instituted with extracts of various rat tissues, as given in table 3. It is clear that kidney has a powerful glutaminase, and that pyruvate increases still further the yield of ammonia, but the percentage increase in ammonia production on the addition of pyruvate to the mixture of tissue extract and glutamine is greatest in the case of liver.

The data in the following tabulation show that an optimal effect of pyruvate on the desamidation of glutamine is achieved in digests with rat liver at a concentration of about 2.5 mg. of pyruvate per cubic centimeter of stock solution, or roughly

[2] The extract concentration was equivalent to 333 mg. tissue per cubic centimeter; the pyruvate concentration, 3 mg. per cubic centimeter stock solution; and amino acid amides at 1.4 x 10^{-2} M stock solution. Pyruvate was determined as 2:4 dinitrophenylhydrazone as before (*3*).

TABLE 3.—*Ammonia nitrogen evolved in digests of rat tissue extracts with various substrates* [1]

Tissue	Ammonia N evolved from [2] —	
	Glutamine	Glutamine and pyruvate
	Micrograms	*Micrograms*
Liver	10	98
Kidney	120	183
Spleen	27	32
Brain	20	40
Pancreas	45	47
Muscle	9	25

[1] Same as in table 1. Incubation period 4 hours at 37° C.

2.7×10^{-2}-M. The ammonia nitrogen was evolved under the same conditions as in table 1, except for the changing concentrations of pyruvate.

Milligrams pyruvate per cubic centimeter stock solution:	*Ammonia N evolved (in micrograms)*
10	18
5	88
2.5	100
1.25	90
0.62	72
0.31	48
0.15	23
0.07	18
0.03	10
0.015	10
0.008	10
0.004	10
0.002	10
0.001	10
0.0	10

In order to see whether the ammonia evolved in mixtures of glutamine with pyruvate is accompanied by a corresponding decrease in the amide nitrogen of the glutamine, experiments were performed in which free ammonia and amide nitrogen were determined on aliquots of the same digest. The amide nitrogen was determined by treating the digest with 1 cc. of 5 percent trichloracetic acid, centrifuging off the precipitated protein, and adding concentrated hydrochloric acid to 1N concentration. The mixture was then gently boiled for 5 minutes, cooled, treated with alkali, and aerated in the usual manner into sulfuric acid traps, which were subsequently nesslerized. The values of ammonia so obtained were the sum of the free ammonia formed by enzymatic desamidation of the amide plus the residual amide nitrogen of the glutamine. When by independent measurement on another aliquot of the same digest, the amount of free ammonia formed enzymatically is known, the residual amide nitrogen can be calculated by difference. The results noted in table 4 show that the increase in free ammonia in the presence of pyruvate is accompanied by a corresponding decrease in amide nitrogen of the glutamine.

TABLE 4.—*Change in amide nitrogen of glutamine incubated with pyruvate in presence of rat liver extracts* [1]

Substrates	Period of incubation	Ammonia N	Residual amide N	Total N [2]
	Hours	*Micrograms*	*Micrograms*	*Micrograms*
Glutamine	0	.....	190	190
Glutamine + pyruvate	0	.....	190	190
Glutamine	4	6	182	188
Glutamine + pyruvate	4	98	88	186

[1] Mixtures prepared as in table 1.
[2] Theoretical maximum amide nitrogen 196 micrograms.

DISCUSSION

The data in table 2 support but do not necessarily prove the concepts of the mechanisms postulated in the Introduction, that the α keto acids exert their effect upon the desamidation of the amino acid amides (1) by condensing to form a dehydropeptide, and (2) by the subsequent splitting of the susceptible dehydropeptide by dehydropeptidase. The net result is the desamidation of the amino acid amide and the recovery of the original keto acid in nearly the same concentration in the presence or absence of amide. It is probable that the dehydropeptide is split as fast as it is formed, and hence changes in pyruvate concentration would not be readily observable. The final proof of the hypothesis will involve among other things the reproduction of the findings with purified enzyme systems.

Neither alanine nor alanylglycine yields much ammonia under the conditions used, which eliminates the possibility that transamination reactions may be concerned to any significant extent. Virtanen and Laine (*8*) noted that the transamination of pyruvic acid with glutamine and asparagine occurred after the amide group was split, and thus the amide group does not participate in the transamination reactions (enzymes from vegetable sources).

The enormous rise in ammonia on adding pyruvate to a digest of glutamine or asparagine in rat-liver extract is rather extraordinary and to our knowledge has not been reported before. That this ammonia is derived almost completely from the amide group is suggested by the relatively small amount of desamination which takes place with glutamate or with aspartate, whether pyruvate is present or not (table 2), and by the corresponding decrease in amide nitrogen of glutamine (table 4).

There is a definite asparaginase in rat liver which appears to be even more active than the accompanying glutaminase. This finding has been consistently observed.

The figures given in table 2 for the ammonia noted in digests of either glutamine or asparagine with pyruvate, 102 or 135 micrograms of ammonia

nitrogen, respectively, represent roughly one-half of the theoretical amide nitrogen available. The data in table 1 suggest that further incubation beyond this 4-hour period would result in the production of more ammonia, but the reaction was not carried beyond 5 hours.

The relatively small effect of pyruvate on the desamidation of glutamine and of asparagine in extracts of the primary hepatoma, as compared with the magnitude of this effect in extracts of normal liver, is interesting in view of the fact that the dehydropeptidase activity of the tumor is greater than that of the liver (*5*). If the mechanisms of ammonia production are actually as postulated in the Introduction, then it would appear that it is the primary condensation of the amides with pyruvate which is relatively weak in the tumor.

The failure of pyruvoylglycine to lead to increased ammonia production may be explained on the basis either of failure to condense with the amide or of actual condensation with the amide to form a tripeptide, in which the dehydroalanyl residue is in the middle of the chain and hence unreactive toward dehydropeptidase (*2*). Such small increases in ammonia production as noted in table 2 might have been due to a slight hydrolysis of pyruvoylglycine to pyruvic acid and glycine.

Implicit in the concepts advanced is the formation of a peptide bond at the γ carbon of glutamic acid and at the β carbon of aspartic acid. The former type of bond is present in glutathione. Condensation of glutamine in the fashion indicated with pyruvoylglycine would lead to a desthiolglutathione, and work on this possibility is in progress.

The increase in ammonia in the digests of glutamine with pyruvate in extracts of tissues stands in marked contrast with the disappearance of added ammonium salts in the presence of pyruvate and tissue slices. Thus, slices of bird liver form glutamine from ammonium salts and pyruvate (*9*), and urea is formed from ketoglutaric acid and ammonium salts in the presence of rat-liver slices (*10*). In connection with the last-mentioned experiments, it was noted that urea appears to be synthesized from glutamine and ammonium salts plus a source of carbon dioxide.

SUMMARY

Aqueous extracts of certain normal rat tissues exhibit a relatively weak activity in splitting the amide bond of glutamine and asparagine. When pyruvate is added, the desamidation is enormously increased without change in the final pyruvate concentration. This increase has been interpreted as being due essentially to two consecutive steps: (1) A condensation of the amide with pyruvate to form the dehydropeptide, followed by (2) the splitting of the dehydropeptide by dehydropeptidase in the tissues to form products which include pyruvate and ammonia. The increase in ammonia is accompanied by a corresponding decrease in amide nitrogen.

The dipeptide, pyruvoylglycine, does not exhibit this effect, and this fact has been interpreted on the basis either of its failure to condense with the amide or of its actual condensation with the amide to form a tripeptide in which the dehydroalanyl residue is in the middle of the chain and hence insusceptible to dehydropeptidase.

Extracts of the primary rat hepatoma do not show the pyruvate effect on amide desamidation to anywhere near the extent shown by extracts of normal liver, a phenomenon interpreted on the basis of the relative failure of the amide to condense with the keto acid in the former tissue.

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Monosodium Glutamate Studies. II.

Evaluation of a Possible Flavor Stabilizing Effect of Glutamate in Frozen Foods and of the Stability of Glutamate to Commercial Canning Process

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Use of glutamate in processed foods has raised questions concerning its stabilizing or antioxidant effect reported for frozen foods and its stability to processing conditions encountered in canned foods. It is generally assumed that glutamate is not altered in the canning process, but sufficient evidence supporting this view has been lacking. Critical analysis of conflicting published reports on whether monosodium glutamate has a flavor stabilizing effect on frozen foods has failed to resolve the question. Of three studies on frozen pork sausage, two *(7, 11)* report that glutamate has some antioxidant properties and another *(10)* indicates a possible prooxidant effect. The first study also reported an antioxidant effect on frozen poultry.

Poultry was selected for further study because of the authors' interest and previous studies on its stability under varying conditions. Cooked poultry products in addition to raw frozen poultry were included because of the growing importance of "convenience" foods, the lesser stability of cooked poultry products *(6)*, and the ease with which glutamate could be incorporated into them. Pork sausage was selected because of the conflicting reports from previous studies, because it was expected to develop off-flavors readily in frozen storage, and because glutamate could be incorporated uniformly.

One problem encountered in analysis of the published studies is that panel *preference* has not always been clearly distinguished from retardation of off-flavor development. This situation has occurred when samples with and without added glutamate are compared directly, and results showed an initial preference as well as a preference after storage for the samples to which glutamate has been added. Obviously, such results cannot be interpreted as an indication that glutamate has a stabilizing effect in frozen storage, since they may simply indicate that this panel preferred foods with glutamate added, in the presence as well as in the absence of a storage induced off-flavor. With tests of this type Norton *et al (11)* reported that poultry and other meats and fish "retained their original high quality for longer periods in freezing storage" when glutamate was used than when not used. The panel, consisting of 5 or 6 persons, apparently made a single comparison of samples with and without added glutamate at each test period. Comments indicating that rancid flavor developed in the samples without glutamate encouraged the opinion that glutamate has some antioxidant properties, but chemical tests to provide supporting evidence were not made.

In a study by Kemp *(7)* a panel of 8 to 14 members found no differences in palatability among pork sausage samples stored less than 4 months at 0° F with and without added seasonings and glutamate (0.1 and 0.3%). Samples stored 4 to 12 months with seasonings but no glutamate were consistently rated lower in palatability than those with neither seasonings nor glutamate, and those with both seasonings and glutamate. When judged for palatability, all samples had added seasonings (to three it was added before and to the other three it was added after storage). Some undesirable palatability change during storage of the seasoned samples stored without glutamate is indicated which either did not occur or was not detected in the seasoned samples stored with glutamate and in the unseasoned samples stored with no glutamate. No difference was found between samples containing the two levels of glutamate. No chemical tests were conducted. A darkening in color was reported in all seasoned samples (with or without glutamate) during storage.

Neill and Page *(10)* tested raw and cooked unseasoned sausage containing 0, 0.10, 0.15, and 0.30% glutamate for acceptability and peroxide development after storage at −10° F. Acceptability ratings showed an original preference for glutamate-containing samples that was unaffected by storage for 1 year. Significant peroxide development occurred only in the raw pork series after 18 months' storage. It was slightly higher in the glutamate treated samples than in the control. This was interpreted by the authors as indicating a slight prooxidant effect of glutamate.

Addition of glutamate is recommended for a large number of foods that are to be subjected to a canning process at 240–250° F. Military specifications include glutamate for the following canned products: soups, codfish patties, turkey loaf, tuna and noodles, hamburgers, and sausage patties *(3)*. It is generally assumed that this processing treatment does not materially influence the effect of the added glutamate, but the evidence is inconclusive. Galvin *(4)* reported that in canned soups the glutamate taste is not much

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affected by the heat process—the flavor of foods changes more than glutamate. Anson *(1)* reported that the flavor of glutamate itself is not greatly influenced by processing and storage. In analyses of several fruits and vegetables prior to and following canning, Mahdi *et al (8)* found that the heat process did not decrease the level of free natural glutamic acid. The possibility that added glutamate would cause flavor changes by increasing the rate of the browning reaction has been considered by Anson *(1)* and Melnick *(9)*. This phase of the problem was not considered in the present study, as the products studied had a considerably higher water content than the 10 to 15% at which the rate of browning is normally at a maximum *(1)*.

METHODS

Two types of panels were required for the sensory tests used because of the differences in objectives of these studies. For the test of effectiveness of glutamate as a flavor stabilizing agent (antioxidant) in frozen foods, the judges were selected for their ability to detect the stale and rancid off-flavors that may develop in frozen poultry and sausage products. For the test of the stability of glutamate to the canning process, the judges were those selected in a previously reported study for their ability to detect small flavor differences between products with and without added glutamate.

Sensory tests for effectiveness of glutamate as a flavor stabilizing agent in frozen foods. These sensory tests were based on the extent to which the panel could detect off-flavors in foods stored at 10° or 20° F. in the presence and in the absence of glutamate. No direct comparison of samples with and without glutamate was made; thus questions of preference or acceptability were eliminated.

The general plan for testing was a comparison between samples stored at 10° or 20° F and controls held at −30° F after various storage periods. Samples without glutamate held at elevated temperatures and at −30° F were compared directly, as were samples with glutamate held at elevated temperatures and at −30° F. Judges were asked to indicate which of each pair of coded samples had more off-flavor. They were also asked to comment on the type of off-flavor (staleness or rancidity) as an aid in interpreting the importance of the flavor change.

Extent of detection of off-flavors in the foods without glutamate held at elevated temperatures was compared with the extent of detection of off-flavors in foods with glutamate to determine whether off-flavors developed more rapidly or were detected more readily in the products that did not contain glutamate. If off-flavors were distinguished more readily in the products with no glutamate than in those with glutamate, two conclusions would have been possible. Either glutamate prevented the chemical changes responsible for off-flavor development or it masked changes which occurred. If off-flavors were distinguished to approximately the same extent, it could be concluded that glutamate did not show a stabilizing effect on flavor under the conditions of this experiment.

The panel of judges evaluated each set of samples 4 or 5 times at each storage period. Each comparison is thus based on at least 32 judgments. Samples compared were from the same production lots. The order of presenting pairs containing no glutamate and pairs containing glutamate was randomized at each storage period.

Sensory tests for stability of glutamate to the canning process. For the test of stability of glutamate to the canning process, samples of the broths to which glutamate was added before and after processing were evaluated by the panel selected for its ability to detect small differences in flavor, using the duo-trio test described in the previous paper *(5)*. The control sample was the sample with glutamate added before processing. Coded samples containing glutamate added before and after processing were presented with the control. The panel was asked to indicate which of the two coded samples was like the control. Samples were randomized and tests were replicated as in the previous tests.

Sample preparation for frozen food tests. *Fried Chicken.* Sixty chickens weighing approximately 2 lb (ready-to-cook) were cut into the following pieces: wing, breast, thigh (including part of backbone), and drumstick. Pieces were rolled in a dredge consisting of flour or a mixture of flour and an amount of glutamate needed to yield a concentration of 0.15% or 0.35% glutamate in the uncooked product (chicken, dredge, and batter). The amount of glutamate needed was based on preliminary experiments to determine the average pickup of dredge and batter by chicken parts of this weight. The dredged chicken parts were dipped in a batter of the following composition:

Ingredient	Per cent
Flour	28
Shortening	2.5
Milk	54.6
Salt	1.4
Eggs	13.5

The chicken was transferred rapidly to a deep fat fryer containing peanut oil and fried for 10 min at 145° C (293° F), keeping chicken below the fat level. One half of each chicken was stored at 10° F in a polyethylene bag placed in a carton, and the other half was stored at −30° F in a N_2 atmosphere.

In preliminary experiments, in which glutamate was added to the batter, birds with glutamate showed more browning during cooking than those to which glutamate was not added. Therefore, glutamate was added with the dredge, since increased browning did not occur when the glutamate was applied to the layer beneath the crust.

Turkey. Sixty small Beltsville turkeys were eviscerated at the laboratory and injected while warm with solutions containing gelatin and glutamate as recommended by Ellis *(2)*. They were divided into 4 groups of 15 each. One group received no injection, one a 2.5% gelatin solution, two a solution containing 2.5% gelatin and glutamate. The solutions were injected through a hypodermic needle connected to a pipetting machine, 5 ml per injection, with enough injections (25–30) to give approximate concentrations of 0.15 and 0.35% glutamate in the last two groups, distributed as evenly as possible in the breast, thigh, and leg. Birds were chilled in slush ice in polyethylene bags, frozen, sawed in half, and repackaged for storage. Half of each was wrapped in cellophane for storage at 10° F; the other half was packed in Cry-o-vac and held at −30° F as a control.

Turkey a la king was prepared according to the method described in the previous paper *(5)*. The product was divided into three parts, and glutamate added to two of these to give concentrations of 0.15 and 0.35%. Experimental samples were stored in MSAT cellophane bags in cartons at 10° F and control samples at −30° F in sealed cans in a N_2 atmosphere.

Pork sausage was prepared according to the method described in the previous paper. The pork trimmings were divided into 3 parts. NaCl (1%), the seasonings, and glutamate (for the 2 parts containing 0.15 and 0.35% glutamate) were added before grinding and mixing. For lot 1, sausage was divided into ½-lb portions, packaged in cellophane or canned as required, and frozen at −30° F. The packaged samples were stored at 10° F. This sausage contained 60% fat.

A second lot of sausage was prepared by the above method. Experimental samples were packed loosely in cans with air in the headspace approximately equal to the volume of the sausage, hermetically sealed, and stored at 20° F. Control samples were canned, N_2 packed, and stored at −30° F. This lot of sausage contained 50% fat.

Sample preparation for canning tests. *Chicken broth, cream of chicken soup,* and *green snap beans* were heat processed with and without added glutamate. The broth and cream of chicken soup were prepared as described previously with two levels of glutamate, 0.10 and 0.25%. The NaCl level in the broth was 0.75% and in the cream of chicken soup 1.125%. They were processed in No. 303 glass jars, 400 g per jar, at 240° F for 25 min. A second lot of the cream of chicken soup was subse-

quently prepared with a lower NaCl content (0.75%) to guard against the possibility that the relatively high salt level in the first batch might have masked any slight flavor differences. The second batch was processed in 403 x 612 cans, approximately 1,170 g per can at 240° F for 50 min. The green beans were purchased fresh from a local supermarket. After washing, ends were removed and beans were cut into 2 inch pieces, blanched at 180° F for 1½ min, cooled, packed in 209 x 313 cans to which brine containing NaCl and glutamate was added as required. They were processed at 240° F for 20 min.

Chemical tests. Tests for peroxide development were made on the frozen foods at each temperature at intervals of 2 weeks or more to indicate whether chemical changes occurred in either the meat and skin (with coating) of the fried chicken, in cooked turkey meat and turkey a la king, and in raw and cooked pork sausage. The method used has been described by Pool *et al (12)*.

Glutamate determinations on the broth and cream of chicken soup were made on samples to which glutamate was added after canning as well as before canning and on the bean samples to which glutamate was added before canning by the enzymatic decarboxylation method described in Part I of this paper *(5)*.

RESULTS

Possible flavor stabilizing effects of glutamate in frozen foods. Monosodium glutamate, added in concentrations of 0.15 and 0.35% of the product weight had no beneficial effect in preventing or masking off flavor development in frozen fried chicken stored at 10° F for 4 months in turkey a la king or raw turkey stored at 10° F for 9 months, or in pork sausage stored at 10 and 20° F for 4 months. Flavor differences between controls held at −30° F and samples held at 10 or 20° F were detected approximately equally in samples with and in samples without glutamate (Table 1). Flavor testing was discontinued after changes were consistently recognized by the judges. Comments of the judges indicated that staleness developed to some extent in all of the products at the elevated temperatures and that rancidity developed in the fried chicken at 10° F and pork sausage at 20° F (Table 2). Comments for samples containing the two levels of glutamate are reported together since they were very similar. Also, comments for all storage periods have been combined for the sake of simplicity although there were a few more adverse comments on samples stored two months or longer than those stored less than 2 months. Since a rancid type of off-flavor is considered a more serious defect than staleness, an illustration of the approximately equal distribution of comments among the 3 glutamate levels (0, 0.15, and 0.35%), for fried chicken, the product that received the most rancidity comments, has been plotted (Figure 1).

Peroxide tests were continued after flavor tests had been stopped to see whether any beneficial effect of glutamate developed with longer storage. The addition of monosodium glutamate had no effect on peroxide developments in these products at 10° F for

TABLE 1

Effect of glutamate on off-flavor development in stored frozen foods

Product and storage temperature	Storage time	Per cent judgments indicating more off-flavor in samples stored at 10° or 20° F than in controls at −30° F[1]		
		Added glutamate		
		0	0.15%	0.35%
	Mo			
Fried Chicken 10° F	1	66	69	78
	2	87	81	87
	4	97	84	87
Turkey a la king 10° F	3	63	81	63
	6	66	75	69
	9	100	78	84
Turkey 10° F	3[2]	56		
	3[3]	66	53	72
	6[2]	66		
	6[3]	69	84	56
	9[2]	58		
	9[3]	75	75	86
Pork Sausage 10° F	0.5	47	72	63
	1	75	59	63
	2	81	78	72
	4	53	59	69
Pork Sausage 20° F	1	69	31	81
	2	72	72	63
	4	78	66	75

[1] Total number of judgments, paired comparison test = 32 (all tests except turkey, 9 mo.). Per cent required for statistical significance is 69, 75, and 81 for the 5%, 1%, and 0.1% levels of probability, respectively.

Total number of judgments, turkey, 9 months only = 36. Per cent required for statistical significance is 67, 72, and 78 for the 5%, 1%, and 0.1% levels of probability, respectively.

[2] No injection of birds before freezing.

[3] Gelatin injected with or without glutamate as indicated.

TABLE 2

Adverse comments on frozen foods stored with and without glutamate addition

Product	Per cent adverse comments by panel[1]			
	−30° F controls		Stored samples	
	Glutamate		Glutamate	
	−	+	−	+
Staleness				
Fried chicken +10° F	3	4	36	25
Turkey +10° F	8	4	12	13
Pork Sausage +10° F	3	4	9	14
Pork Sausage +20° F	6	5	10	11
Rancidity				
Fried chicken +10° F	3	4	19	21
Turkey +10° F	0	0	2	2
Pork Sausage +10° F	1	0	2	3
Pork Sausage +20° F	4	4	7	13

[1] Total tastings for each sample ranged from 96 to 128 for the unsupplemented samples and from 192 to 256 for the glutamate samples.

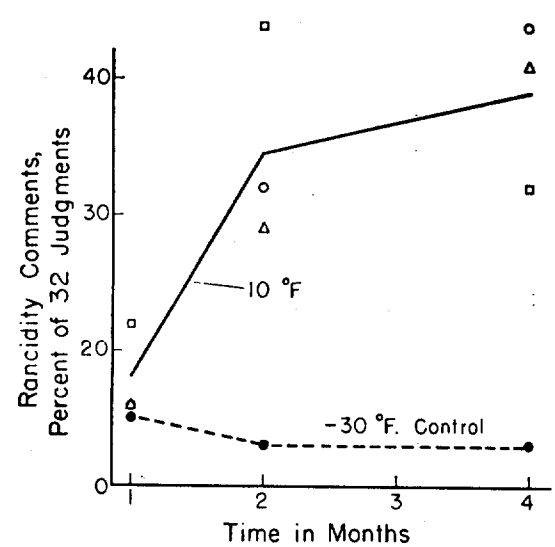

Figure 1. Similarity of rancid flavor development in frozen fried chicken with and without added monosodium glutamate. Triangles, circles, and squares represent no glutamate, 0.15%, and 0.35% glutamate, respectively.

the following storage periods: fried chicken, 1 year; turkey a la king, 15 months; raw turkey, 9 months; pork sausage, 9 months; and in pork sausage at 20° F for 6 months. The peroxide values for all products were relatively low even after the longest storage periods. Increases in peroxide values of the above products during the indicated storage times were as follows: fried chicken, 6 to 11; turkey a la king, 8 to 12; raw turkey, 5 to 7; pork sausage at 10° F, 3 to 10; pork sausage at 20° F, 2 to 6, expressed in meq/kg. Values for pork sausage stored at 20° F include glutamate levels of 1 and 2% in addition to the 0, 0.15, and 0.35% levels used in the other products. Peroxide values are shown separately for the three glutamate levels used in fried chicken (Figure 2) to illustrate the similarity among values found in all products.

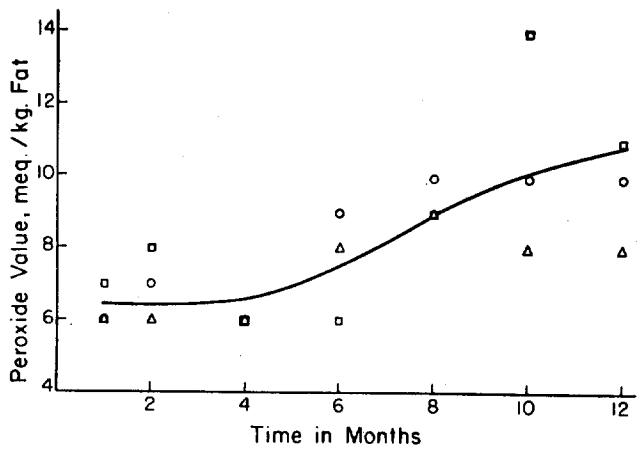

Figure 2. Similarity of increase in peroxide values in frozen fried chicken at 10° F, with and without added monosodium glutamate. Triangles, circles, and squares represent no glutamate, 0.15% glutamate, and 0.35% glutamate, respectively.

No difference was observed in this study in sausage color related to glutamate level, contrary to a previous report *(11)*.

Stability of glutamate to heat processing. Glutamate determinations by the enzymatic decarboxylation method described in the previous paper *(7)* showed no loss in added glutamate in chicken broth, cream of chicken soup, or green beans due to commercial canning conditions (Table 3).

TABLE 3

Effect of commercial canning conditions on glutamate retention

Product	Added glutamate	Monosodium glutamate found[1]		
		Glutamate added		
		Before processing		After processing
	%	%		%
Chicken Broth	0.10	0.126		0.123
	0.25	0.272		0.269
Cream of Chicken Soup	None	0.020		
	0.10	0.116		0.122
	0.25	0.275		0.263
		Beans	Brine	
Green Beans	None	0.009	0.005	.....
	0.15	0.117	0.131	.....
	0.30	0.266	0.296	.....

[1] Values are averages of three or more determinations per can on 2 to 6 cans per product.

TABLE 4

Effect of adding glutamate before or after canning on flavor of chicken broth and cream of chicken soup

Product	Concentration of added glutamate	Number of judgments[2]	Judgments identifying sample with glutamate added before canning[1]
	%		%
Chicken broth	0.10	24	25
	0.25	21	52
Chicken Soup (cream of)	0.10	126	44
	0.25	117	49

[1] Duo-trio test judges were asked to identify sample in each pair which was identical to control sample to which glutamate had been added before canning.

[2] Results are combined for the two processing runs made on cream of chicken soup.

Evaluation of chicken broth and cream of chicken soup showed that the panel of judges selected to detect small flavor differences was unable to identify the samples of these products to which glutamate was added before processing when they were compared directly with identical control samples and samples to which glutamate was added after processing (Table 4).

SUMMARY OF RESULTS AND CONCLUSIONS

Monosodium glutamate at 0.15 or 0.35% did not prevent or delay off-flavor development at 10° F in fried chicken held 4 months; turkey a la king, 9 months; raw turkey, 9 months; or pork sausage, 4 months; or in pork sausage held 4 months at 20° F. Nor did it retard peroxide development at 10° F in fried chicken held 1 year; turkey a la king, 15 months; uncooked turkey, 9 months; or pork sausage, 9 months; or pork sausage held 6 months at 20° F. The presence of glutamate neither prevented peroxide development nor did it mask the flavor changes that occurred.

No loss in glutamate content was found in chicken broth, cream of chicken soup, or green beans subjected to commercial canning conditions. No flavor differences were detected between two samples of chicken broth or cream of chicken soup prepared by adding equal amounts of glutamate before and after canning, respectively.

Since glutamate is stable to heat processing and since it does not affect the usual processing changes in the product as judged from this limited study, addition prior to canning, as is generally recommended, is indicated to assure uniform distribution, especially in heterogeneous products.

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INTERNATIONAL MINERALS & CHEMICAL CORPORATION

Partial Translation of

a Paper Entitled

"Observations of Pharmacological Actions and Toxicity of Sodium Glutamate, with Comparisons between Natural and Synthetic Products"

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II. Materials and Methods

The experimental sample, MSG was provided by Ajinomoto Company. Natural L-sodium glutamate was designated as NL, synthetic L-sodium glutamate as SL, and synthetic D-sodium glutamate as SD. The chemical formula is shown in Figure 1. The concentrations of solutions tested were 0.2%, 2% and 20%. Figure 1 shows the chemical formula of MSG. The experimental animals used were goldfish, frog, Wister male rat, rabbit, cat and dog. Plant parts were used for study of the effect on plants, and for an experiment in taste, the human tongue was used. All other experimental methods will be described in Experimental Results Section.

III. Experimental Results

A. Studies on the toxicity of various MSG. For toxicity studies by forced feeding of test material in rats, it is very important to observe the details of general phenomena in addition to the growth curve for the materials which are expected to give a very small amount of toxicity. In order to obtain a most suitable growth curve it is desirable to use immature and healthy animals. Therefore, we used the 120 g Wister male rat. Many times the required number of these animals were grown for two weeks under control conditions and better or healthier animals were selected for this experiment. The feeding was done by a type of constant temperature feeding box, at temperature of 22°C±1°. Five animals per cage per group and 10 groups were used. This is shown in Drawing 1. The feed, Oriental NMF, was used (Table 2).

Three drops of vitamin B complex was added to drinking water. Water was changed daily. Fresh vegetables were given weekly.

For testing material NL, SL and SD at concentrations of 0.2%, 2% and 20% solution were divided into three and given to rats at 1 cc per 100 g per day for 90 days at a set time in day according to the Zonday(?) method of forced feeding. In addition, for control experiments 20 mg of sodium chloride per Kg was force fed to rats. This amount represents the corresponding equivalent amount of MSG per 50 Kg body weight(?).

Body weights were measured daily at a certain time using an automatic balance with 0.5 g sensitivity. At the same time the feed consumption and water consumption were both recorded. In addition the motion of the animal, the appearance, the hair growth, nose breathing or not, change in appearance of eyes and other general phenomena were also made and recorded. Moreover during forced feeding, if the compounds were pushed into the lung by mistake these animals were removed from the experiment. After 90 days of forced feeding the rats were sacrificed immediately by bleeding and the weight and volume of intestinal organs were measured and compared with the control. The various intestinal organs were preserved in formalin solution and pathological studies were made.

1. The growth experiments of rat by forced feeding of various MSG.

   1) Control experiment. Average body weight 190 g prior to start of experiment. Experimental animals after

90 days weighed 336.2 g and showed good daily body weight gains. This growth curve was used for the standard and the general conditions as well as water and feed consumption. Condition of feces and urine was all recorded in detail, and compared with other experimental animals.

2) The forced feeding experiment with NL.

i) With the basic amount of NL at 20 mg/Kg, the body weight of rats increased after forced feeding. Average body weight at the start was 115 g; after 90 days it had increased to 319.6 g. No abnormal appearance of general conditions were observed during this period and no differences were observed when compared to the control (Table 4 a,b and Table 13).

ii) With 10 times amount of the basic NL (200 mg/Kg) the body weight after forced feeding still continued to increase and increased smoothly. That is, the body weight curve increased linearly and was not different from controls. The average body weight at the beginning, 160.2 g, reached 336.6 g after 90 days. (Table 5 a,b and Table 13). The water and feed were all normal and the hair growth appeared normal.

iii) With 100 times that of NL basic amount (2000 mg/Kg) the body weight of the rats increased from 156.8 g to 302.6 g after 90 days. During that period no abnormal symptoms were seen, the appetite of the animals was

the same as controls, the appearance of the hair was normal. No differences were observed in the eyes, nose. However, after 5 days feed in some cases a sudden increase in body weight was seen and in other cases slight suppression in the rat of body increases were shown after 60 days. However, from average values of growth curves no meaningful differences were observed (Table 6 a,b and Table 13).

3) SL Feeding

i) With the basic amount of SL (20 mg/Kg) the body gain increases were normal from an average of five animals of 131.8 g the body weight reached 306.2 g after 90 days. No differences can be made when compared with control (Table 7 a,b and Table 13). During this period a temporary decrease in body weight for 5 rats was observed but this was found to be due to the leaking in the water supply. General conditions of the animal, behavior, hair growth, eye appearance and skin condition were normal.

ii) With 10 times that of basic SL (200 mg/Kg), the body weight of the rats increased daily. The average weight of the rat at the beginning was 155.2 g and increased to 334 g after 90 days. During this time, general conditions were excellent and appeared to be superior to that of the basic amount of SL (20 mg/Kg). No differences were observed when compared with control (Table 8 a,b and Table 13).

iii) With 100 fold that of basic SL (2000 mg/Kg) feeding the body weight of the rats increased daily from 158.4 g average to 288.4 g after 90 days. The general conditions of the rats were good during this time. No abnormality was observed with regard to eyes, nose, or any sign of sickness. However, after 13 days, a slight and temporary loss in body weight was observed due to the water loss. This was however recovered after 15-20 days and normal growth rate was observed until 90 days (Table 9 a,b and Table 13).

4) SD force feeding experiment.

i) At 20 mg/Kg feeding the body weight increased from 126.8 g to 298.3 g in 90 days. During 60 days some weight loss was observed and some animals showed reduced growth development but no abnormality (any symptom) was shown when compared with the control experiment. No meaningful differences were recognized. (Table 10 a,b and Table 13).

ii) Feeding at 200 mg/Kg, the body weight increased from 145 g to 305.4 g in 90 days. No abnormalities were found and hair growth conditions, activities and appetites were normal. No symptoms were observed (Table 11 a,b and Table 13).

iii) Feeding at 2000 mg/Kg the body weight increased from 165 g to 287.8 g after 90 days. During this period the activity and the hair growth were normal. No change in eye appearance and no nose breathing

phenomena were seen. No abnormal symptoms were observed in skin. Some reduction in body weights were seen when compared to that of control group. However, a meaningful difference cannot be obtained when compared with the control group (Table 12 a,b and Table 13).

2. Studies on the effect of feeding on the weight and volume of intestinal organs in rats. After 90 days continuous feeding all the rats were sacrificed by bleeding. Their organs, that is brains, heart, lung, stomach, kidney, liver and spleen (were removed) and weight and volume of various organs were measured and recorded. The results were shown in Table 14 a,b,c. No differences were seen between control group and experimental group. Also, no differences were seen between the experimental groups.

3. Pathological studies. After the organs were preserved in formalin solution, the pathological studies of the organs were performed by hematoxylin-eosin staining. The results show that in stomach the complete and normal gland structure was seen and the upper epidermic tissue cells and tissue structure were normal. Similarly, the tissues and gland structure of the small and large intestine were normal and no swelling or abnormality were seen. In the intestinal structure (the layer of viscous(mucous) membrane) no increase in free cells were recognized in the tissues under the mucous, and no swelling or blood clotting symptom were recognized. In liver, no abnormal fatty liver tissue or other abnormality

was seen. The perfect normal liver structure was maintained. In spleen, no abnormal cell number or blood clotting or tissue fattening phenomenon were observed. In kidney, no abnormality was observed in tissues or cells. Others such as the brain tissue, heart and lung were also studied but no abnormalities were seen.

4. Small Conclusion (?)

B. Studies on the general pharmacological reactions.

1. Toxicity against various animals by force feeding of large amount of samples: One to 10 g per Kg were force fed to dogs and cats. No abnormal symptoms appeared. Vomiting occurred when large amounts were fed to dog. However, when large amounts of sample were mixed with small amounts of rice and vegetable or Miso and cooked, no vomiting was shown. Feeding of 0.5 to 1 g or higher per Kg to rabbits for two weeks by intravenous injection showed no abnormal effect.

2. Effect on plan seed germination: The seed of the "plenm partens"(?) were studied at room temperature for 8 days. These experiments were based pn the Hara method by using 40 to 520 times concentrated(?). The results are shown in Figures 15-17 (Tables). At lower concentration, stimulation on the germination and development were seen by NL, SL and SD forms. However, differences between the effects by NL, SL and SD were not seen.

3. Effect on small fish: Three fishes per group of 5 centimeters length were used in solutions containing 0.01% to 10% of NL, SL and SD. Goldfish were placed in the solutions. General phenomena were observed during 24 hours. Table 18 shows the result of this experiment. The fish in the NL solution increased their mobility at the concentration of 0.05%. The fish mobility slightly increased in SL and SD solution at 0.01%. At 0.1 to 3% the motion stopped after the increased activity. At the concentration above 4%, spasm-like motions were observed immediately and (the fish) floated to the top and stopped moving after a while. After 10 hours those fish are dead. Therefore this kind of phenomena increased when the concentration increased and the time required for this type of phenomena decreased on increased concentration. Moreover, the SD form showed greater effect when compared with NL and SL forms.

4. Studies on the taste by human tongue: The NL and SL both have taste - no difference. SD does not have taste or flavor.

5. Studies of effect on isolated frog heart: 0.1% to 1% MSG solution were perfused into the isolated frog heart. No reactions were shown with NL, SL and SD. At 10% an apparent reduction in the pulse and temporary pulse stoppage was observed. This probably is due to the over-concentration of MSG which caused some physical damage.

6. Effect on respiration and blood pressure in rabbit: Urethane-treated rabbits were used for respiration and blood pressure studies. Intravenous injection at 1 mg/Kg showed no effect with NL, SL and SD. At 10 mg/Kg with SD, the respiration slightly increased. At 100 mg/Kg, NL, SL and SD all show some slight increase in respiration rate, and increase in blood pressure. At 500 mg/Kg increase in respiration and blood pressure were observed but respiration did not stop at 800 mg/Kg.

7. Effect on smooth muscle: Isolated rabbit intesting was used. The Magnus method was used. No effect was seen at 0.01%. At 0.05% only SD showed some decrease in tension. At 5%, NL, SL and SD all showed slight temporary decreases in tension and decreases in pulse.

8. Small Conclusion.

C. Effect on the brain nerve pulse. (EEG?) Cat of 2.5 to 3 Kg body and Wister male rats of about 300 g weight were used. (See Methodology)

1. Study in cat
2. Cats were used for neurological tests (experimental detail is omitted).
3. Neuropotential study by injection of MSG solution into skull (Detail omitted).
4. Conclusions.

D. Effect on metabolism. Effect on blood sugar metabolism and blood electrolytes, sodium, potassium, calcium, chloride and protein metabolism, total blood protein, protein fraction

changes, and lipid metabolism (blood cholesterol) were measured. The experimental animal was the mature male rabbit weighing 2.5 Kg. We have already described the general pharmacology reactions at 100 mg and 500 mg/Kg. Injection of MSG was intravenous into urethane treated rabbits. Respiration and blood pressure raises and these amounts are the minimum required to induce this reaction(?). Therefore at these doses, or dosed continuously for 10 days, injection was made and the metabolic changes were studied(?).

1. Blood sugar studies. Sugar was measured by the Somogyi method. SD, after intravenous injection of 100 mg/Kg, a slight increase in blood sugar was seen and this started to decrease after 45 minutes. With SL, after 60 minutes from injection, the blood sugar increased slightly and showed no difference from that of NL. Both SL, NL and SD showed slightly decreased blood sugar after 120 minutes, when 500 mg/Kg were injected. Similar phenomena of sugar decrease was observed with SD after 50 minutes. With SL, the blood sugar increased and reached a maximum at 60 minutes but showed a similar effect to that of 100 mg/Kg. With the NL solution, similar results are obtained.
2. Blood electrolyte studies. Fluorometric analysis was used for sodium, potassium, calcium. The Sharles and Sharles method was used for chloride determination. The change in electrolyte concentration was obtained by measurement of electrolytes before and after the injection.

In the case of 100 mg/Kg dosage, injection of SD showed an increase of sodium and chloride, calcium decreased after 15 minutes but sodium increased after 30 minutes. Potassium decreased at 15 and 30 minutes but after 120 minutes recovered to normal. Both calcium and chloride recovered to normal value after 120 minutes but sodium increased at the rate of 5% for 240 minutes. With SL sample, the tendency of increase of sodium and chloride was slightly less than SD, calcium and potassium changes were similar to that of SD sample (Figure 10). With 500 mg/Kg sodium, calcium and chloride increased immediately after injection with SD. The increase of sodium became more pronounced at 120 minutes which gives the increased rate of 10%. The decrease in potassium became more profound than that of 100 mg/Kg and the rate of decrease reached about 13% after 120 minutes and apparently had an opposite effect to that of sodium. The rate of calcium increase was next to sodium. After 240 minutes a 5% increase still appeared. The change in chloride was very small and recovered to normal after 120 and 240 minutes. The effect of SL is similar to that of SD, in that sodium, calcium and chloride increased but potassium decreased.

3. Blood cholesterol. Blood cholesterol was determined by Zak's method. In the case of a single intravenous injection of 100 mg/Kg there was an immediate decrease of blood cholesterol, with NL, SL and SD. The decrease of cholesterol was more profound with SD and reached 18% after 30 minutes, and continued until 240 minutes. The effects of SL and NL are similar

but a 10% decrease was maintained for 240 minutes. At 500 mg/Kg dosage, SD and SL both caused a rapid decrease of cholesterol, at the rate of 20% at 15 minutes, but temporarily showed some increase in cholesterol at 60 minutes but after that showed a decrease again. With NL, the decrease of cholesterol was not rapid. The rate of decrease however reached maximum at 60 minutes. After that slight tendency of increase in cholesterol was shown. All three MSG samples showed decrease of cholesterol up to 240 minutes.

4. Blood protein. 500 mg/Kg was continuously injected for 10 days. The blood protein fractions were studied.

1. Total blood protein. The protein content before injection was 6.25 to 6.6 g/100 ml. Injection of NL, SL and SD did not effect the total protein level. Sometimes however, there was a slight increase.

2. Blood protein fractions. Paper electrophoresis was used for protein fractionation. Decrease in albumin fraction was shown with NL, SL and SD and at the eleventh day, 0.43 to 0.60 g/100 ml decrease was shown. The alpha-globulin fraction tended to increase with NL and SD. SD showed a slightly higher increase rate but no change was seen with SL. Little change in beta-globulin fraction was noticed with NL, SL and SD. Increase in gamma-globulin fraction was seen with NL, SL and SD and reached 0.2 to 0.3 g/100 ml at the end of feeding. The increase was highest with SD. Therefore the common effect of these forms of MSG would suppress the albumin formation and

tend to increase the alpha-globulin and gamma-globulin and have no effect on beta-globulin formation. Therefore, a decrease in albumin and increase in globulin resulted in reversed value of A/G ratio.

5. Effect of continuous intravenous injection on the body weight. The body weight and general condition after injection with 5 mg/Kg of NL, SL and SD showed very little difference in the increase of body weight among the three groups. After 11 days injection, a 5 to 7% increase in body weight was seen and no evidence was shown that injection of relatively large amounts of MSG decreased body weight.

Conclusions and Summary omitted.

Relations between Plasma Glutamic Acid Level and Liver GOT, GPT Activities in Rats and Mice in Respect to their Ages.

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Liver GOT (Glutamic Aspartic Transaminase) and GPT (Glutamic Alanine Transaminase) activities were examined and the corelation between the activities and the plasma glutamic acid level after MSG oral administration (1g/kg b.w.) was examined.

Experimental Procedure

1) Assay of enzyme activity

Rats of Wistar-Imamichi strain and mice of 4CS strain were used throughout. Animals were killed by decapitation, and their livers were removed and frozen immediately. The frozen livers were minced and homogenized in a Waring homogenizer with 10 volumes of ice-cold 0.01 M phosphate buffer (pH 7.6). In a 10ml glass centrifuge tube were placed 0.2ml of the homogenate, 0.5ml of the 0.1M amino acid solution, and 1.5ml of the phosphate buffer. After the mixture was kept at 38°C for 3 to 5 minutes, 0.5ml of the 0.1 M α-ketoglutaric acid, previously neutralized to pH 7.6 and kept at 38°C, was added to the mixture.

The tubes were shaken, and incubation was continued for 60 minutes.[1] At the end of the incubation period, 1 drop of trichloroacetate solution (100g/dl) was added to each tube to stop the enzymatic reaction. The tubes were centrifuged and the clear supernatant was employed for pyruvic and oxaloacetic acids determination.

Determination of pyruvic acid and oxalacetic acid[2)3)].

The amount of pyruvic and oxaloacetic acids formed, necessary for the determination of GOT and GPT activities, were measured by the following procedure.

0.5ml of 2,4-dinitrophenylhydrazine was added to each supernatant, and the mixture was standed on for 5 minutes. Then, 2ml of toluene was added, and centrifuged for 5 minutes. Aliquots of 1ml of toluene layer was shaken with 3ml of 2.5% (w/v) potassium hydroxide solution in 95% (v/v) ethanol. After 15 minutes the color intensity of 2,4-dinitrophenylhydrazone of pyruvic acid was measured at 500mμ by photometer.

Determination of oxaloacetic acid was carried out after oxaloacetic acid was changed into pyruvic acid by citrate aniline solution (50%, w/v with respect to citrate and 50%, v/v with respect to aniline).

Specific activity of GPT and GOT were expressed as μ mole pyruvic acid and oxaloacetic acid, formed per 1 hour per 1g of fresh liver (38°C).

2) Determination of plasma glutamic acid in plasma

Determination of glutamic acid in plasma after MSG oral administration (1g/kg) was carried out in the same manner as described in Appendix B, but non-fasting animals were used throughout the experiments.

2. Results

Experimental results are shown in the attached figures.

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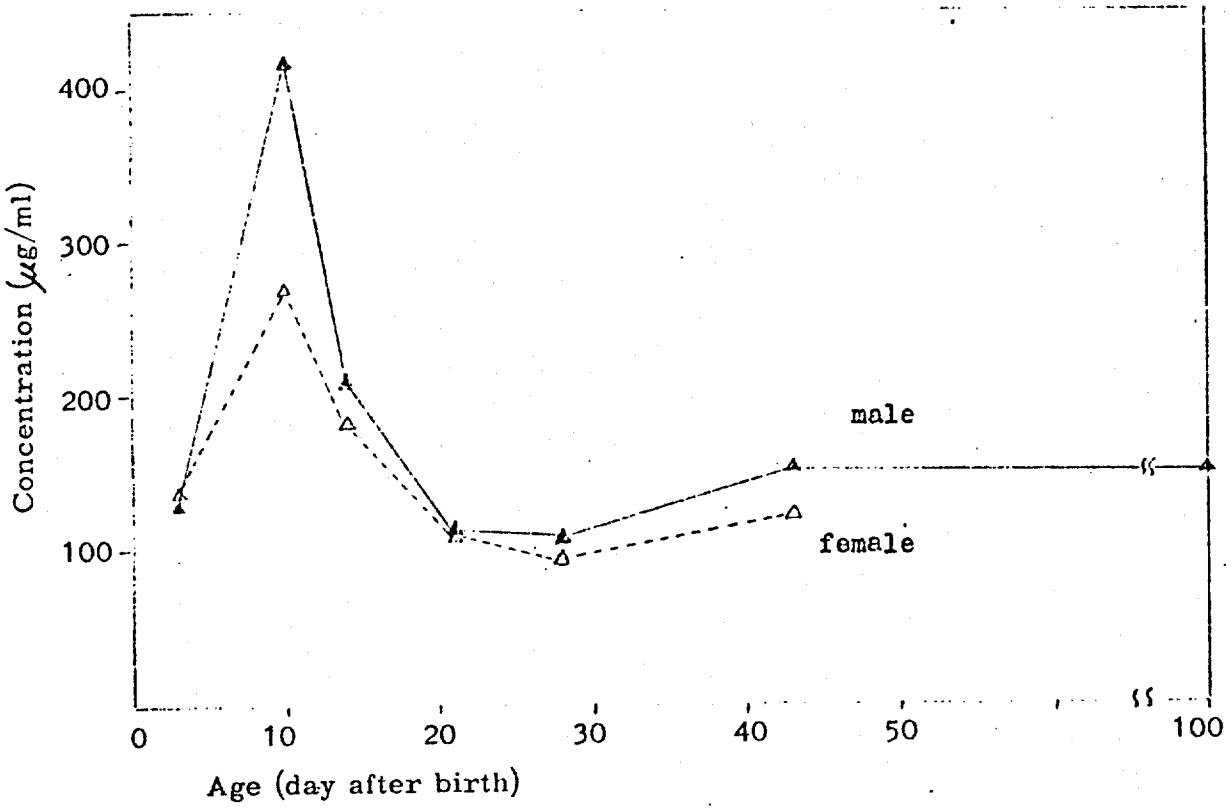
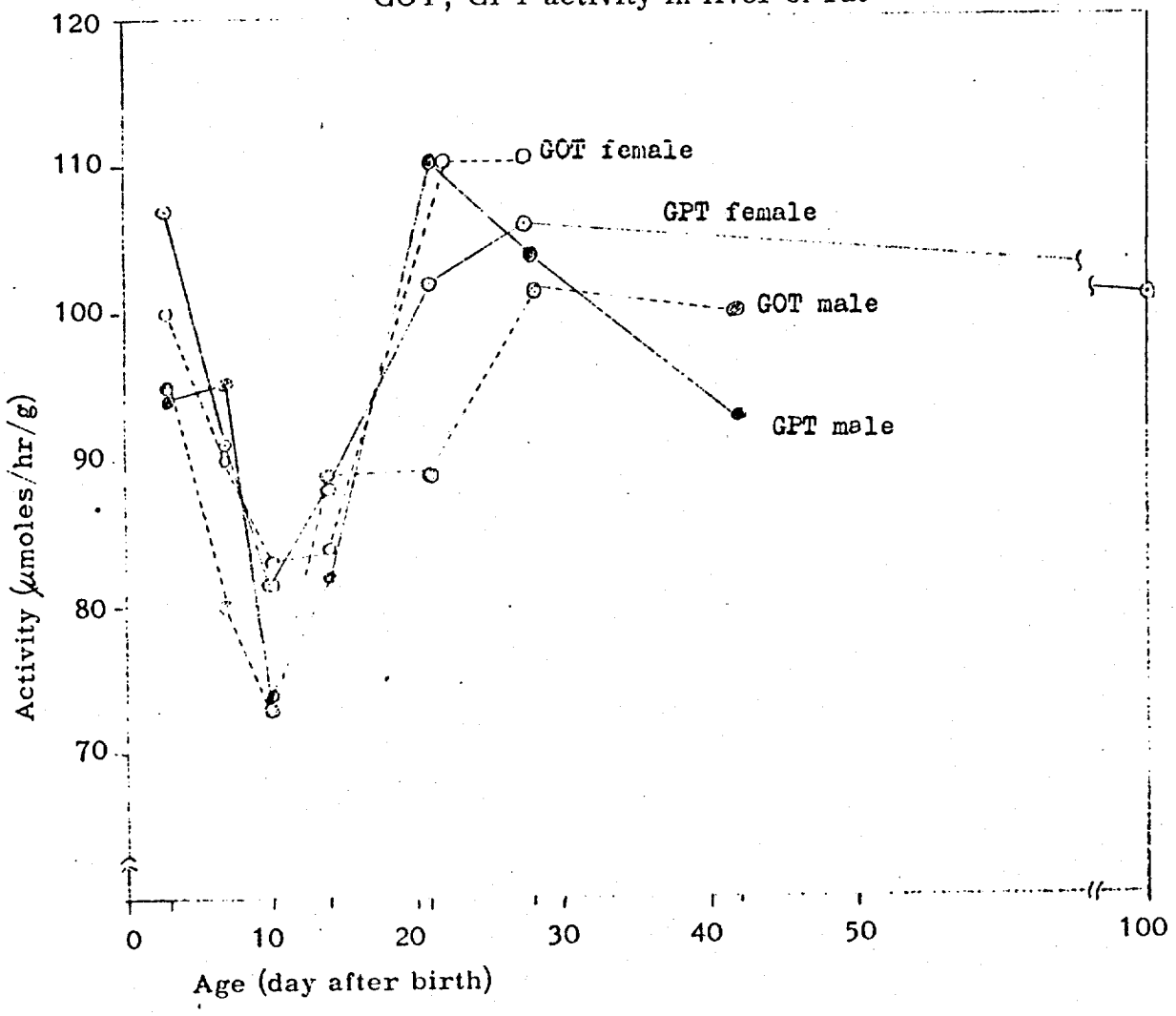
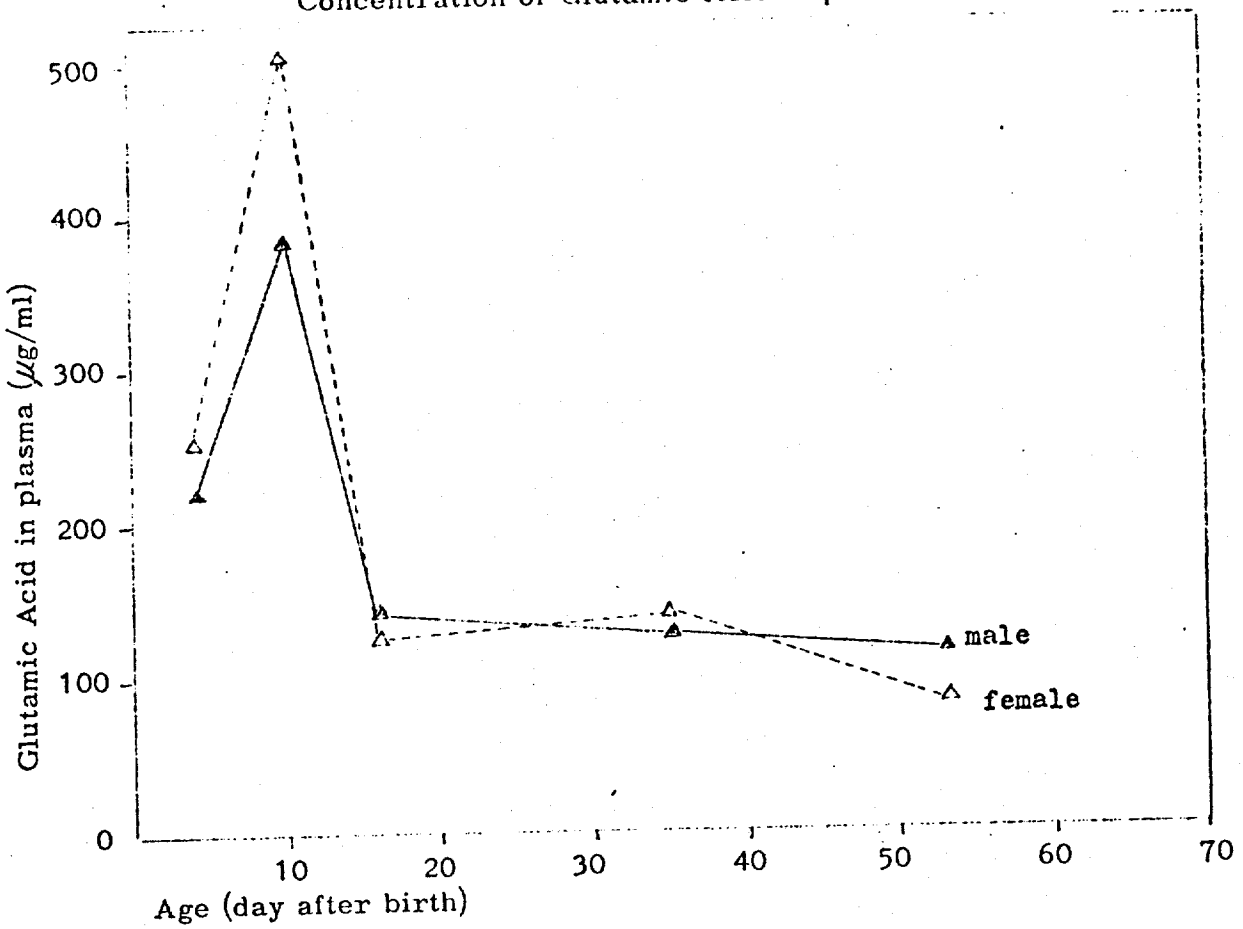
Concentration of Glutamic Acid in plasma of rat



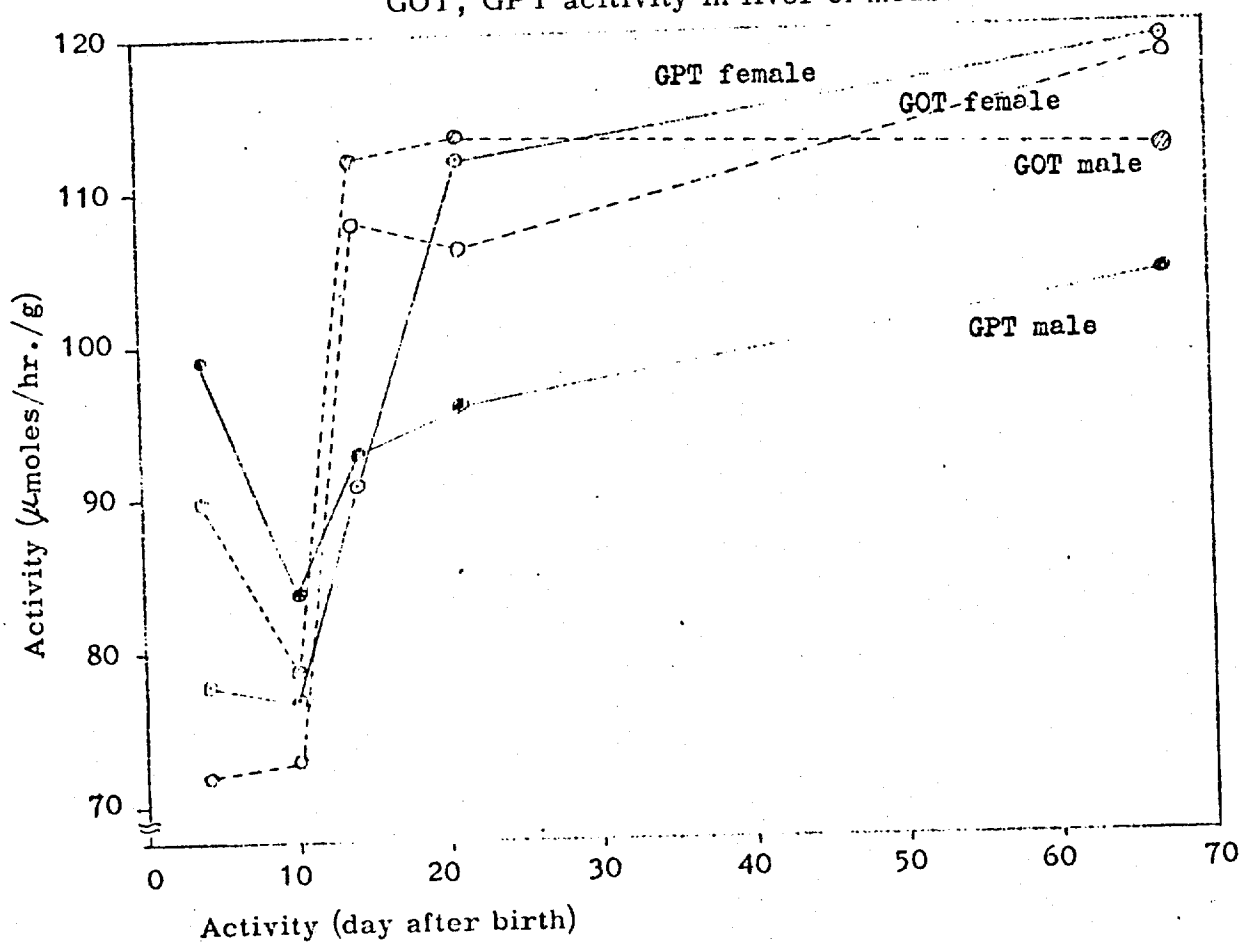
GOT, GPT activity in liver of rat



Concentration of Glutamic Acid in plasma of mouse



GOT, GPT acitivity in liver of mouse



SUPPLEMENTAL PATHOLOGY REPORT

ADDENDUM TO FINAL REPORT DATED NOVEMBER 3, 1966
(REPRODUCTION STUDY - RABBITS)

PROJECT NO. 466-117

Submitted to

International Minerals & Chemical Corporation
Skokie, Illinois

HAZLETON LABORATORIES, INC.

A SUBSIDIARY OF TRW INC.
P.O. BOX 30, FALLS CHURCH, VIRGINIA 22046

December 10, 1969

HAZLETON LABORATORIES, INC.
TRW LIFE SCIENCES CENTER

Sponsor: International Minerals & Chemical Corporation Date: December 10, 1969

Material: Monosodium Glutamate

Subject: SUPPLEMENTAL PATHOLOGY REPORT
Reproduction Study - Rabbits
Project No. 466-117

SUMMARY

Previously hematoxylin- and eosin-stained sections of brain tissue from rabbit pups from does receiving monosodium glutamate at a level of 8.25% of the diet were examined. The present study consisted of the histopathologic examination of brain sections from 10 pups (five males and five females) from the does receiving monosodium glutamate at the low (0.1%) and intermediate (0.825%) levels. Transverse sections of the brain were examined at the level of the hypothalmic nuclei and pituitary gland. Slight artifactual distortion of the tissue incurred during processing was occasionally noted in sections from pups of both treatment levels. Evidence of neuronal necrosis or other pathologic alterations was not apparent in the sections examined.

Submitted by Richard W. Voelker, Jr.
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Staff Pathologist

njm

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Clinical and Laboratory Note

Seizures and the Dose of L-Glutamic Acid in Rats

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Numerous experimental models of epilepsy have been devised to test anticonvulsant drugs and to investigate the site of origin, the physiopathogenesis and the morphological concomitants of seizures. The experimental convulsions have been classified according to the agent (Swinyard 1949; Swinyard *et al.* 1952; Toman 1951; Woodbury and Davenport 1952) as:

(1) Electrically induced seizures.

(2) Chronic epileptogenic lesions (Gastaut *et al.* 1958; Kopeloff *et al.* 1944, 1954, Openchowski 1883).

(3) Sensory-reflex convulsions (Quadbeck 1956; Servit 1963).

(4) Chemically induced convulsions (Gastaut *et al.* 1958; Goodmann *et al.* 1953; Orloff *et al.* 1949; Radomski and Woodard 1949; Walker *et al.* 1945).

The chemically-induced cerebral seizures can be evoked either by extraneous drugs or by metabolites of normal brain metabolism. L-glutamate (L-glu.) injected into the cortical grey matter or intracisternally (i.c.) can evoke seizures in animals of various species (Hayashi 1954; Hennecke 1968; Wiechert 1968; Wiechert and Göllnitz 1970 b). We are reporting here the effect of the dose of L-glu. on the complex motor symptomatology of the seizures induced by it.

METHODS

The animals were male and female albino rats of the Wistar strain weighing 130 to 200 g and about 5 months old. Animals were discarded which developed audiogenic seizures (ultrasound waves, 60 sec, 15 and 25 kHz, 110 db).

We injected equal volumns of L-glu. acid (pH 7.0) into the cisterna cerebellomedullaris with a Terumo-Mikrolitersyringe of the type MSN-100*. The doses given to groups of 36 rats were 2.94 mg/kg; 3.92 mg/kg; 4.9 mg/kg; 5.88 mg/kg; 6.86 mg/kg; 7.84 mg/kg; 9.8 mg/kg. After the injection the animals were observed for 10 min. The

* Made by the firm J. Terumo Co., Ltd. Tokyo (Japan)

CD_{50} for running seizures, for clonic jerks of the front paws, and for generalized tonic-clonic seizures was estimated by the graphic method of net probabilities*.

RESULTS

Group 1: 36 animals injected i.c. with L-glu. 2.9 mg/kg.

Nineteen percent of the animals exhibited running seizures after a latency of up to 5 sec. The rats began to run forward, then in more or less large circles and avoiding obstacles at first. The running then increased in speed, it became erratic and the rat no longer avoided obstacles. Convulsive jumping then occurred at intervals and the animals could become aggressive. After 3–10 sec of activity the animals fell motionless for some seconds, when a new running seizure occurred. Eighty-one percent of the rats exhibited episodes alternately of restlessness and inactivity but no true running seizures.

Group 2: 36 animals injected i.c. with L-glu. 3.92 mg/kg.

Sixty-four percent of the animals developed far more violent running seizures immediately after the injection, often with rotation of the body about its vertical axis. At the end of a seizure the running was less coordinated, and brief clonic jerks followed by spasms of the forepaws could occur, as well as automatisms of brushing and scratching and clonic twitching of the tail. After these seizures, 20% of the animals developed clonic fits of one or both forelegs resulting, in 3% of the animals in generalized tonic–clonic seizures. In 36% of the animals there was no behavioural change at all.

Group 3: 36 animals injected i.c. with L-glu. 4.9 mg/kg.

Ninety-four percent of the animals developed running seizures so violent as to obscure any initial clonus and tonus innervation of the forelegs. At 5–20 sec after the injection 50% of the animals developed clonic jerks of the forelegs which progressed to tonic-clonic seizures in 11%. The behavior of 6% of the animals was not altered in any way.

Group 4: 36 animals injected i.c. with L-glu. 5.88 mg/kg.

All animals developed running seizures which progressed in 75% to clonic jerks of the forelegs, in 23% to generalized tonic–clonic seizures.

Group 5: 36 animals injected i.c. with L-glu. 6.86 mg/kg.

All animals developed running seizures, accompanied in 89% by clonic jerks of the forelegs. In 44% the seizures progressed to tonic–clonic seizures.

Group 6: 36 animals injected i.c. with L-glu. 7.8 mg/kg.

Clonic jerks of the forelegs occurred in 100% and culminated in generalized tonic–clonic seizures in 75%. When this high dose was given running seizures seldom occurred.

* Copyright 1952, Schaefers Feinpapier, Plauen (Vogtl.) (GDR).

Generalized tonic–clonic seizures could occur immediately or after clonic jerks of the forelegs.

Group 7: 36 animals injected i.c. with L-glu. 9.8 mg/kg.
Generalized tonic–clonic seizures constituted the whole seizure in 97%.

Fig. 1 shows the dose/seizure ratio for three of the convulsive states delimited by us:
CD_{50} for running seizures: 3.6 mg/kg of rat
CD_{50} for clonic jerks of the forepaws: 4.9 mg/kg of rat
CD_{50} for generalized tonic–clonic seizures: 7.0 mg/kg of rat.

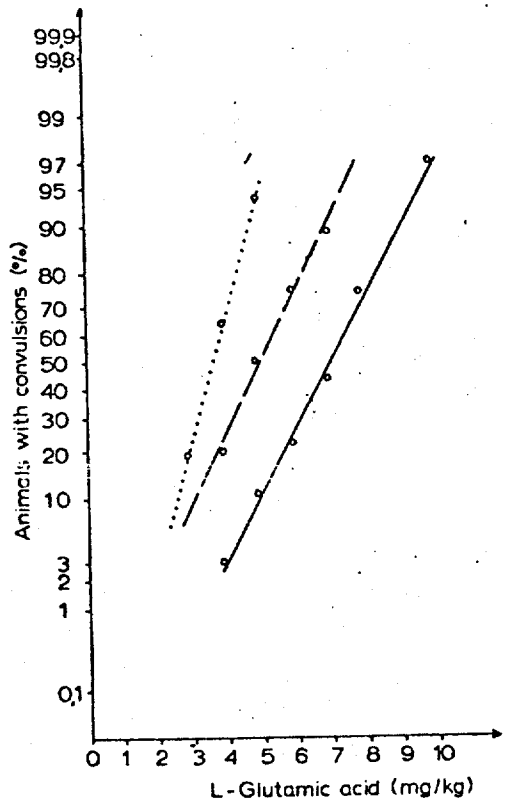

Fig. 1. Graph showing the dose for the three convulsive states delimited by us; ·····: running seizures; ----: clonic jerks of the forepaws; ——: generalized tonic–clonic seizures. Ordinate: % of animals affected scaled according to the integral of Gauss; abscissa: dose of intracisternal L-glutamic acid in mg/kg, linear scale.

DISCUSSION

L-glu. plays an important role in brain metabolism. Whether it is a true transmitting agent in the central nervous system (Krnjevic 1965; Meyer 1965) or whether it has nonspecific excitatory action is not yet known. Our study shows that the seizure manifestations vary with the dose of L-glu. injected into the cerebellomedullary cistern. Small doses produce running seizures comparable to those described by Gastaut *et al.* (1958) and Silver *et al.* (1947) and to the audiogenic seizures of rats (Quadbeck 1956; Servit 1963). High doses induce generalized tonic–clonic seizures.

Intermediate doses cause seizures resembling the phenomena seen in psychomotor epilepsy in man.

The possibility of inducing cerebral seizures of varying severity by administering a constituent of brain metabolism makes it possible to investigate the releasing mechanism of these forms of seizure (Hennecke 1968; Wiechert 1968; Wiechert and Göllnitz 1970 a,b; Wiechert and Herbst 1966).

The neurochemistry of epilepsy and especially the metabolism in the preconvulsive phase and in various stages of seizure may be more accessible to investigation by the experimental model described than has been possible by other models. An attempt to find substances which influence the metabolism of glutamic acid before and during the seizure so that the balance of L-glutamic acid to gamma-amino-butyric acid is preserved seems promising.

SUMMARY

L-glutamic acid injected intracisternally in rats causes various seizures according to the dose. Low doses cause running seizures and automatisms (CD_{50}: 3.6 mg/kg of rat). Medium doses cause clonic jerks of the forelegs (CD_{50}: 4.9 mg/kg of rats). High doses cause generalized tonic-clonic seizures (CD_{50}: 7.0 mg/kg of rat).

This model of seizures promises to be useful to investigate metabolic events preceding seizures and to test anticonvulsant substances.

RÉSUMÉ

L'acide L-glutamique injecté dans les cisternes des rats, provoque des crises différentes selon la dose. Des faibles quantités provoquent des crises pendant lesquelles l'animal se met à courir et présente des automatismes (CD_{50}: 3.6 mg/kg de rat). Des quantités moyennes provoquent des myoclonies des pattes antérieures (CD_{50}: 4.9 mg/kg de rat). Des doses élevées provoquent des crises généralisées tonico-cloniques (CD_{50}: 7.0 mg/kg de rat).

Ce moyen expérimental de provoquer les crises, promet d'être utile pour l'étude des manifestations métaboliques qui précèdent les crises, ainsi que comme test de produits anti-convulsivants.

(C. A. TASSINARI, Marseilles)

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Streptococcus bovis was selected as one test organism because of its universal association with the rumina of cattle; *S. bovis* phages could be routinely isolated[7]. Attempts to obtain phages active against another test organism, *Bacteroides ruminicola*[8], have not been successful[9]; however, difficulty was encountered in obtaining appropriate lawns for plaque detection under anaerobic conditions.

Although many factors that influence the number and kinds of microorganisms in the rumen have been elucidated[10], the role of bacteriophages in the rumen remains to be determined[11].

Zusammenfassung. Die Isolierung der im Pansen vorkommenden Bakteriophagen wird erstmals beschrieben. Bakteriophagen, die *Streptococcus bovis* und Pansen *Serratiae* spp. angreifen, wurden aus vielen Pansen-Proben isoliert und eingehend untersucht; *Bacteroides ruminicola* Phage wurde dabei nicht aufgefunden.

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Departments of Bacteriology and Animal Science, Iowa State University, Ames (Iowa 50010, USA), June 1, 1966.

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Zur Wirkung parenteral verabfolgter L-Glutaminsäure auf die bioelektrische Aktivität des Gehirns

Seit den Untersuchungen von QUASTEL und WHEATLEY, WEIL-MALHERBE, ROBERTS et al., TOWER, HIMWICH et al.[1] über die Bedeutung der Aminosäuren für den Hirnstoffwechsel hat das Interesse für die bioelektrische Aktivität des Gehirns erheblich zugenommen. Besonders die Glutaminsäure stand lange im Vordergrund der Untersuchungen, da man dieser Aminosäure auch bei enteraler Verabfolgung eine therapeutische Wirkung bei den verschiedensten Krankheitsbildern, insbesondere bei den Oligophrenien (ALBERT, HOCH und WAELSCH; ZIMMERMAN und BURGEMEISTER; ZIMMERMAN, BURGEMEISTER und PUTNAM; SCHACHTER[2]), aber auch eine antikonvulsive Wirkung bei cerebralen Krampfanfällen (PRICE, WAELSCH und PUTNAM; WAELSCH und PRICE; ZIMMERMAN, BURGEMEISTER und PUTNAM[3]) zuschrieb. Dagegen beobachteten YAMAMURA et al.[4] nach Injektion von 2%-igem Natriumglutamat in die A. carotis communis bei Epileptikern eine Zunahme der Krampfanfälle. HAYASHI[5] fand 1954, daß durch intracisternale Applikation von L-Glutaminsäure sowohl bei Tieren als auch bei Menschen tonisch-klonische Konvulsionen ausgelöst werden können. Dieses Anfallsgeschehen wurde von uns später eingehend tierexperimentell untersucht und elektroencephalographisch verfolgt (WIECHERT und HERBST[6]). Die vorliegenden Untersuchungen sollen zeigen, ob bei hochdosierter parenteraler Applikation von L-Glutaminsäure Veränderungen auch im Hirnstrombild von Hunden hervorgerufen werden können.

Methodik. Als Versuchstiere dienten ausgewachsene Hunde beiderlei Geschlechts von 9–12 kg Körpergewicht aus eigener Zucht. Die EEG-Ableitungen erfolgten mittels Nadelelektroden von der intakten Kopfhaut der nicht narkotisierten, durch Tricuran[7] relaxierten Tiere bipolar von frontal nach occipital beiderseits mit Hilfe eines 8-Kanal-Direktschreibers der Firma Schwarzer. Wir infundierten kontinuierlich L-Glutaminsäure als Natrium-Glutamat 0,69 g/kg Hund innerhalb von 50 min in die Vena saphena parva entsprechend einer Dosis von 20 g/die, wie sie üblicherweise bei Kindern in der Klinik verordnet wurde. In weiteren Versuchen wurde die Dosis der L-Glutaminsäure auf das Doppelte (1,35 g/kg Hund) gesteigert. Als Modifikation der Versuchsanordnung wurde diese Menge auch in die Carotis comm. in einem Zeitraum von etwa 25 min kontinuierlich infundiert. Die Konzentration der Glutaminsäure vor und während des Versuches wurde im Serum der Tiere und nach Ende der Infusion auch im Liquor entsprechend der früher beschriebenen Methode (WIECHERT[8]) durch die zweidimensional aufsteigende Papierchromatographie bestimmt.

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Ergebnisse. Wie aus dem Vergleich der Figuren 1 und 2 hervorgeht, lassen sich trotz eines hohen Glutaminsäurespiegels im Serum nach i.v. bzw. intraarterieller Infusion in die Carotis bei gesunden Hunden keine Veränderungen der bioelektrischen Aktivität im Hirnstrombild nachweisen. Lediglich in 2 Fällen fanden sich Abweichungen von der Norm. Bei beiden Hunden bestand eine Vorschädigung des Gehirns, die sich in der Leer-Ableitung des EEGs durch Amplitudenänderung und Verlangsamung der Frequenzen dokumentierte. In einem Fall kam es unter der Infusion zu einer deutlichen Desynchronisation, während im anderen Fall sogar das Auftreten von paroxysmal aufschießenden «spikes» beobachtet werden konnte.

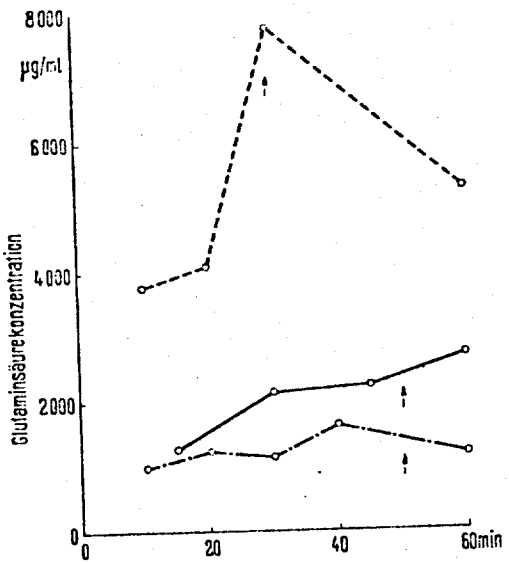

Fig. 1. Glutaminsäurekonzentration im Serum nach Infusion von L-Glutaminsäure (L-Glu) —·—·— = 0,69 g/kg L-Glu i.v.; —— = 1,38 g/kg L-Glu i.v.; ---- = 1,38 g/kg L-Glu i. carotis; ↑ = Ende der Infusion.

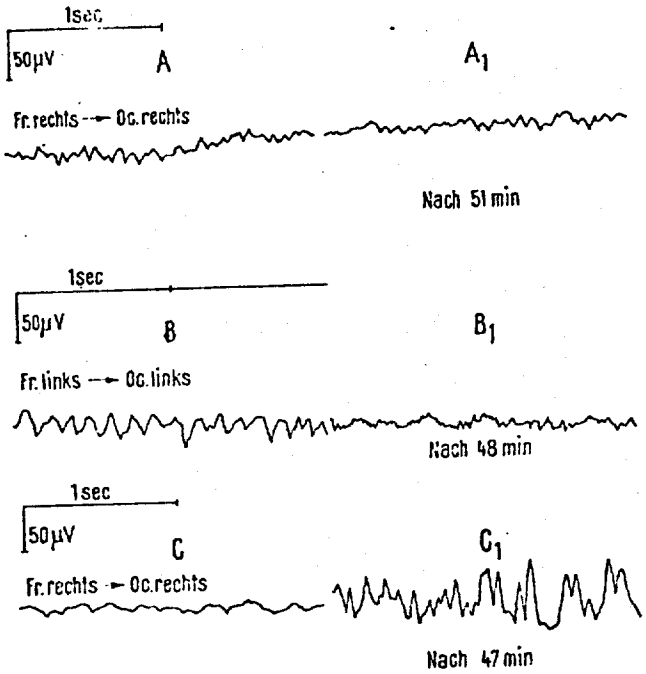

Fig. 2. EEG-Registrierung von der Schädeloberfläche (bipolar) bei nicht narkotisierten, mit Tricuran relaxierten Hunden. (A) Leerableitung (gesunder Hund). (A1) Nach i.v. Tropf (50 min) von 1,38 g L-Glutaminsäure/kg Hund; nach 51 min keine Änderung (häufigster Befund). (B) Leerableitung (vorgeschädigter Hund). (B1) Nach i.v. Tropf von 1,38 g L-Glutaminsäure/kg Hund; nach 48 min deutliche Desynchronisation. (C) Leerableitung (vorgeschädigter Hund. (C1) Nach i.v. Tropf von 1,38 g L-Glutaminsäure/kg Hund; nach 47 min Auftreten von paroxysmalen spannungshohen «spikes».

Diskussion. Die Frage nach der Wirkung enteral und parenteral verabfolgter Glutaminsäure auf das Gehirn ist immer noch umstritten. GRÜNDIG und BRETSCHNEIDER[9] fanden nach oraler Applikation ein teilweises Ansteigen des bei hirngeschädigten Kindern erniedrigten Liquor-Glutaminsäurespiegels. Sie nehmen einen durch Glutaminsäure gesteigerten Kohlenhydratstoffwechsel an und führen die therapeutische Wirkung der L-Glutaminsäure auf ein vermehrtes Angebot von α-Ketoglutarsäure an das Gehirn zurück (GRÜNDIG, SALVENMOSER und BRETSCHNEIDER)[10]. Typische Veränderungen nach Applikation von Glutaminsäure im Sinne des Auftretens von Krampfpotentialen konnte HEBER[11] beobachten. HAYASHI[12] fand bei Hunden eine verbesserte Differenzierung des bedingten Speichelreflexes 20 min nach i.v. verabfolgter Glutaminsäure, die mehrere Stunden anhält.

Bei unseren Versuchen ergab die i.v. bzw. intraarterielle Infusion von L-Glutaminsäure in die Carotis auch bei sehr hoher Dosierung keine sichtbare Wirkung auf die über mehrere Stunden abgeleiteten Hirnpotentiale. Ebenso fand sich trotz hoher Glutaminsäurekonzentration im Serum keine nachweisbare Zunahme dieser Aminosäure im Liquor. Lediglich bei den vorgeschädigten Tieren traten während der Infusion pathologische Veränderungen im EEG auf, die in einem Fall mit erhöhter Liquorglutaminsäurekonzentration korrelierten. Auf Grund der von uns durchgeführten Versuche ist anzunehmen, daß bei gesunden Hunden keine faßbaren Veränderungen der hirnelektrischen Aktivität im EEG auftreten. Damit scheint auch eine direkte aktivierende Wirkung enteral oder parenteral verabreichter Glutaminsäure auf das ZNS nur geringfügig zu sein. Es zeigte sich jedoch, daß bei Hunden mit einer voraufgegangenen Hirnschädigung nach parenteraler Glutaminsäureapplikation Krampfpotentiale im EEG registriert werden konnten.

Diese Tatsache sollte auch für die Indikation der Glutaminsäuretherapie beim Menschen Beachtung finden, da es nicht ausgeschlossen scheint, daß insbesondere bei Kindern unter hoher Glutaminsäuregabe entsprechende Komplikationen auftreten können.

Summary. A high dose of a parenteral application of L-glutamic acid does not cause any demonstratable change in the EEG of dogs. During the infusion, variations in the EEG occur in previously cerebrally damaged animals, for instance, an increased discharge of seizures.

A. HERBST, P. WIECHERT und H. HENNECKE

Abteilung für Psychiatrie der Universitäts-Nervenklinik Rostock (DDR), 7. Juli 1966.

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Development of Urea-Cycle Enzymes in the Juvenile Rat: Effect of Long-Term Administration of L-Monosodium Glutamate and Ammonium Acetate

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REPORTS FROM THIS LABORATORY[1, 2] have shown that various nitrogenous substances altered the specific activities of certain liver and kidney enzymes, when orally administered to experimental rats for prolonged periods of time. During these studies we noted that groups of control rats exhibited marked variability in mean enzyme specific activities at different age or weight levels. This information prompted an experiment to determine whether liver urea-cycle enzyme specific activities were actually changing when measured in the juvenile rat at weekly intervals during a 6-week growth period.

The synthesis of urea represents the major liver mechanism for ammonia detoxification in the intact mammal. Since it has been previously shown that the oral administration of L-glutamic acid results in an increase of citrulline formation without affecting eventual urea synthesis,[3] it was decided to observe the periodic effect of L-monosodium glutamate on individual urea-cycle enzyme activities. In addition, ammonium acetate was administered intragastrically in order to define more precisely the previously observed repression of liver arginase activity at 2 and 4 weeks,[1] and also to determine if any change or adaptive response in liver carbamyl phosphate synthetase might occur, since this enzyme was not measured during ammonium acetate administration in our earlier studies.

The natural biochemical defense of the mammalian organism against metabolic insult from overwhelming amounts of ammonia lies in the ability of the organism to fix or detoxify this toxic substance.[4] A clinical-

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ly adaptable mode of defense designed to lower toxic blood and tissue levels of ammonia might lie in the administration of suitable enzyme inducers (increased concentration of substrate) or coenzymes to increase the capacity (enzyme induction) of those tissue enzymes involved in ammonia fixation.[5] L-Monosodium glutamate has received wide clinical trial as a biochemical agent in lowering toxic blood ammonium concentrations in human patients with far-advanced liver disease associated with the brain syndrome known as hepatic coma.[6-10] L-Monosodium glutamate may lower blood ammonium concentration as a result of increased glutamine synthesis[6] or by preventing the depletion of α-ketoglutaric acid of the Krebs cycle in the brain.[9, 11] These reactions are in part dependent on the enzymes glutamine synthetase and L-glutamic dehydrogenase, respectively.

This experiment suggests evidence for a new hypothesis of the action of L-monosodium glutamate in ammonia detoxification, by demonstrating a marked induction of one of the liver urea-cycle enzymes (carbamyl phosphate synthetase) resulting from the oral administration of L-monosodium glutamate to experimental rats. In addition, the liver urea-cycle enzyme activities of young control rats were found to change during a 6-week growth period, even when the rats were maintained on a normal protein diet which provided a nearly constant rate of growth throughout the experimental period. There was evidence for the early induction of carbamyl phosphate synthetase by the oral administration of ammonium acetate, and confirmation of arginase repression as reported previously.[1] Adaptation of the enzyme carbamyl phosphate synthetase in response to both L-monosodium glutamate and ammonium acetate occurred with prolonged administration of these substances.

METHODS AND MATERIALS

Eighty-five male weanling Sprague-Dawley rats,* with a mean body weight of 38 gm., were placed singly in cages and offered water and a complete diet† containing 27% vitamin-free casein, 59% starch, 10% vegetable oil, 4% salt mixture (USP XIV), and a vitamin fortification mixture. After 3 days, the mean body weight increased to 52 gm. At this time, the rats were divided into 3 groups and the oral administration of L-monosodium glutamate and ammonium acetate began. Group 1 served as the control group. Group 2 received a water solution of L-monosodium glutamate by stomach tube twice daily (2.0 gm. per kilogram of body weight, per dose) and Group 3 received a water solution of ammonium acetate by stomach tube twice daily (0.47 gm. ammonium nitrogen per

*Obtained from Northwest Rodent Company, Pullman, Wash.
†Obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

kilogram of body weight, per dose). Five controls were sacrificed by decapitation at 0 and 1 weeks. Thereafter, 4 control rats were sacrificed weekly. At the end of the first week, 4 rats from experimental Group 2 and 3 from experimental Group 3 were sacrificed. This pattern of weekly sacrifice was continued for 6 weeks. During the first weeks of the experiment, 13 rats died from the effects of stomach-tube insertion. These rats were observed to have ruptured esophagi at autopsy. No deaths occurred during the remaining weeks of the experimental period. Rats were sacrificed 48 hr. after the last dose of ammonium acetate or L-monosodium glutamate, to avoid any residual effect from a single dose.

At sacrifice, the livers were removed; tissue protein content and the specific activities of the urea cycle enzymes carbamyl phosphate synthetase, ornithine transcarbamylase, arginine synthetase, and arginase were measured as described previously.[1] All rats were weighed twice per week and the doses of L-monosodium glutamate and ammonium acetate calculated and administered accordingly. The total volume administered of either test solution did not exceed 1.2 ml., even at 6 weeks.

A unit of enzyme specific activity is defined as that amount of enzyme which catalyzes the production of 1 μmole of product per hour, per milligram of tissue protein under the assay conditions for that enzyme.

RESULTS

Figure 1 depicts the weekly development of liver urea-cycle enzymes in young control rats fed a complete and constant diet ad libitum for a

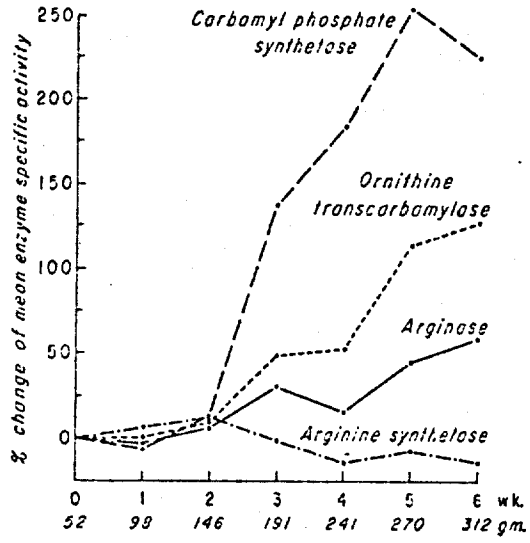

Fig. 1. Development of urea-cycle enzymes in liver of juvenile rat.

period of 6 weeks. Percentage changes of mean enzyme specific activities were calculated from mean individual zero-week urea-cycle enzyme specific activities. The results demonstrate changes of urea-cycle enzyme activity during the observed growth period. All of the liver urea-cycle enzyme activities remained relatively constant during the first 2 weeks of growth, even though body weight doubled the first week and increased threefold the second week (Table 1). The mean growth rate of the control rats continued to remain relatively constant until the fifth week. Carbamyl phosphate synthetase, ornithine transcarbamylase, and arginase increased in specific activity at the end of the third week. Ornithine transcarbamylase and arginase continued to increase in specific activity throughout the 6-week period of observation, while carbamyl phosphate synthetase specific activity increased for 5 weeks and then decreased slightly during the last week of observation. The specific activity of liver

TABLE 1. GROWTH AND SACRIFICE PATTERN OF CONTROL AND EXPERIMENTAL RATS

	No. of rats			*Mean body wt.*		*Mean growth rate*
Week	*Start*	*Sacrifice*	*Death*	*Gm.*	*±S.D.*	*(gm./week)*
			Group 1 (controls)			
0	30	5	0	52	7	—
1	25	5	0	98	11	46
2	20	4	0	146	9	48
3	16	4	0	191	14	45
4	12	4	0	241	15	50
5	8	4	0	270	17	29
6	4	4	0	312	23	42
			Group 2 (L-monosodium glutamate)			
0	30	0	0	53	7	—
1	30	4	6	94	12	41
2	20	4	0	132	13	38
3	16	4	0	170	16	38
4	12	4	0	212	23	42
5	8	4	0	248	21	36
6	4	4	0	268	32	20
			Group 3 (ammonium acetate)			
0	25	0	0	56	7	—
1	25	3	5	87	14	31
2	17	3	1	127	16	40
3	13	3	1	163	19	36
4	9	3	0	202	25	39
5	6	3	0	249	19	47
6	3	3	0	268	32	19

arginine synthetase did not change appreciably during the observed growth period, and in fact, decreased slightly (13%) during the last 4 weeks of growth. Carbamyl phosphate synthetase increased 251% at 5 weeks before dropping to 226% at 6 weeks; ornithine transcarbamylase increased 129%, and arginase increased 60% at 6 weeks. The percentage increase of liver arginase specific activity was approximately one-fourth of carbamyl phosphate synthetase activity, and that of ornithine transcarbamylase, one-half; liver arginine synthetase remained relatively unchanged.

Table 1 summarizes the weekly mean body weight, growth rate, and sacrifice pattern of the 3 groups of rats studied. Rats receiving intragastric L-monosodium glutamate (Group 2) and rats receiving intragastric ammonium acetate (Group 3) exhibited slightly lower weekly increases in mean body weight, and consequently lower mean growth rate values, than the control rats of Group 1. The mean growth rate of Group 1 and 2 was relatively constant, even though the mean growth rate of Group 2 was slightly lower until after the fourth week. Diminishing numbers of rats due to weekly sacrifice probably accounts for the erratic changes observed during the fifth and sixth weeks, even in Group 1. The mean growth rate of Group 3 was also less than Group 1, except in the fifth week, and was more variable than either Group 1 or 2 throughout the experiment.

The mean body weight of both experimental and control rats sacrificed at weekly intervals agreed closely with the mean body weight of surviving rats.

Figure 2 demonstrates the individual weekly response pattern of liver urea cycle enzyme activities in Group 2 experimental rats which received intragastric L-monosodium glutamate for six weeks as compared with that of Group 1 rats which received no L-monosodium glutamate during the experimental period. Each point on each graph represents the mean enzyme specific activity, derived from 4 or 5 rat livers for that specific enzyme. The standard deviations of mean enzyme specific activities are indicated only when the difference between experimental and control values are significant ($P < 0.05$). Chronic intragastric administration of L-monosodium glutamate to experimental rats had no significant effect on liver ornithine transcarbamylase, arginine synthetase, or arginase specific activities during the 6 weeks of observation, as compared with controls. However, the specific activity of liver carbamyl phosphate synthetase was markedly and significantly increased as compared with controls after 1 week (97%, $P < 0.05$), 2 weeks (179%, $P < 0.001$), and 3 weeks (76%, $P < 0.01$) of L-monosodium glutamate administration.

After 4 weeks of continued administration, the specific activity of carbamyl phosphate synthetase had returned toward control values. The remaining fifth and sixth weeks' observations failed to show a significant enzyme activity difference from control values.

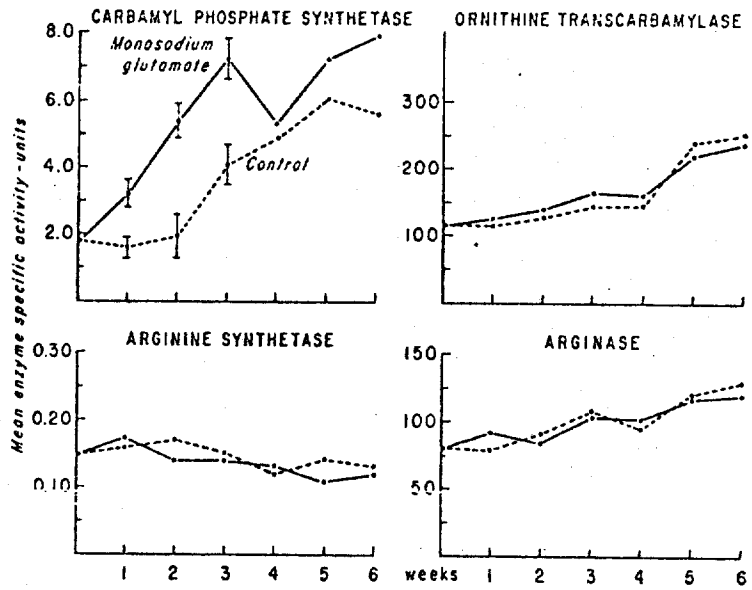

Fig. 2. Effect of L-monosodium glutamate on liver urea-cycle enzyme activity.

The intragastric administration of ammonium acetate to experimental rats of Group 3 decreased liver arginase specific activity 22% below the arginase activity of control rats at 5 weeks. Ornithine transcarbamylase and arginine synthetase specific activities did not significantly differ from those of control rats of Group 1 throughout the 6-week period of observation. These data are in essential agreement with our previous observations.[1] However, carbamyl phosphate synthetase specific activity was increased 98% above the control level ($P < 0.02$) after 1 week of ammonium acetate administration. Thereafter, the specific activity of carbamyl phosphate synthetase decreased to 64% at 2 weeks, 33% at 3 weeks, and returned to the control value at 4 weeks. The specific activity values remained indistinguishable from control values during the remaining 5 and 6 weeks of observation. With the exception of the first week, the percentage changes in carbamyl phosphate synthetase specific activity, although relatively large, were not significantly different from the control levels. This lack of statistical significance may be due to the small number of rats used. Nevertheless, a twofold increase of carbamyl phosphate

synthetase specific activity occurred after 1 week of intragastric ammonium acetate administration, and the return toward control activities, with prolonged exposure, indicates adaptation to the orally administered substrate.

DISCUSSION

There are apparently few, if any, data available in the literature that demonstrate a relationship between liver urea-cycle enzyme activities and increased age or body weight in the developing juvenile rat maintained on a normal and relatively constant protein diet. Markedly enhanced activity of liver urea-cycle enzymes in anuran tadpoles during metamorphosis has been reported.[12–15] Allison *et al.*[16] found that the growth rate of rats fed diets of different protein and nitrogen content correlated with serum and liver ribonuclease activity. Ribonuclease activity of serum and liver decreased as growth rate and protein intake increased. Muramatsu and Ashida[17] studied the effect of different levels of dietary protein on growth and liver enzyme activities of rats. Ross and Batt[18] investigated the relationship between certain liver enzyme activities and age of rats maintained on diets which differed in amounts of protein and dextrose. In all age groups studied, the liver enzymes alkaline phosphatase, cathepsin, histidase, and D-amino acid oxidase were found to vary with the protein content of the diet and with age. Barrows and Roeder[19] reported on the concentration of certain liver and kidney enzymes in rats fed normal, protein-depleted, and protein-repleted diets. They found the most striking age-dependent enzyme changes to occur between 1 and 3–5 months, but no marked age differences in enzyme activities (> 15%) were found in rats fed a normal protein diet, except for increased catheptic activity in the oldest (24-month) animals.

Since the original work of Krebs and Henseleit, the urea cycle has been modified as follows: The formation of carbamyl phosphate from carbon dioxide and ammonia in the presence of ATP, Mg^{++}, and acetyl glutamic acid via the enzyme carbamyl phosphate synthetase represents the initial step in the eventual synthesis of urea and pyrimidines.[20] Ornithine plus carbamyl phosphate is transcarbamylated to citrulline.[21] Argininosuccinic acid is formed from the condensation of citrulline and aspartic acid and then split into arginine and fumaric acid by the condensing and cleavage enzymes of arginine synthetase, respectively.[22, 23] Fumaric acid enters the citric acid cycle and ultimately forms oxaloacetic acid. After transamination, oxaloacetic acid regenerates aspartic acid for continued use in the urea cycle. Arginine is converted to urea for excretion in the kidney and to ornithine for reuse in the urea cycle.

Our measurements of carbamyl phosphate synthetase and ornithine

transcarbamylase activities were based on the colorimetric determination of citrulline[24, 25] by the method of Brown and Cohen.[26] In spite of the fact than an exogenous source of beef liver ornithine transcarbamylase was not added to the carbamyl phosphate synthetase assay medium,[1] liver carbamyl phosphate synthetase specific activity was markedly increased (as compared with controls) as a result of the oral administration of L-monosodium glutamate, while liver ornithine transcarbamylase remained unaffected even though both enzymes were measured directly as the rate of citrulline formation. These observations indicate that increased carbamyl phosphate synthetase specific activity and not ornithine transcarbamylase activity was responsible for the increased formation of citrulline, and probably provided increased formation of precursor, carbamyl phosphate, prior to citrulline formation.

Tissue and body fluid concentrations of urea and urea-cycle intermediates were not measured in this experiment, so the over-all production of urea nitrogen from increased carbamyl phosphate synthetase specific activity is not known. However, ornithine transcarbamylase, arginine synthetase, and arginase specific activities were not increased, suggesting that the capacity for urea formation was not increased by the oral administration of L-monosodium glutamate. Koritz and Cohen[3] previously demonstrated in rats that urea synthesis from increased citrulline formation due to dietary glutamic acid and carbamyl-L-glutamate was unaffected when measured at 8 or 9 weeks.

If one assumes that carbamyl phosphate (precursor of pyrimidines and urea) is increased as a consequence of the observed increased carbamyl phosphate synthetase specific activity resulting from oral L-monosodium glutamate administration, then the above data could suggest the possibility of temporary accelerated pyrimidine synthesis via enzyme induction. This pathway of ammonia fixation, as yet relatively uninvestigated, might well provide additional evidence for the therapeutic use of L-monosodium glutamate to lower abnormally elevated blood ammonium levels in patients with hepatic coma. Direct measurement of selected pyrimidines and urea during L-monosodium glutamate administration would be helpful in establishing the biochemical role of this compound in ammonia fixation and its relative usefulness as a therapeutic agent in the treatment of hepatic coma.

A significant increase in liver L-glutamic dehydrogenase was previously demonstrated in rats following intragastric administration of L-monosodium glutamate and L-arginine for 3 weeks.[1] From those data and the observations reported here (Fig. 2), it is believed that L-monosodium glutamate could lower blood ammonium by several biochemical path-

ways: firstly, by increasing the activity of L-glutamic dehydrogenase, thereby stimulating the reductive reamination of α-ketoglutarate with ammonia to form glutamic acid; and secondly, by the fixation of ammonia by induction of carbamyl phosphate synthetase to form more carbamyl phosphate, which is the precursor necessary for the synthesis of urea and pyrimidines. An increase in liver glutamine synthetase activity during L-monosodium glutamate administration was not demonstrated in our previous study,[1] and since other investigators have been unable to demonstrate a significant increase in blood glutamine formation from the combination of glutamic acid and ammonia during L-monosodium glutamate administration,[9, 27] this third biochemical mechanism of ammonia fixation may possibly assume a role of lesser importance in the liver than in other body tissues. Warren and Shenker[28] have considered that glutamine formation is the major pathway of ammonia detoxification in tissues other than the liver. However, any one or all of these mechanisms of ammonia detoxification should be considered potentially operative until more precise information is known concerning the role of the kidney in the rate of glutamine deamination, the capacity of the muscle mass to form and store glutamine, and the mechanism by which ammonia enters into the cerebral metabolic cycles of the brain.

The principle of substrate and coenzyme concentrations regulating the degree of enzymic activity in vivo, although not a new concept in the clinical treatment of metabolic disorders, represents a challenge that should eventually provide additional insight into the treatment of a variety of diseases associated with molecular changes within the cell.

The clinical use of such compounds as L-monosodium glutamate,[27, 29] arginine,[30] or arginine-glutamate[31] as an effective treatment of ammonia intoxication has been discouraging. However, those patients who have responded to this type of therapy by awakening from deep ammoniagenic coma with decreased blood ammonium concentrations[31–34] have provided dramatic evidence for the continued use and investigation of this principle of substrate loading, at least as an adjunctive therapeutic measure.

Since the control rats gained more weight than the experimental animals, and the urea-cycle enzymes are sensitive to starvation,[35] pair feeding in the course of this study would have been a helpful control. However, the *selective* rise of carbamyl phosphate synthetase suggests that diet was not a critical factor. The possible in-vitro effect of acetate as such, chemically affecting the methods of enzyme assay, would have been of additional value as a control measure.

It must be emphasized that application of these data obtained from normal rat liver to the normal or diseased liver of man must be with ex-

treme caution. In addition, sodium glutamate, where used, is currently administered to man over short periods of time (hours to days), not usually on a long-term basis. Unfortunately, data were not obtained during the course of this study for periods of time less than 7 days.

SUMMARY AND CONCLUSIONS

Liver urea-cycle enzyme specific activities increase with age and body weight of the developing juvenile rat, with the exception of arginine synthetase. The failure of rat liver arginine synthetase to respond in a similar fashion during the observed growth period is unexplained.

Liver carbamyl phosphate synthetase specific activity was markedly and significantly increased during the early oral administration of both L-monosodium glutamate and ammonium acetate. Prolonged administration of these substances resulted in a return toward control values, indicating adaptation of carbamyl phosphate synthetase to the administered substrates. Liver arginase specific activity was decreased afer 5 weeks of ammonium acetate administration. Liver ornithine transcarbamylase, arginine synthetase, and arginase specific activities did not significantly differ from those of controls during 6 weeks of intragastric L-monosodium glutamate administration.

The induction of rat liver carbamyl phosphate synthetase during L-monosodium glutamate administration suggests a hypothesis for ammonia detoxification not previously considered in the literature. The response of rat liver carbamyl phosphate synthetase is related to current concepts of ammonia intoxication which may occur in human patients with far advanced liver disease.

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An Oral LD_{50} Value of MSG in Mice

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An Oral LD_{50} Value of "Monosodium L-glutamate" in Mice

Test Material: Monosodium L-glutamate monohydrate (Commercially available)

Experimental Procedure:

The experiment was carried out in July, 1968.

Eight groups of ten male mice of dd-strain which were in the body weight range of 14.0 g to 16.0 g were used, and each dose group was put in one cage, room temperature being at 23 ± 2°C, humidity at 60 ± 10%.

Solid feed for rats: CE-2 produced by CLEA JAPAN, INC. was used as a basal diet. The feed and water to drink were freely given.

All doses were administered orally in aqueous solutions of which concentrations were adjusted to be administered at a constant dosage volume of 0.4 ml/10 g of body weight. Control animals were administered with the same volume of water alone.

During the observation period of one week, mortalities were recorded.

The oral median lethal dose (LD_{50}) and its 95 percent confidence limit were calculated by the method of Litchfield, J.T., and Wilcoxon, F (1949), J. Pharmac. Exp. Ther., 96, 99.

Results

Relationship between the time elapsed after dosing and mortality in each dosed group and the control group is shown in the following table.

Table

Time after dosing (hr.)	Numbers of Survival Animals										Mortality ratio (%) after one week
	0	2	4	24	48	72	96	120	144	168	
Dosage (g/kg)											
0 (Control)	10	10	10	10	10	10	10	10	10	10	0
8.00	10	10	10	10	10	10	10	10	10	10	0
10.00	10	10	10	10	9	9	9	9	9	9	10
12.0	10	9	9	9	9	9	9	8	8	8	20
14.0	10	10	10	8	8	8	8	7	7	7	30
17.0	10	8	8	7	7	7	7	7	7	7	30
20.0	10	3	3	3	3	3	3	2	2	2	80
24.0	10	2	2	0	0	0	0	0	0	0	100

The oral median lethal dose (LD_{50}) was calculated to be 16.2 (14.2 - 18.4) g/kg of body weight.

Clinical Experiments on Chinese Restaurant Syndrome.

Part 1. Double Blind Studies with Orally Administered Monosodium Glutamate.

Part 2. Effects of MSG-Intake on the Blood Pressure, the Electrocardiogram and the Concentration of Glutamic Acid in the Blood.

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Clinical Experiments on Chinese Restaurant Syndrome

Part 1 Double Blind Studies with Orally Administered Monosodium Glutamate.

I. Introduction

Recently, the cases called chinese restaurant syndrome (CRS) were reported,[1] and it was observed to be caused by the dietary intake of Monosodium Glutamate (MSG).[2] [3]

However, the symptoms (numbness at the back of the neck which gradually extends to both arms and the back, general weakness and palpitation, etc)[1] reported by Kwok as CRS seem not to always co-incide with those(burning sensation, facial pressure and chest pain)[3] which were observed by Schaumburg in the cases of the administration of MSG.

The authors made it clear epidemiologically in the previous paper that MSG did not cause any special symptom under the circumstances of normal dietary intake in Japan.[4] In this paper, the clinical experiment on CRS was carried out by a double blind method on 61 healthy male volunteers to confirm the observations reported previously, and also the nature of symptoms, the condition for their appearance and their intensity were dealt with.

II. Method

1. Subject: Sixty-one healthy men of 35 - 54 years old.
2. Sample: To examine the effect of MSG-intake by a double blind method, both MSG and Lactose (placebo) were prepared in the form of capsules which could not be distinguished by appearance from each other.

   MSG-sample: Monosodium L-Glutamate Monohydrate (MSG), 435mg per capsule.

   Lactose-sample: Lactose, 425mg per capsule.
3. Time and method of intake

   3-1 Intake in the non-empty stomach condition: The samples were administered in the following 4 combinations to each of the 61 persons 30 min after lunch.

i. Placebo (20 lactose-capsules)

ii. MSG-2.2g (5 MSG-capsules + 15 lactose-capsules)

iii. MSG-4.4g (10 MSG-capsules + 10 lactose-capsules)

iv. MSG-8.7g (20 MSG-capsules)

Method of intake: The 4 combinations of samples mentioned above were put into paper-envelopes marked with random sampling numbers respectively, and given to every person to take one at a time in an arbitarary order during any 4 days in a week convenient to the subject.

The person was allowed to drink about one cup of water at the time of intake.

3-2 Intake in the empty stomach condition (1): The samples were administered in the following combination at 10 a.m. every morning to each of 57 subjects who had taken no food but water after supper on the previous day.

i. Placebo (10 lactose-capsules)

ii. MSG-2.2g (5 MSG-capsules + 5 lactose-capsules)

iii. MSG-4.4g (10 MSG-capsules)

The samples were given to the persons in the same way as mentioned in 3-1 to take one by one in an arbitary order during any 3 days in a week convenient to the subject.

3-3 Intake in the empty stomach condition (2): The samples were administered in the following combinations to 48 persons in the same way as mentioned in 3-2.

i. Placebo (20 lactose-capsules)

ii. MSG-8.7g (20 MSG-capsules)

4. Methods of observation and description

The conditions of bodies after the intake were described by the subjects themselves in blank forms distributed with the samples as follows;

i. Change in pulse rate: The pulse was examined 5 min. before, and 15, 30, 45 and 60 min. after the intake.

ii. Symptom: Experience of the following symptoms - burning sensation, pressure sensation, chest pain, headache, nausea, thirst, feeling of weakness; Times of their appearance and disappearance. Besides these 7 symptoms, every kind of abnormal sign was described if it was felt.

III. Result

1. Symptoms caused by the intake of samples and their frequencies

The results of the above-mentioned 3 experiments of MSG-intake are tabulated in Table 1.

The frequency, the times of appearance and disappearance of every symptom, and the body parts where the symptom was felt are shown by the 7 symptoms mentioned above in Tables 2 - 8.

Intake in the non-empty stomach condition: The results of the intake 30 min. after lunch showed the following frequencies classified by symptoms; pressure sensation 1, thirst 2 and others 8 in the lot of Placebo; the 7 symptoms 0, others 3 in the lot of MSG-2.2g; pressure sensation 1, chest pain 1, headache 1, thirst 1, feeling of weakness 2, others 8 in the lot of MSG-4.4g; burning sensation 1, headache 1, thirst 2, others 9 in the lot of MSG-8.7g.

In total, the number of persons who showed any symptoms is 10 (16.4%) in lot placebo, 3(4.9%) in Lot MSG-2.2, 11 (18%) in Lot MSG-4.4 and 11 (18.0%) in Lot MSG-8.7. The frequency is not significantly higher in every MSG-lot than in the Placebo-lot. And the frequencies of CRS-like symptoms * of

Schaumburg et al. were 1(1.6%), 0(0%), 2(3.3%) and 1(1.6%) in the 4 lots, respectively, showing no significant difference between Placebo-lot and the others.

Intake in the empty stomach condition (1) -- The effect of intake was tested every morning on 57 persons who had taken no food after supper on the previous day. The frequencies of symptoms in this experiment were : nausea 1, feeling of weakness 1 and others 2 in Lot Placebo; headache 2, thirst 3 and others 7 in Lot MSG-2.2g; pressure sensation 1, chest pain 2, headache 5, thirst 1, feeling of weakness 1 and others 15 in Lot MSG-4.4g.

* According to Schaumburg et al., CRS-symptomes caused by the intake of MSG are: a burning sensation usually beginning over the chest and spreading subsequently to the neck, shoulders, forearms, the abdomen, and occasionally the thighs; facial pressure; and chest pain. In this paper, all the cases showing a burning sensation, a pressure sensation and a chest pain were counted in the respective symptoms regardless of the body parts where they were felt. So the CRS-like symptoms in the present paper include more other cases than the CRS-symptoms.

The total number of persons showing the symptoms is 4(7.0%) in Lot Placebo (15.8%) in Lot MSG-2.2g, 18(31.6%) in Lot MSG-4.4g, indicating that the appearance of symptoms increases with the dose of MSG-intake. But the frequencies of CRS-like symptoms are 0(0%), 0(0%) and 3(5.3%) in these lots, respectively, being too low to discuss the significance of difference among the lots.

Intake in the empty stomach condition (2) -- The second experiment of intake in the empty stomach condition was conducted on 48 persons, excluding 9 who rejected the test because of the experience of fasting in the first experiment. The results were as follows: Nausea 1, thirst 1 and others 7

in Lot Placebo; burning sensation 3, pressure sensation 4, headache 2, nausea 5, thirst 3, feeling of weakness 4 and others 20 in Lot MSG-8.7g. In total the number of persons showing any symptoms are: 9(18.7%) in Lot Placebo-20 and 27(56.2%) in Lot MSG-8.7.

The symptoms included in others were mainly heartburn, a little warm feeling of body and sleepiness throughout the 3 experiments. And most of them were non-specific and rather insignificant.

2. When and where symptoms appeared

The frequency of each symptom mentioned above was classified by the times of appearance and disappearence of the symptom and by the body parts where it was felt as shown in Tables 2-8.

Time of the appearance of symptoms -- As the samples were all administered in the form of capsules which were expected to be dissolved in 5-6 min after the intake, the absorption of MSG seemed to be slower in these experiments than in the intake in the form of uncapsulated crystals or a solution.

The time length from the intake to the appearance of symptoms showed comparatively uniform distribution in the 3 ranges, 10-20, 20-30 and more than 30 min in the 7 symptoms, but it was mainly distributed in 2 ranges, less than 10 and more than 20 min, in the CRS-like symptoms. If the rate of absorption is taken into consideration, it is doubtful that the symptoms manifested within 10 min are due to the intake of MSG.

Body parts where the CRS-like symptoms were felt ---

If the sppearance of the symptoms was examined in a wider area of the body than in the case reported by Schaumburg, the burning sensation was experienced in the face, neck, arms and axilla. The pressure sensation was felt in the neck, orbit, cheek and chest. The body part where chest

pain was felt was described as the fore-thorax by the subjects, but one case was a stomach-ache.

3. Change in pulse rate

The pulse rate was examined 5 min before, and 15, 30 and 60 min after the intake by the subjects themselves in every case. No remarkable change was observed after the intake.

IV Consideration

The effects of MSG-intake in the non-empty and empty stomach conditions were tested. The results obtained are considered in relation to the appearance of CRS-like symptoms as follows:

In the experiment on the non-empty stomach condition, the number of persons showing CRS-like symptoms was 1/61, 0/61, 2/61 and 1/61 in the lots of Placebo, MSG-2.2g, MSG-4.4g and MSG-8.7g, respectively, indicating no difference between the lot of Placebo and the others. In fact, there is no symptom corresponding to the cases reported by Schaumburg in the 4 CRS-like cases obtained if they are judged by both the time of appearance and the body parts where they were felt. And neither of the two CRS-like symptoms observed in lot MSG-4.4g were experienced in Lot MSG-8.7g, that is, there was no reproducibility of them. From these results, it can be said that MSG-intake in the full stomach condition did not cause any CRS-like symptom even if the dose was as large as 8.7g. On the other hand, the number of persons showing CRS-like symptoms were 0/57 (and 0/48), 0/57, 3/57 and 5/48 in the lots of Placebo, MSG-2.2g, MSG-4.4g and MSG-8.7g, respectively, in the experiments on the empty stomach condition. But if they are judged by time, body parts and reproducibility, none of the cases observed in lot MSG-4.4g correspond to CRS-like ones, and only 3 cases, burning sensation 1 and pressure sensation 2, in Lot MSG-8.7g

are counted in them.

There were only 2 subjects, in the lot of MSG-8.7g in the empty stomach condition, who felt two CRS-like symptoms at the same time, and there was no person who experienced 3 symptoms at the same time throughout the experiments even if the appearance of symptoms were examined in a wider range of the time of appearance and a wider area of the body.

The initial symptoms of CRS reported by Kwok et al. are mainly numbness, palpitation and general weakness. The numbness and the palpitation were not found at all in the authors' experiment.

The feeling of weakness alone was observed in a few cases, but its occurrence seemed to be more frequent only in Lot MSG-8.7g in the empty stomach condition than in the lot of Placebo. These results suggest that the CRS-symptoms observed to occur in Chinese restaurants are not always the same as the CRS-like symptoms caused by the intake of a large dose of MSG. And the latters, even if they are due to the intake of a large dose in the empty stomach condition, appear to be not specific ones, but physiological changes experienced usually, being out of the question to be considered clinically.

Table I. Frequencies Classified by symptoms and the number of subjects showing the symptoms.

Condition	Sample	Subjects No.	Frequencies classified by symptoms								Total number of subjects appeared symptoms	Subjects appeared CRS-like symptoms at the time	Subjects appeared 2CRS-like symptoms at the same time	Subjects appeared 3CRS-like symptoms at the same time
			burning sensation	Pressure sensation	Chest pain	Head-ache	Nausea	Thirst	Feeling of weak-ness	Others				
Non-empty stomach	Placebo	61	0 (0)	1 (1.6)	0 (0)	0 (0)	0 (0)	2 (3.3)	0 (0)	8 (13.1)	10 (16.4)	1 (1.6)	0 (0)	0 (0)
	MSG-2.2	61	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (4.9)	3 (4.9)	0 (0)	0 (0)	0 (0)
	MSG-4.4	61	0 (0)	1 (1.6)	1 (1.6)	1 (1.6)	0 (0)	1 (1.6)	2 (3.3)	8 (13.1)	11 (18.0)	2 (3.3)	0 (0)	0 (0)
	MSG-8.7	61	1 (1.6)	0 (0)	0 (0)	1 (1.6)	0 (0)	2 (3.3)	0 (0)	9 (14.8)	11 (18.0)	1 (1.6)	0 (0)	0 (0)
Empty stomach (I)	Placebo	57	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.8)	0 (0)	1 (1.8)	2 (3.5)	4 (7.0)	0 (0)	0 (0)	0 (0)
	MSG-2.2	57	0 (0)	0 (0)	0 (0)	2 (3.5)	0 (0)	3 (5.3)	0 (0)	7 (12.3)	9 (15.8)	0 (0)	0 (0)	0 (0)
	MSG-4.4	57	0 (0)	1 (1.8)	2 (3.5)	5 (8.8)	0 (0)	1 (1.8)	1 (1.8)	15 (26.3)	18 (31.6)	3 (5.3)	0 (0)	0 (0)
Empty stomach (II)	Placebo	48	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.1)	1 (2.1)	0 (0)	7 (14.6)	9 (18.7)	0 (0)	0 (0)	0 (0)
	MSG-8.7	48	3 (6.2)	4 (8.3)	0 (0)	2 (4.2)	5 (10.4)	3 (6.2)	4 (8.3)	20 (41.7)	27 (56.2)	5 (10.4)	2 (4.[illegible])	0 (0)

( ) = rate of frequencies

Table 2. Time and part of the appearance of burning sensation

		Sample	Subjects No.	Burning sensation +	Time of appearance				Time of disappearance				Duration (min)				Part		
					≦ 10	10-20	20-30	30<	≦ 20	20-40	40-60	60<	≦10	10-20	20-30	30<	Face	neck	Arms and axilla
Non-empty stomach		Placebo	61																
		MSG-2.2	61																
		MSG-4.4	61																
		MSG-8.7	61	1 (49)				1 (49)			1 (49)		1 (49)				1 (49)		
Empty stomach	(I)	Placebo	57																
		MSG-2.2	57																
		MSG-4.4	57																
	(II)	Placebo	48																
		MSG-8.7	48	3 (13)(29)(51)	1 (29)	1 (51)	1 (13)				2 (13)(51)	1 (29)			2 (13)(51)	1 (29)	1 (51)	1 (13)	1 (13)

(No.) = Subject number

Table 3. Time and part of the appearance of pressure sensation

	Sample	Subjects No.	Pressure sensation +	Time of appearance				Time of disappearance				Duration (min)				Part			
				≦10	10-20	20-30	30<	≦20	20-40	40-60	60<	≦10	10-20	20-30	30<	Cheek	neck Orbit		chest
Non-empty stomach	Placebo	61	1 (43)				1 (43)				1 (43)				1 (43)				1 (43)
	MSG-2.2	61																	
	MSG-4.4	61	1 (29)	1 (29)					1 (29)				1 (29)				1 (29)		
	MSG-8.7	61																	
Empty stomach (I)	Placebo	57																	
	MSG-2.2	57																	
	MSG-4.4	57	1 (34)				1 (34)			1 (34)					1 (34)			1 (34)	
Empty stomach (II)	Placebo	48																	
	MSG-8.7	48	4 (29)(34) (44)(51)	2 (29) (51)		1 (44)	1 (34)		1 (51)	1 (29)	2 (34) (44)			1 (51)	3 (29) (34) (44)	1 (44)	1 (29)	2 (34) (51)	

(No.) = Subject number

Table 4. Time and part of appearance of chest pain

	Sample	Subjects No.	Chest pain +	Time of appearance				Time of disappearance				Duration (min)				Part
				≦10	10-20	20-30	30<	≦20	20-40	40-60	60<	≦10	10-20	20-30	30<	Chest
Non-empty stomach	Placebo	61														
	MSG-2.2	61														
	MSG-4.4	61	1 (52)	1 (52)				1 (52)				1 (52)				1 (52)
	MSG-8.7	61														
Empty stomach (I)	Placebo	57														
	MSG-2.2	57														
	MSG-4.4	57	2 (15)(52)	1 (52)		1 (15)		1 (52)	1 (15)			1 (15)			1 (52)	2 (15)(52)
Empty stomach (II)	Placebo	48														
	MSG-8.7	48														

(No.) = Subject number

Table 5. Time of appearance of headache

		Sample	Subjects No.	Headache +	Time of appearance				Time of disappearance				Duration (min)			
					≦10	10–20	20–30	30<	≦20	20–40	40–60	60<	≦10	10–20	20–30	30<
Non-empty stomach		Placebo	61													
Non-empty stomach		MSG-2.2	61													
Non-empty stomach		MSG-4.4	61	1 (15)		1 (15)				1 (15)			1 (15)			
Non-empty stomach		MSG-8.7	61	1 (15)				1 (15)			1 (15)		1 (15)			
Empty stomach	(I)	Placebo	57													
Empty stomach	(I)	MSG-2.2	57	2 (6)(52)	1 (52)		1 (6)				1 (52)	1 (6)		1 (52)		1 (6)
Empty stomach	(I)	MSG-4.4	57	5 (6)(15)(27)(53)(56)		1 (53)	1 (56)	3 (6)(15)(27)		1 (15)		4 (6)(27)(53)(56)	1 (15)	2 (27)(53)	1 (6)	1 (56)
Empty stomach	(II)	Placebo	48													
Empty stomach	(II)	MSG-8.7	48	2 (6)(51)			2 (6)(51)			1 (51)	1 (6)		1 (51)	1 (6)		

(No.) = Subject number

Table 6. Time of appearance of nausea

		Sample	Subjects No.	Nausea +	Time of appearance				Time of disappearance				Duration (min)			
					≦10	10–20	20–30	30<	≦20	20–40	40–60	60<	≦10	10–20	20–30	30<
Non-empty stomach		Placebo	61													
		MSG-2.2	61													
		MSG-4.4	61													
		MSG-8.7	61													
Empty stomach	(I)	Placebo	57	1 (32)	1 (32)											
	(I)	MSG-2.2	57													
	(I)	MSG-4.4	57													
	(II)	Placebo	48	1 (6)				1 (6)				1 (6)			1 (6)	
	(II)	MSG-8.7	48	5 (6)(9) (13)(30) (51)		2 (30) (51)	2 (9) (13)	1 (6)		2 (13) (51)	1 (9)	2 (6) (30)	1 (13)	1 (51)	2 (6) (9)	1 (30)

(No.) = Subject number

Table 7. Time of appearance of thirst

		Sample	Subjects No.	Thirst +	Time of appearance				Time of disappearance				Duration (min)			
					≦10	10–20	20–30	30<	≦20	20–40	40–60	60<	≦10	10–20	20–30	30<
Non-empty stomach		Placebo	61	2 (6)(27)				2 (6)(27)			1 (27)	1 (6)			1 (6)	1 (27)
		MSG-2.2	61													
		MSG-4.4	61	1 (6)				1 (6)				1 (6)				1 (6)
		MSG-8.7	61	2 (6)(51)				1 (6)				1 (6)				1 (6)
Empty stomach	(I)	Placebo	57													
	(I)	MSG-2.2	57	3 (19)(29)(32)		1 (32)	1 (19)	1 (29)		1 (19)		1 (29)				1 (29)
	(I)	MSG-4.4	57	1 (27)			1 (27)				1 (27)				1 (27)	
	(II)	MSG-P-20	48	1 (58)												
	(II)	Placebo	48	3 (29)(32)(58)		1 (32)					1 (32)					1 (32)

(No.) = Subject number

Table 8. Time of appearance of feeling of weakness

		Sample	Subjects No.	Feeling of weakness +	Time of appearance				Time of disappearance				Duration (min)			
					≦10	10–20	20–30	30<	≦20	20–40	40–60	60<	≦10	10–20	20–30	30<
Non-empty stomach		Placebo	61													
		MSG–2.2	61													
		MSG–4.4	61	2 (34)(37)		1 (34)	1 (37)			2 (34) (37)			1 (37)	1 (34)		
		MSG–8.7	61													
Empty stomach	(I)	Placebo	57	1 (8)				1 (8)							1 (8)	
		MSG–2.2	57													
		MSG–4.4	57	1 (34)		1 (34)				1 (34)				1 (34)		
	(II)	Placebo	48													
		MSG–8.7	48	4 (8)(29) (44)(52)	1 (52)	1 (29)	2 (8) (44)		1 (52)		1 (8)	2 (29) (44)	1 (52)		1 (8)	2 (29)(44)

(No.) = Subject number

Part II Effects of MSG-Intake on the Blood Pressure, the Electrocardiogram and the Concentration of Glutamic Acid in the Blood.

I. Introduction

The experiments on MSG-intake dealt with in Part I showed that the administration of MSG in the non-empty stomach condition did not cause any significant effect on the frequency on CRS-like symptoms, even if the dose was as large as 8.7g. A few CRS-like symptoms were observed only in Lot MSG-8.7g, the largest dose examined, when it was administered in the empty stomach condition. These results, however, are mere information obtained by experiments based on subjective observations. For the consideration of the safety of MSG as a flavor enhancer, it is necessary to investigate whether or not any symptom caused by MSG-intake is such an important sign as it must be taken up from a clinical viewpoint which is based on objective observations.

The following experiments were conducted to approach this problem: MSG was administered in the empty stomach condition to the subjects who had not felt any symptom (Negative group) and those who had experienced some symptoms (Postive group) in the experiments stated in Part I, and examinations were made on the concentration of glutamic acid and sodium in the blood, the blood pressure, the pulse rate and the electrocardiogram to investigate the relation between the changes of the values and the subjective symptoms of the persons who were subjected to the tests.

II. Methos

1. Subject: Five persons who had not felt any symptom (Negative group) and 9 persons who had experienced some symptoms (Positive group) in the previous experiments (Table 1)
2. Intake of MSG and the dose: The subjects were prohibited to take any food except water or green tea after supper, and 8.7g of MSG(20 MSG-

capsules)* was administered to them with a cup of water at 10-11 a.m. in the next morning. (* In one case MSG-4.4g(10 MSG-capsules)

3. Self-observation and description: Subjective symptoms were described by the subjects themselves in the same way as mentioned in the Part I.

4. Blood pressure and electrocardiogram:

Blood pressure - Arterial pressure was measured in the recumbent position 5 min before, and 30, 60 and 90 min after the intake of MSG.

Electrocardiogram - An electrocardiogram recorded by chest lead method 35 - 40 min after the intake was compared with that recorded in an ordinary condition.

5. Estimation of glutamic acid content in the blood plasma

5-1 Preparation of blood plasma for the estimation of glutamic acid.

About 5ml of blood, taken from the vein of either a left or a eight arm before. (control) and 30, 60 and 90 min after the intake of MSG, was heparinized and centrifuged to separate blood plasma. To 0.5 - 1.0ml of the plasma was added 3 volumes of methanol and stirred. The mixture was allowed to stand overnight at $0^{o}C$, and then centrifuged for 10 min at 4,000 r.p.m. to remove the protein. The supernatant was dried under reduced pressure. The product was dissolved in fixed amount of distilled water to obtain the sample solution for the determination of glutamic acid content.

5-2 Analytical procedure of plasma glutamic acid

Fifty µl of the deproteinized plasma solution thus prepared was subjected to paper electrophoretic analysis (Conditions; Toyo Roshi No.50 paper, pyridine-acetic acid-water = 1:10:289, pH3.6, 345V/cm, 120 min) to obtain glutamic acid as a single spot, which

was eluted and measured by the ninhydrine-colorimetric method of Yemm and Cooking [5)6)] at 570 mμ.

6. Determination of sodium in the plasma

Ten ml of concentrated hydrochloric acid was added to 0.2ml of plasma, and the mixture was diluted with distilled water to make the sample solution of 100ml for analysis.

The sodium content was directly determined by atomic absorption spectroscopy. (λ: 5890A°)[9)].

III Result

1. Subjective symptom

A MSG (8.7g) capsule was administered to each of the 14 subjects (one of them was given 4.4g of MSG)in the empty stomach condition, and let him record his subjective symptoms himself in the same way as in the previous experiments. The results are shown in Table 1.

The negative group of 5 persons chosen from among the subjects who had not experienced any symptom throughout the previous 3 experiments showed no symptom in this experiment, too.

On the other hand, each member of the positive group reacted to the MSG-intake as follows: No. 1 was a person especially chosen for the reason that he was an only subjects who had felt slight dizziness (after the intake of 4.4g of MSG in the empty stomach condition) but not any of the 7 symptoms listed in the previous experiments. He experienced a burning sensation in this experiment, though it lasted only for a very short time (5 min). No. 9: No symptom against nausea in the previous experiment (8.7g in the empty stomach condition). No. 27: burning sensation and chest pain against headache and thirst in prev. (4.4g empty). No. 29: none of the 7 symptoms this time against burning sensation, pressure sensation, thirst and feeling of weakness in prev.

(8.7g. empty). No. 30: burning sensation and nausea against nausea in prev. (8.7g. empty). No. 34: burning sensation against pressure sensation (not in the face) and feeling of weakness in prev. (4.4g, empty). No. 44: pressure sensation (not in the face) against pressure sensation and feeling of weakness in prev. (8.7g, empty). No. 51: headache and nausea against burning sensation, pressure sensation (not in the face) and thirst in prev. (8.7g, empty). No. 15: chest pain for a short time this time (4.4g) against chest pain and headache in prev. (4.4g, empty). The symptoms felt by individual persons in this experiment were thus considerably different from those experienced by them in the previous experiments, showing that there was nearly no reproducibility of the symptoms. In addition, a doctor, a third person, examined the subjects after the intake of MSG and found no external abnormality in them, even in the persons who felt subjective symptoms.

2. Blood pressure, pulse rate and electrocardiogram

The blood pressure was examined 5 min before, and 30, 60 and 90 min after the intake of MSG in every subject. The result shows that there was no remarkable difference as well as no definite tendency of change between the pressures examined before and after the intake (Table 2). There was also no difference in the pressure between the negative and positive groups. No noticeable difference was found among the pulse rates examined before and 5, 30, 45 and 60 min after the intake (Table 2). Electrocardiographic examinations were made 35 - 40 min after the intake in 3 persons (Nos. 21, 23 and 31) of the negative group and in 6 persons (Nos. 1, 27, 30, 34, 44 and 15) of the positive group. The results did not show any abnormality in the cardiograms as compared with the records obtained in ordinary conditions.

3. Changes of the concentration of glutamic acid and sodium in the blood

The concentration of glutamic acid in the plasma was determined before

and 30, 60 and 90 min after the intake as shown in Table 3. The concentration was 6 - 16 μg/ml before the intake, being 8.6 μg/ml in the negative group and 11.6 μg/ml in the positive group in average. The positive group thus tended to be a little higher in the content than in the negative one, but the difference was not significant. The concentration of glutamic acid in the plasma was clearly higher 30, 60 and 90 min after the intake of 8.7g of MSG than in the control (the value determined before the intake). The value had a tendency to reach the maximum 60 min after the intake, though the maximum value was attained 30 or 90 min after the intake in some cases. The maximum value was 74 μg/ml (60 min after) even in the highest case, being mostly 40 - 60 μg/ml.

And the value was generally 4 - 6 times as high as the control, though it reached 9 times in a case and was only 1.5 times in another. The increase of the concentration was very small when a smaller dose, 4.4g, of MSG was administered. But this dose was tested only in one case.

No significant difference was observed between the negative and positive groups in the change of the glutamic acid content with the lapse of time after the intake of MSG (8.7g).

The sodium ion content of the blood plasma was examined before, and 30, 60 and 90 min after the intake of 8.7g of MSG, showing no change after the administration.

IV. Consideration

Five persons who had been the subjects in the previous three experiments conducted by a double blind method and had never experienced any symptom throughout the experiments were selected to make a group, negative. And 9 persons among the subjects who had felt some CRS-like symptoms in the

same experiments as above were chosen as highly reactive subjects to MSG-intake to make another group, positive. The two groups of persons were subjected to this examination to investigate subjectively and objectively the effect of MSG-intake at a large dose in the empty stomach condition (8.7g except a case of 4.4g). The result proved that there was no reproducibility in the appearence of nearly all the symptoms examined, suggesting that the symptoms caused by the intake of a large amount of MSG were subjective and variable according to the physical and mental conditions of the subjects. Among the CRS-like symptoms, chest pain was only one (No. 15) sign which was felt in both the previous and this experiment. Though the symptoms caused by MSG-intake are thus highly subjective, it is probable that they are accompanied by some objective and clinical changes, such as blood pressure, pulse rate and electrocardiograms, if they are sufficiently serious. Examinations of these values and records, however, showed that there was no difference between the data obtained before and after the MSG-intake. Furthermore, no abnormality was observed in the electrocardiogram of a subject who was feeling a chest pain. The symptoms caused by MSG-intake, therefore, do not seem to be such important ones that they must be especially taken up from a clinical viewpoint.

On the other hand, if the symptoms are caused by MSG-intake, it is expected that the appearance of symptoms are closely related to the concentration of glutamic acid or sodium in the plasma. But the concentrations determined before and after the intake showed that there was no close relation between them: firstly, it can be said that MSG-intake did not cause any change in sodium content of the plasma; secondly, the appearance of symptoms is not due to variation of the absorption and metabolism of MSG, because there is no difference between the negative and the positive groups in the glutamic acid content of the blood after MSG-intake.

In fact, while No. 15 took 4.4g of MSG and showed a very small increase of glutamic content in the blood, he felt a chest pain. And the MSG-intake had no influence on the blood pressure and the electrocardiogram even in the time when he was feeling a chest pain. So it is doubtful whether the pain was actually due to MSG-intake or not in this case.

All these results of objective observations prove that the symptoms caused by MSG-intake are very subjective ones and not such serious signs that their appearance must be taken up as a clinically important subject.

Summary

To investigate the symptoms which are said to be caused by the intake of a large amount of MSG, their importance in judgement of the safety of MSG as a flavor enhancer, and the relation between Kwok's and Schaumburg's CRS symptoms and the CRS-like symptoms caused by MSG-intake, clinical experiments were conducted on 61 voluntary subjects in the non-empty and a empty stomach conditions.

When MSG was administered in the non-empty stomach condition, the appearance of CRS-symptoms was not more frequent in each of the experimental lots of MSG-intake at the doses of 2.2g, 4.4g and 8.7g than in the lot of placebo.

When MSG was administered in the empty stomach condition after 15 hours' fasting, the appearance of Schaumburg's CRS-symptoms was not found in the lots of MSG-2.2g and MSG-4.4g, but only three cases (about 6.1) were observed to be corresponding to them in the lot of MSG-8.7g.

Kwok et al. reported serious cases of CRS-symptoms, but such a case could not be observed throughout the authors' experiments.

In the next place, to examine objectively the effect of MSG-intake on the changes of blood pressure, pulse rate, electrocardiograms, concentrations

of glutamic acid and sodium in the plasma, an experiment was conducted in two groups of subjects, negative and positive. The negative group included 5 persons chosen from among the subjects of the abovementioned experiments and had never felt any symptoms throughout the experiments. The positive group had 9 members who had experienced some symptoms in the same experiments as above.

Blood pressure, pulse rate and electrocardiograms thus examined showed no change even in the lot of MSG-8.7g irrespective of the appearance of symptoms. Glutamic acid in the blood increased after MSG-intake, but its increase showed nearly no difference between the positive and the negative groups. There were also some cases in which the appearance of symptoms was accompanied by nearly no increase in the concentration of glutamic acid, suggesting that the effect of placebo must be rather taken into consideration as a factor of the appearance of symptoms in these cases.

The appearance of CRS-like symptoms was observed only in the positive group. But there were some subjects who did not felt any symptoms in this group, and detailed investigations revealed that most of the symptoms appearing in the second series of experiments (Part II) were different from those appearing in the previous experiments (Part I), showing that there is nearly no reproducibility in their appearance.

From the results of the two series of experiments, it is noticed that the symptoms caused by MSG-intake, especially CRS-like symptoms, are observed when MSG is administered at such a large dose as 8.7g to very susceptible persons in the empty stomach condition. But the symptoms are only subjective ones, which are variable according to the conditions at the time of intake and not alway reproducible, being objectively not abnormal. Consequently, they are considered to have no problem at all as to the safety of MSG which is in daily use as a flavor enhancer.

*1 The person especially was chosen, because of experience of slight dizziness at the previous experiments.

*2 The pressure sensation is not facial pressure.

Table 1. List of subjects and their subjective symptoms experienced.

Group	Subject No.	Age	Sex	Body weight (kg)	Subjective symptom throughout the previous 3 experiments							MSG (g)	Subjective symptom in this experiment ( ) = time of appearance of symptom						
					Burning sensation	Pressure sensation	Chest pain	Headache	Nausea	Thirst	Feeling of weakness		Burning sensation	Pressure sensation	Chest pain	Headache	Nausea	Thirst	Feeling of weakness
Negative group	21	41	male	72.8								8.7							
	23	37	"	67.5								"							
	31	38	"	68.0								"							
	42	37	"	56.0								"							
	57	38	"	45.0								"							
Positive group	1*1	48	"	50.5								"	+ (45-50)						
	9	44	"	67.0					+			"							
	27	39	"	53.4				+		+		"	+ (15-40)		+ (14-40)				
	29	44	"	66.0	+	+				+	+	"							
	30	40	"	59.7					+			"	+ (25-55)				+ (25-50)		
	34	39	"	59.0		(+)*2					+	"	+ (45-90)						
	44	45	"	57.5		+					+	"		(+)*2 (40-60)					
	51	53	"	51.0	+	(+)*2				+		"				+ (25-50)	+ (40-50)		
	(15)	39	"	49.2			+	+				4.4			+ (35-40)				+ (35-45)

Table 2. Blood pressure, pulse rate and electrocardiogram

Group	Subject No.	Blood pressure max/min (mm Hg)				Pulse rate (/ min)					Cardiogram (35-40 min after intake)
		Before	After			Before	After				
			30min	60min	90min		15min	30min	45min	60min	
Negative group	21	132/84	124/76	126/86	124/76	64	63	52	58	58	O.B.
	23	130/90	130/92	134/94	130/92	69	71	64	70	71	O.B.
	31	124/90	126/88	136/86	(132/86)*2	68	66	68	68	68	O.B.
	42	120/74	118/82	118/82	120/82	75	70	72	71	71	-
	57	128/68	128/76	126/70	124/74	88	80	80	75	72	-
Positive group	1	124/76	124/80	120/72	120/78	72	74	75	72	72	O.B.
	9	130/80	130/82	130/84	134/82	70	65	72	68	68	-
	27	116/66	116/66	112/64	110/64	76	72	72	72	68	O.B.
	29	128/86	124/84	124/84	128/86	68	70	70	66	66	-
	30	98/64	114/72	106/66	-	51	52	52	55	49	O.B.
	34	116/70	114/72	116/82	118/74	53	52	53	53	60	O.B.
	44	118/78	128/78	124/76	118/80	66	67	69	68	68	O.B.
	51	136/86	132/88	128/88	128/88	72	71	72	72	71	-
	(15)*1	106/68	100/64	104/70	110/78	58	60	60	64	60	O.B.

*1 ... MSG 4.4g *2 ... after 30 min

Table 3. The concentration of glutamic acid in plasma

Group	Subject No.	Glutamic acid in plasma (mg/ml)			
		Before	After 30min	After 60min	After 90min
Negative group	21	8(100)	12(150)	32(400)	27(338)
	23	12(100)	44(367)	33(275)	17(142)
	31	11(100)	38(345)	63(573)	56(509)*1)
	42	6(100)	9(150)	54(900)	29(483)
	57	6(100)	17(283)	27(450)	26(433)
	Mean ± S.E.	8.6±1.3 (100)	24.0±7.2 (279)	41.8±6.7 (486)	24.8±2.6*2) (288)
Positive group	1	8(100)	50(625)	45(563)	15(188)
	9	14(100)	23(164)	32(229)	44(314)
	27	11(100)	47(427)	71(643)	24(218)
	29	11(100)	32(291)	42(384)	53(482)
	30	15(100)	33(220)	49(327)	-
	34	11(100)	18(164)	74(673)	50(455)
	44	16(100)	23(144)	24(150)	23(144)
	51	9(100)	29(322)	38(422)	45(500)
	Mean ± S.E.	11.9±1.0*3) (100)	31.9±4.2 (268)	46.9±6.2 (394)	36.3±5.7*4) (305)
	15*5)	9	12	14	15

( ) = rate of value after/before
*1) after 80min *2) mean value of 4 persons except No. 31.
*3) 11.6±0.9 in the case of 9 persons with No. 15.
*4) mean value of 7 persons except No. 30.
*5) MSG-intake 4.4g

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The Effects of Oral Administration of MSG on Plasma Glutamic Acid Level in Rats and Mice

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Changes in the plasma glutamic acid level at intervals after the oral administration of MSG were examined in rats and mice, and the difference of changes were compared between neonates and adults.

1. Experimental Methods

1) Animal

Adult rats: Male adult rats of Wistar-Imamichi strain (11-14 weeks after birth, 300-350g body weight) were served after the starvation for 24 hours.

Neonatal rats: Male neonates of Wistar-Imamichi strain (2-3 days after birth, 6.0-8.0g body weight) were served immediately after separation from the mother.

Adult mice: Male mice of 4CS strain (11-14 weeks after birth, 25-30g body weight) were served after 24 hours starvation.

Neonatal mice: Male adult mice of 4CS strain (3-4 days after birth, 1.5-2.0g body weight) were served immediately after separation from the mother.

2) Administration Method

Adults: MSG solution of different concentrations was administered (1.0ml/100g b.w. until 4g/kg b.w. dosing but 2.0ml/kg b.w. for 8.0g/kg b.w. dosing) by a stomach tube. (Solvent; physiological saline solution for rats, and water for mice).

Neonates: MSG solution in water of corresponding concentrations was administered by a polyethylene-tube at the rate of 0.01ml/g.b.w.

3) Method of collecting blood

15-20 minutes after the administration, blood was collected by the following procedure.

Adult rats: Immediately after the slight anesthesia with ether, the chest was opened and blood was taken by heart puncture.

Adult mice, Neonatal rats and mice: Axillary artery was cut and blood was taken therefrom by the use of a heparinized capillary tube for hematocrit determination.

4) Determination of glutamic acid in plasma

Adult rats: about 5ml of blood was heparinized and centrifuged to separate plasma. 3 volumes of methanol was added to 0.5ml of the plasma and sterred. The mixture was allowed to stand overnight at 0°C, and then centrifuged for 10 min at 4,000 r.p.m. to remove the protein. The supernatant was dried under reduced pressure. The product was dissolved in a fixed amount of distrilled water to obtain the sample solution for the determination of glutamic acid.

20 µl of the deprotenized plasma solution thus prepared was subjected to paper electrophoretic analysis (Conditions: Toyo Roshi No.50 filter-paper,

buffer mixture; pyridine-acetic acid - water = 1 : 10 : 289, pH3.6, 345 V/cm, 120 min) to obtain glutamic acid as a single spot, which was eluted and measured by the ninhydrine-colorimetric method of Yemm and Cooking [1)2)] at 570 mμ.

Adult mice, neonatal rats and mice: The blood was centrifuged to separate plasma. On 20 μl of the plasma thus obtained was followed the same procedure as explained above.

3. Results

Results are shown in the attached figures.

References

1) Yemm, E.W. and Cocking, E.C., Analyst 80, 209 (1955)

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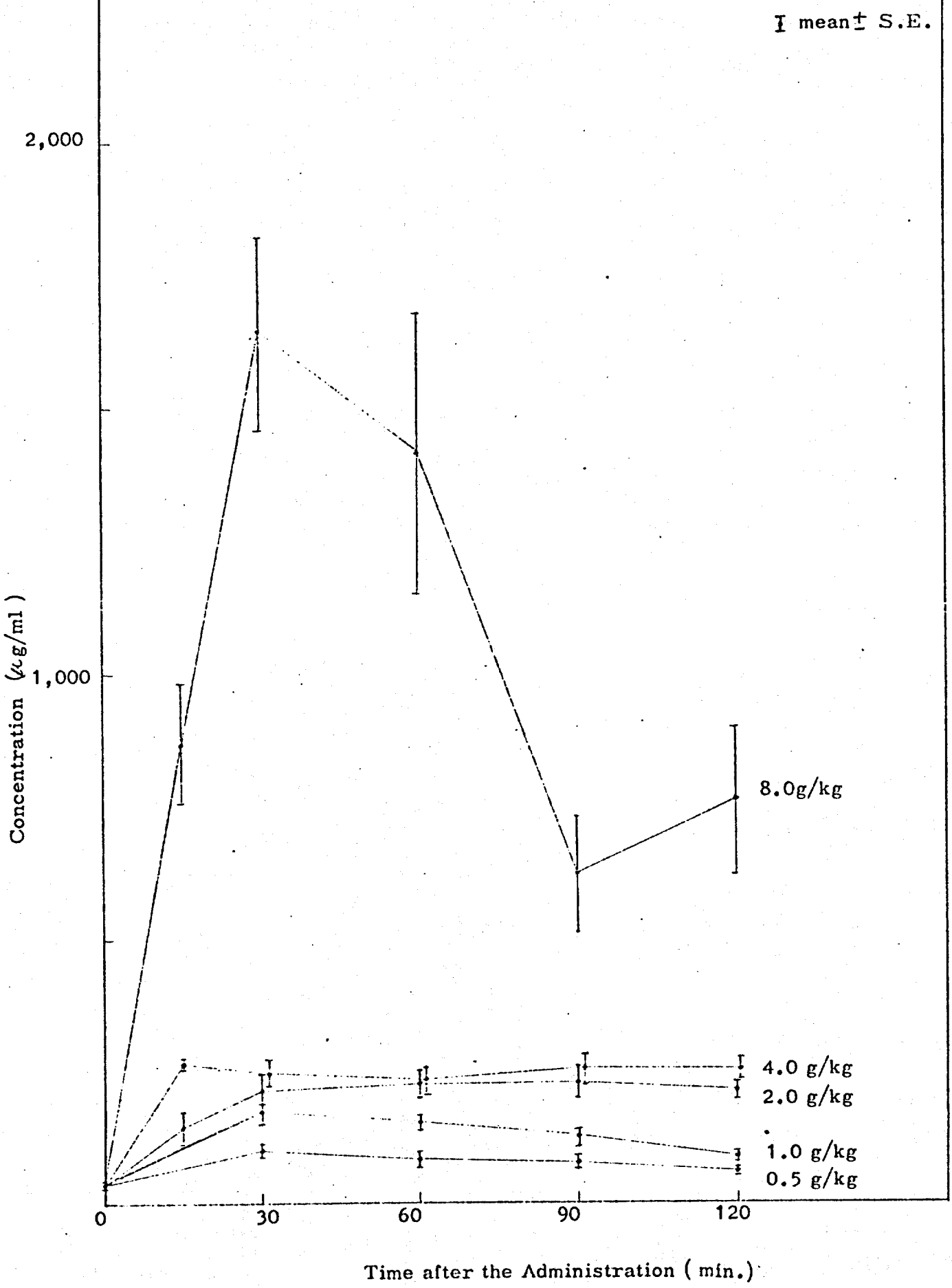
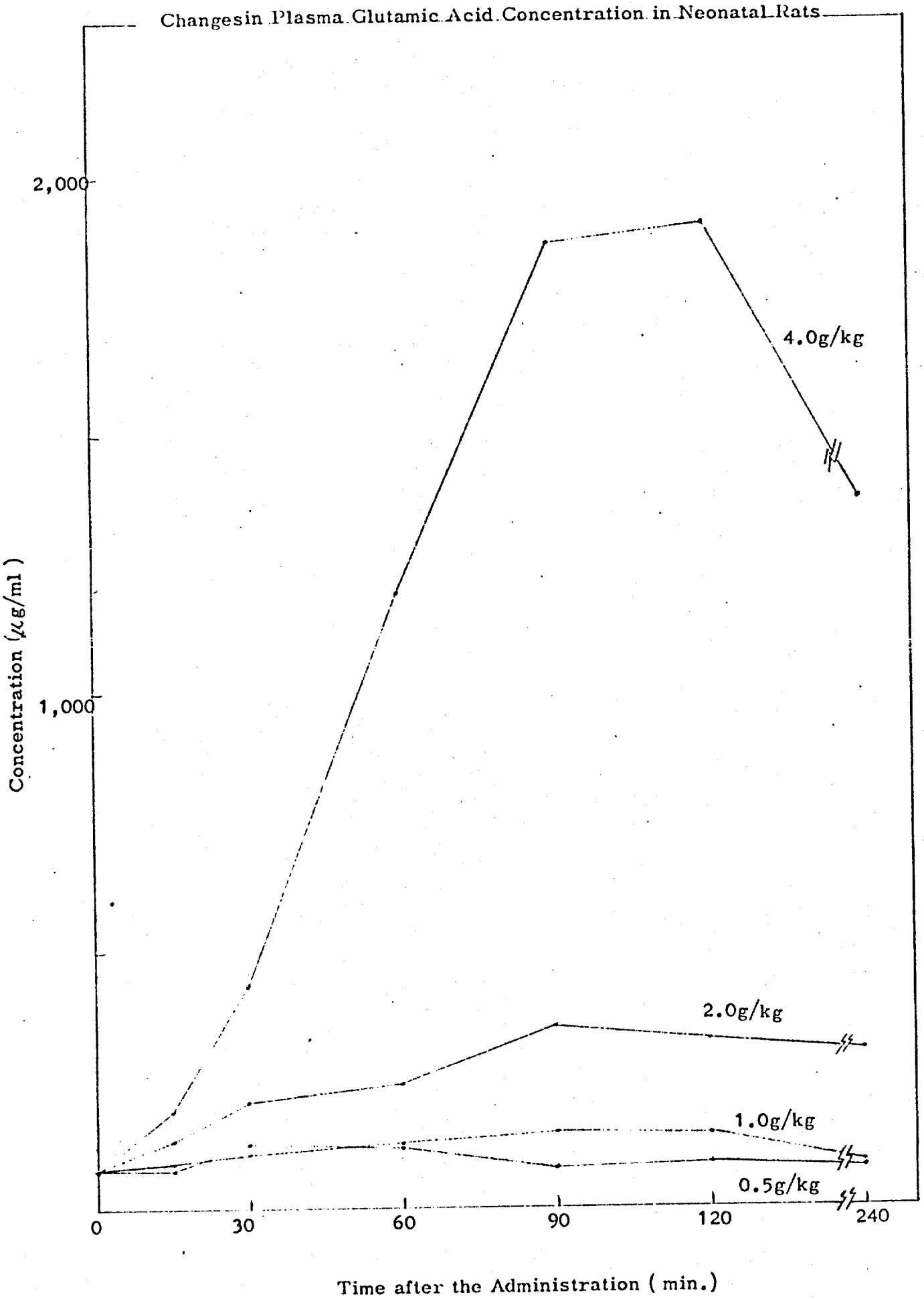
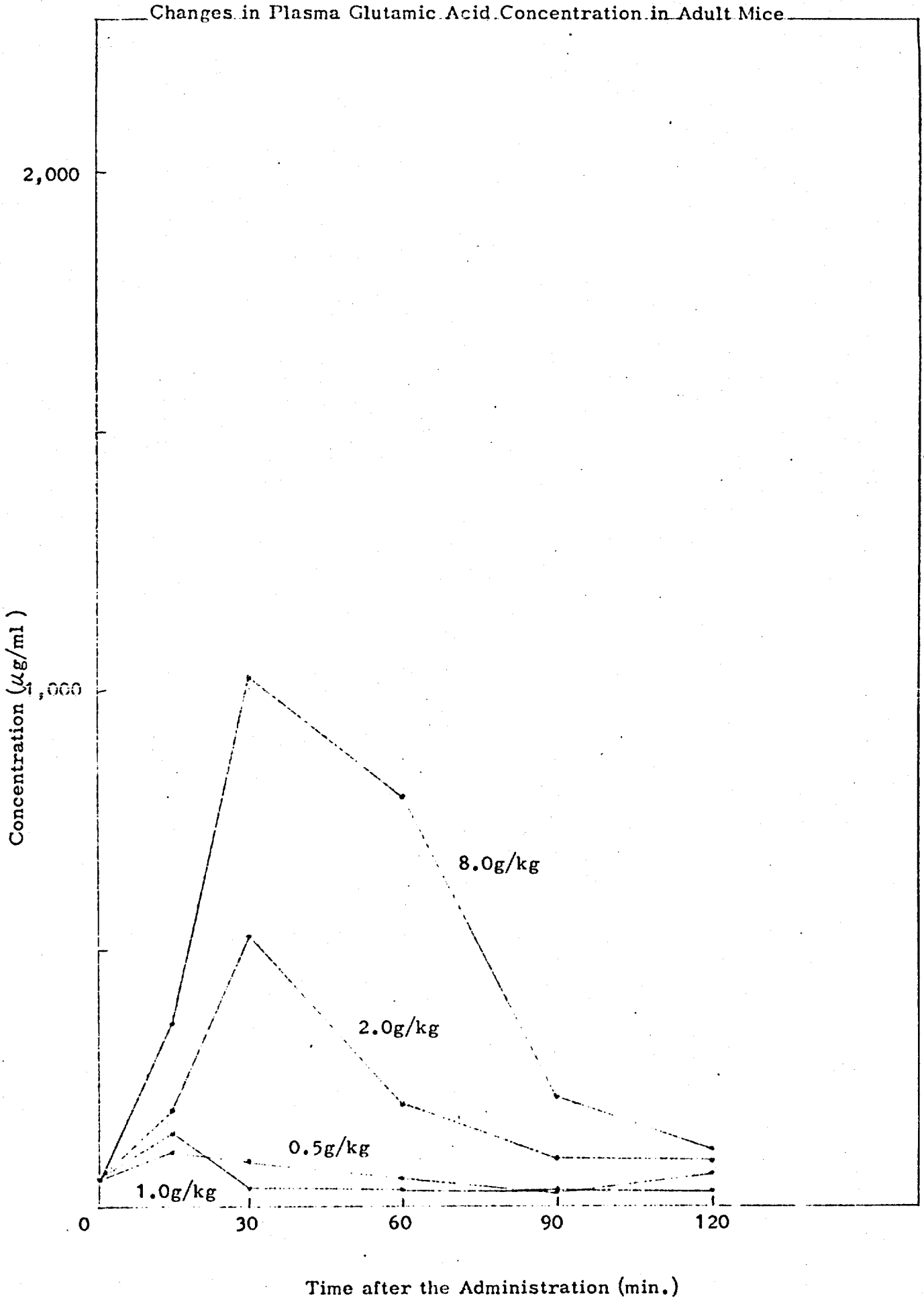
Changes in Plasma Glutamic Acid Concentration in Adult Rats



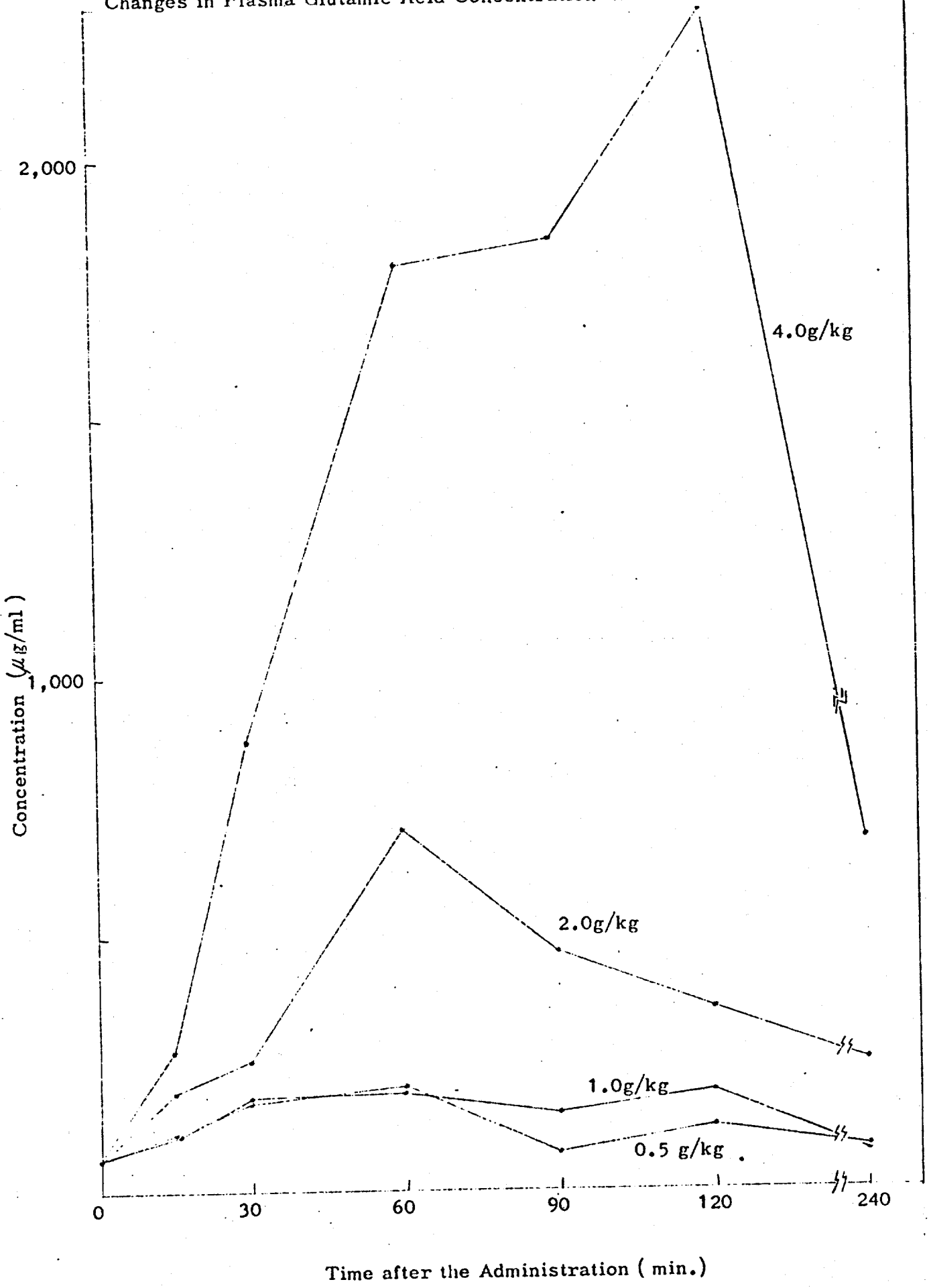
Changesin Plasma Glutamic Acid Concentration in Neonatal Rats



Changes in Plasma Glutamic Acid Concentration in Adult Mice



Changes in Plasma Glutamic Acid Concentration in Neonatal Mice



Epidemiological Investigations on the Chinese Restaurant Syndrome (CRS)

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1. Introduction

Strange symptoms called Chinese restaurant syndrome (CRS) have been observed recently in U.S.A.[1]. It is said that in these cases some persons had a numbness at the back of the neck, gradually extending to both arms and the back, general weakness and palpitation, 15-20 minutes after the beginning of taking meals at Chinese restaurants. H.H. Schaumburg et al found by chance during the studies on the cause of these symptoms that when persons were administered a large amount of monosodium glutamate (MSG) in the empty stomach, they had such symptoms as a burning sensation at a portion from the chest to the neck, shoulder and arms, a facial pressure and a chest pain, and he inferred that the MSG contained in chinese food was probably the substance which caused CRS.[2] [3]

Since MSG is a sodium salt of glutamic acid, which is generally present in many natural foods, we have been convinced that MSG is one of the most safe food additives. Accordingly, in relation to this finding, it is necessary from an epidemiological view point to clarify whether such symptoms are actually observed or not in Japan, where MSG has been used as a flavor enhancer since more than 60 years ago, and the average per-capita intake of MSG is estimated at about 2g per day.

In this survey was used Instant Ramen (Prepared Oriental Type Noodle), one of the food stuffs which is popular and highest in MSG content. The investigations were conduced on the experience of some symptoms by 912 persons who took Instant Ramen, in relation to their physical constitution and the past record of taking the noodle to examine whether such symptoms as CRS are actually observed or not. The results are reported in this paper.

II. Method and Items for Investigation

1. Time of investigation: August, 1969
2. Place of investigation: Kawasaki City, Kanagawa Prefecture
3. Persons examined: Employees of the Central Research Laboratories of Ajinomoto Co., Inc., both male and female, ranging from 18 to 60 in age. The classification by sex and age is shown in Table 1.
4. Method of investigation: Information was obtained through a questionnaire delivered to each person. To ensure the accuracy of investigations, the persons to be examined were only informed that the data were necessary for sales promotion in future (Single blind test).
5. Items for investigation

   (1) Physical Constitution: Weight, height, type of blood, digestive anamnesis, alcohol tolerance, allergic diathesis (If asthma, urticaria or chemical allergy is found in grandparents, parents, brothers or sisters, the person was tentatively considered to have the disposition), and the ataxic diathesis of digestive system.

   (2) Record of taking Instant Ramen: Amount per month and per serving, time of taking, choice in seasoning, addition of MSG to the noodle at the time of intake, brand of the noodle, (The contents of MSG in their noodle were shown in Table 2) intake level of the Ramen soup, amount of MSG taken per month.

(3) Experience of some symptoms caused by the intake of Instant Ramen:

The following symptoms presumed to be due to the intake of Instant Ramen were selected, and inquiries were made about them in the questionnaire to consider in relation to CRS.

* Stomach-ache, oppressive feeling in the stomach, heavy sitting on the stomach, nausea, thirst, palpitation, headache, dizziness, perspiration, abdominal pain, diarrhea, and others (concretely).

6. Tabulation and analysis of the data

The results of investigations were noted down on cards by individuals and put on record in an electronic computer for analysis. First, taking every reasonable combination into consideration, χ^2- tests were carried out abnormal signs ($p<0.05$ or $p<0.01$), and secondly multiple correlation with examined regarding these factors and CRS-like symptoms to make clear the main causes of the significance.

III. Results and Consideration

1. Experience of some symptoms caused by the intake of Instant Ramen

The number of persons who had ever had one or more experiences of some symptoms caused by the intake of Instant Ramen was 495 (52.3%) among the 912 persons. It was classified by the symptoms as shown in Table 3.

Among the symptoms, the case of thirst is the largest in number, accounting for 34% of all cases. It is followed by heavy sitting on the stomach (28%), perspiration (17%) and stomach-ache, abdominal pain and diarrhea (less than 10%). In regard to the symptoms of involving autonomic nervous systems in CRS, the case of warm feeling represented 4.0%, and the cases of palpitation, headache and dizziness less than 1%.

The others are made up of the cases of cructation, dyspepsia and inflammation of the oral cavity, etc.

The relations between the sex and age of persons and the occurrence of these symptoms were examined by the χ^2-test, which showed a tendency that the occurrences of diarrhea, perspiration and abdominal pain were a little more frequent in the male than in the female and those of oppressive feeling in the stomach, heavy sitting on the stomach, nausea and diarrhea were often experienced by young people.

2. Relation between the occurrence of abnormal symptoms after the intake of Instant Ramen and the constitutional factor.

As shown in Table 3, each symptom showed nearly no sexual difference in frequency, so further analysis were made on the total number of both sexes. To avoid a decrease of frequency in each group and to simplify the analysis, simple tabulation was made in disregard of age. The result is shown in Table 4.

(1) Relation to the allergic diathesis

The persons and their family of relatives in the first and second degrees were examined to see the relations between the occurrence of abnormal symptoms after taking Instant Ramen and the asthma, urtiaria, eczema and chemical allergy as a manifestation of allergic diathesis. The results show that the symptoms of thirst, oppressive feeling in the stomach and perspiration tend to occur frequently in persons with allergic diathesis (Table 4).

(2) Relation to the ataxic diathesis of digestive system

The persons and their family of relatives in the first and second degrees were examined to know whether there are any relations between the cases of abnormal symptoms and the ataxic diathesis of digestive system manifested by food poisoning, diarrhea and constipation. The results show that the ataxic diathesis has relations to the occurrences of heavy sitting on the atomach,** thirst,** abdominal pain,** oppressive feeling in the stomach and diarrhea,* respectively (Table 4).

The occurrences of oppressive feeling in the stomach, ** heavy sitting on the stomach, ** nausea,** and thirst** are 100% more frequent in constipated persons than in the controls respectively. The cases of heavy sitting on the stomach ** and thirst ** are 50% more frequent in the persons habitually suffering from diarrhea. The case of diarrhea * shows the same tendency. The symptoms of oppressive feeling in the stomach * and heavy sitting on the stomach * tend to be the same in the case of food poisoning.

(3) Relation to type of blood, alcohol tolerance, digestive anamnesia and circulatory abnormalities (hypertension and hypotension).

The relations between the occurrences of the symptoms and type of blood, alcohol tolerance, (digestive anamnesia and the constitutional abnormality of circulatory system manifested by hypertension or hypotension observed in family histories were examined, respectively. There are no correlation between them (Table 5.).

(4) Brief account

Heavy sitting on the stomach, thirst and perspiration, which were abnormal signs frequent in the above-mentioned simple tabulation, seem to be hardly distinguishable from a sense of satiety, a phenomenon observed when salty soup is taken, and a physiological reaction to the intake of food in hot conditions, respectively.

On the contrary, the symptoms which were lower than 10% in occurrence are certainly due to pathologic irritation of the digestive system.

But CRS-like symptoms as palpitation, headache and dizziness, were only less than 1% in occurrence, respectively.

Though there are many factors which have relations to the symptoms thus taken up or are in need of further examination, the χ^2-tests by simple tabulation proves that the symptoms tend to be frequent in such persons as young, allergic or constitutionally dyspeptic (what is called poor digestion).

3. Relation between the occurrences of the symptoms and the condition of taking Instant Ramen

The results of analysis in the preceding paragraph show that each symptom is affected by respective factors arising from personal diathesis or constitution. It is, however, understood from the table that the difference in occurrence of symptoms is not always greatly influenced by the presence of special diathesis. Therefore, the relation between the condition of taking Instant Ramen and the occurrence of symptoms was examined by simple tabulation as follows:

(1) Relation to the average intake level of Instant Ramen per month

There are some relations between the average intake level of the noodle per month and the occurrence of thirst, ** perspiration, ** warm feeling, * and diarrhea,* respectively. Among the symptoms the case of perspiration seems to be more frequent in occurrence as the daily intake increases. But no definite tendency was observed in the others (Table 6-1).

(2) Relation to the amount of Instant Ramen per serving

There are some relations between the amount of Instant Ramen per serving and the occurrences of heavy sitting on the stomach, * thirst * and perspiration,* respectively, which show a tendency that the larger the amount is the more frequent the occurrences of thirst and perspiration are (Table 6-2).

(3) Relation to the amount of soup taken with Instant Ramen

There are relations between the intake level of Ramen Soup and the occurrences of thirst,** perspiration,** and heavy sitting on the stomach,* respectively, showing a tendency that the larger the amount is, the more frequent the occurrences of thirst and perspiration (Table 6-3).

(4) Relation to the time of taking Instant Ramen

The relations between the degree of hunger and the occurrences of the symptoms were examined (Table 6-4). There is no remarkable correlation between them but the abdominal pain* tends to occur if the noodle is taken into the full stomach. This is probably due to overeating.

(5) Brief account

Examination of the relations between the condition of taking Instant Ramen and the occurrence of symptoms showed that the average intake level per month correlated significantly with such symptoms as thirst, warm feeling, perspiration and diarrhea. But the occurrence of these symptoms can be said to increase clearly only in the groups of more than 10 servings per month, and the main symptoms in this case are warm feeling and perspiration.

As to the intake level of the noodle per serving, it is noticed that the occurrence of heavy sitting on the stomach was rather frequent in the groups of persons taking a smaller amount of the noodle.

Perspiration had no relation to the amount of the noodle taken. Only the occurrence of thirst showed correlation with the amount of the noodle taken.

As to the intake of the soup---symptoms which were proved to be frequent in persons taking the whole amount of the soup with the noodle are thirst, warm feeling and perspiration.

When the taking of Instant Ramen in the empty stomach is compared with that in the full stomach, only abdominal pain was shown to be significantly frequent in the case of taking after a meal (in the full stomach) or between meals (in the half-full stomach).

All symptoms in quenstion but diarrhea are hardly distinguishable, as mentioned above, from physiological phenomena and can not be always said to be caused by the intake of Instant Ramen. Regarding these results, it is noteworthy that there was no significant correlation between the occurrence of CRS-like symptoms and the condition of taking the noodle.

4. Relations between the occurrences of the symptoms and the amount of MSG ingested.

The tabulation in the preceding paragraph was based on the intake of Instant Ramen itself. Then the data will be analyzed as much as possible on the basis of the estimated amount of MSG-intake.

(1) Relation to the MSG content in the soup of Instant Ramen.

The amount of MSG contained in the soup of Instant Ramen varies according to the brand of the noodle. Analysis of the most popular brands shows that its content on the dry basis is 1.3g at maximum and 0.6g at minimum per serving as shown in Table 2. The relations between the MSG content of the soup and the occurrences of the symptoms were examined in each brand of the noodle (Table 7-1), but there was no significant correlation between them.

(2) Relation to the addition of MSG in Instant Ramen at the time of taking.

Relations between the addition of MSG to Instant Ramen at the time of taking (including the additional use in the course of cooking) and the occurrences of the symptoms were examined (Table 7-2). No marked correlation was observed between them, but the occurrence of thirst* was little more frequent in the case of the addition of MSG than in the control.

(3) Relation to the choice in the seasoning (especially the preference of MSG) of Instant Ramen.

Relations between the choice in the seasoning, that is, the preference of MSG, of Instant Ramen and the occurrences of the symptoms were examined (Table 7-3). The result reveals that there is no remarkable correlation

between them. But the occurrence of heavy sitting on the stomach* seems to be a little frequent in the persons who like the noodle not so strongly seasoned with MSG.

(4) Relation to the amount of MSG in ordinary use.

Relation between the average intake level of MSG per month and the occurrence of symptoms was examined. (To obtain the average intake level of MSG for the person in question, the monthly average consumption of MSG taken from "AJI-NO-MOTO" and "Hi-Me" by his family was calculated and divided by the number of the family members. Accordingly, the amount for single persons who live in boarding houses or dine out is understimated. In other words, this value represents only the amount of MSG used in cooking or at table in each family and contains no MSG coming from processed foods. It is, therefore, not an exact intake level of MSG.) Although the examination has no relation to the amount of Instant Ramen taken and the result suggests nothing about the connection between the abnormal signs caused by the intake of the noodle and MSG, it indicates that the amount of MSG in ordinary use has no bearing on the occurrence of abnormal signs as shown in Table 8 for reference.

(5) Brief account

When the occurrence of symptoms was examined from a viewpoint of MSG-intake, no significant correlation was found between the occurrence of them and the brand of Instant Ramen (the MSG content varies according to the brand of the noodle).

But slightly significant connection was observed between thirst and the addition of MSG to the noodle at the time of taking, and also between

the choice in taste of the noodle (the degree of preference of MSG) and heavy sitting on the stomach.

No symptom showed significant correlation with the amount of MSG in ordinary use, and it was made clear that there is no relation between the symptoms caused by taking Instant Ramen and the amount of MSG in ordinary use.

It is noticeable from the above-mentioned results that the intake level of MSG had no relation to the occurrence of CRS-like symptoms.

5. Effects of compound factors

So far as the results of this investigation were surveyed as mentioned above, every factor examined seemed to have no relation to the appearance of CRS-symptoms. In the following, a discussion will be made to examine compound effects of some factors by way of precaution.

The factors taken up in this investigation are so large in number that the examination of every compound of them is substantially impossible, because the numbers of the combination increase factorially with the increase in number of factors to be examined. Therefore two constitutional factors, the allergy and the ataxic diathesis of digestive system, which in the experiment showed significant relation to some of CRS-like symptoms, were subjected to analysis of their relations with CRS-like symptoms in connection with the average quantity of MSG used. There are also some other interesting ones to be examined than the above, but they could not be taken up as the subjects of statistical analysis because of their low frequencies. There are also some other compounds, whose effect

will be easily presumed from the results mentioned below and need no special analysis. Consequently, the subject of discussion here will be limited to the above two constitutions.

Table 9's are contingency tables for the average monthly intake of MSG, the allergic diathesis and the ataxic diathesis of digestive system in 6 symptoms which are regarded as CRS-like ones. In table 9-1 (pressure sensation in the stomach), the figures in parentheses show expected value of frequencies which are expected when the 4 factors including the symptom are independent of each other, and the differences between the expected and the observed values were statistically significant at the level of 1%. Similarly, the differences were also highly significant in each of the other 5 symptoms.

To make clear the location where the significance is present, the presence of factors was evaluated as 1 and the absence of them as 0, though such a classification is not sufficiently proper, to submit the data to multiple regression analysis. Only the cases of the pressure sensation in the stomach and the perspiration are dealt with here, because these symptoms were frequent in appearance.

The equation of regression and the evaluation are as follows:

$$\hat{y}_p = a_0+a_1x_1+a_2x_2+a_3x_3+a_4x_4+a_5x_5+a_6x_6$$

y_1:	Pressure sensation in the stomach	appearance	1,	non-appearance	0
y_2:	Perspiration	"	1,	"	0
x_1:	MSG-intake	much	1,	little	0
x_2:	Allergy	presence	1,	absence	0
x_3:	Ataxic diathesis of digestive system	"	1,	"	0

x_4:	Allergy x Ataxic diathesis of digestive system	positive correlation	1,	negative c.	0
x_5:	MSG-intake x Allergy	"	1,	"	0
x_6:	MSG-intake x Asynergy of digestive organs	"	1,	"	0

In the analysis of the data, it was found that there was some correlation between the variables as follows:

MSG-intake:	Allergy	r=-0.0299
MSG-intake:	Ataxic diathesis of digestive system	r=0.0153
Allergy:	Ataxic diathesis of digestive system	r=0.3065**

Table 10 is a contingency table of the allergic diathesis and the ataxic diathesis of digestive system. Remarkable correlation is observed between them. The correlation was proved to be significant by a χ^2-test, and the appearance of the ataxic diathesis of digestive system seems to be highly frequent in constitutionally allergic persons as clearly seen in the comparison of actual frequencies with the expected values in parentheses.

The effects of the individual factors on the appearance of symptoms are tentatively shown in Tables 11-1 and 11-2, and the results of multiple regression analysis are given in Table 12-1 and 12-2. It is noticeable from these two series of tables that the significant relation which is observed between a factor and the appearance of symptoms by simple regression analysis can not be found in multiple regression analysis at all. This is a phenomenon often observed when correlation between the factors are very notable. For instance, in the case of the pressure sensation

in the stomach, it is evident that 3 factors showing more than 7 per cent of contribution in simple regression, but their contribution is only 11.4% in total in the multiple regression. The same relation is also seen in the case of perspiration. The correlation between the factors is already stated.

The relation between the MSG-intake x allergy and the pressure sensation in the stomach is significant in simple regression analysis. But it is impossible to avoid the correlation between the compound factors in view of the nature of the data, and especially in this case the correlation between them is very significant, r=0.5408. So it can not be concluded that the compound factors have actually such an effect. To investigate this point, a contingency table of the pressure sensation in the stomach was made by the MSG-intake, and the allergy as shown in Table 13-1 and the difference between expected and observed frequencies was analyzed by a χ^2 -test as usual, obtaining the result that the difference can not be rejected statistically. Similar analysis was made in the other constitution and symptoms, finding no significant relation between them as shown in Table 13-2.

Another case of compound factors which need simple regression analysis is the combination of the ataxic diathesis of digestive system and the allergy. As it was known that there was considerably significant correlation between them, their compounds were classified into four groups, ++, +-, -+ and --, and a contingency table was made to analyze the relation between the compounds and each symptom by a χ^2-test, obtaining the result as shown in Table 14, there was no case which shood significant relation

at a level of 5%, though the χ^2-value calculated in the pressure sensation in the stomach was considerably near to the level of significance as shown, for reference, in Table 15. The appearance of the pressure sensation in the stomach seems to be especially frequent in the group susceptive to ataxic diathesis of digestive system and the allergy. The multiple regression analysis suggests that the ataxic diathesis of digestive system has a more noticeable effect than the other do, though the difference is not significant statistically.

Brief account

Investigations were made on the possibility of the induction of CRS-like symptoms by the effect of compound factors in connection with the quantity of MSG intake. It was doubted from contingency tables if there were significant relations with CRS-like symptoms, but further investigations revealed the following facts.

(1) The all cases of statistic significance observed in the contingency tables are mainly due to the significant correlation between the allergy and the ataxic diathesis of digestive system both of which are the constitutional factors taken up in this study.

(2) There is no evidence that the compound factors influence the appearance of the symptoms in connection with the average monthly intake of MSG.

(3) The allergic diathesis and the ataxic diathesis of digestive system when combined show the greatest effect on the pressure sensation in the stomach.

The conclusion (1) will be useful in considering the epidemislogical investigation of this kind. In conclusion (3), though the pressure sensation in the stomach is certainly a CRS-like symptom, the symptom thus picked up singly is only a general manifestation, and it is quite natural that the symptom is induced by the compound effects of the indigestible Instant Ramen, the ataxic diathesis of digestive system and the allergy. Therefore, it is unreasonable to discuss the result in (3) in relation to CRS - Symptoms.

IV Discussion

Relations between the symptoms caused by the intake of Instant Ramen and CRS.

Relations between CRS and the occurrences of abnormal signs after the taking of Instant Ramen, which is representative of the foodstuffs containing large amounts of MSG, were examined to investigate whether CRS-like symptoms are observed or not in such a condition of MSG intake as is usual in Japan and, if any symptom was observed, also whether they are due to CRS or not. The result obtained shows that there is no evidence which suggests relations between them.

The symptoms caused by the intake of Instant Ramen are mainly abnormalities of digestive system, such as stomach-ache, oppressive feeling in the stomach, heavy sitting on the stomach, abdominal pain and diarrhea. It is also probable that they are accompanied by nausea and others. These digestive abnormalities can be easily forecast to occur, because Instant Ramen is mainly composed of α-starch, containing edible oil and salty

water called "Kansui", and is often taken in a form which may cause dyspepsia for the reason of insufficient cooking. Actually, there are many reports on these cases.

The fact that the occurrence of thirst is highest among the symptoms seems to be the manifestation of a normal physiological desire after the taking of food containing much salt and spices.

The occurrence of perspiration is also not strange, because Instant Ramen contains much spices and is taken while it is hot.

The cases of warm feeling, dizziness, headache and palpitation are very rare, and they may be considered to be secondary symptoms accidentally caused by the above-mentioned digestive abnormalities and by the perspiration.

When the symptoms mentioned above are compared with those of CRS, the symptoms of palpitation and headache are analogous, but these are extremely low in the percentage of occurrence. And what are called the three main symptoms, burning sensation, facial pressure and chest pain, which are claimed to be caused by the taking of MSG in large quantities, can not be observed in the cases attributed to Instant Ramen.

The relations between the symptoms caused by the ingestion of Instant Ramen and the intake of MSG were examined to further clarify these problems, but there was no relation between the occurrences of the symptoms and the MSG content of Instant Ramen.

CRS and the CRS-like symptoms caused by the taking of MSG in large quantity are reported to occur after taking into the empty stomach, while the occurrence of symptoms after the taking of Instant Ramen is found to have no relation to the degree of hunger at the time of taking.

This fact also suggests that the symptoms caused by the taking of Instant Ramen have no relation to CRS.

From the results of investigations mentioned above, it is probably unthinkable that CRS occurs in the condition of normal MSG intake in Japan.

V Summary

1. To investigate whether CRS is observed or not in the condition of normal MSG intake in Japan, 912 persons were examined to see if they experienced any symptoms after the intake of Instant Ramen which is representative of the foodstuffs containing MSG in large quantities.

2. It was difficult to trace an analogy between CRS and the symptoms caused by the intake of Instant Ramen.

3. No relation was observed between the symptoms caused by the intake of Instant Ramen and those by MSG.

From the results mentioned above, it can be said that CRS is not induced to occur in the condition of normal MSG in take in Japan.

Table 1. Classification of persons by sexes and ages

	≦ 20 (year-old)	20-30	31-40	41-50	51 ≦	Total
Men	3 (persons)	440	202	40	35	720
Women	54	125	8	5	0	192
Total	57	565	210	45	35	912

Table 2. MSG content in the noodle soup of typical brand

Brand	MSG Content (g/serving)
a	0.94
b	0.79
c	1.36
d	0.87
e	1.33
f	0.61
g	0.92

Table 3. Relations between the sex and age of persons and the occurrence of the symptoms caused by the intake of Instant Ramen.

Occurrence rate of symtoms			Sexes		Ages				
Symptoms \ Number of persons		Total 912	men 720	women 192	≦ 20 57	21-30 565	31-40 210	41-50 45	51 ≦ 35
Stomach-ache	+ (%)	55 (6.0)	43 (6.0)	12 (6.3)	6 (10.5)	40 (7.1)	9 (4.3)	0 (0)	0 (0)
Oppressive feeling in the stomach	+ (%)	100 (10.1)	75 (10.4)	25 (13.0)	7 ** (12.3)	76** (13.5)	17 ** (8.1)	0 ** (0)	0 ** (0)
Heavy sitting on the stomach	+ (%)	257 (28.2)	202 (28.1)	55 (28.6)	12 ** (21.1)	187 ** (33.1)	55 ** (26.2)	1** (2.2)	2 ** (5.7)
Nausea	+ (%)	59 (6.5)	48 (6.7)	11 (5.7)	3 * (5.3)	48 * (8.5)	8 * (3.8)	0 * (0)	0 * (0)
Thirst	+ (%)	313 (34.3)	248 (34.4)	65 (33.9)	21 (36.8)	208 (36.8)	65 (32.2)	11 (24.4)	8 (22.9)
Palpitation	+ (%)	4 (0.4)	3 (0.4)	1 (0.5)	0 (0)	2 (0.4)	2 (1.0)	0 (0)	0 (0)
Headache	+ (%)	5 (0.5)	5 (0.7)	0 (0)	0 (0)	2 (0.4)	3 (1.4)	0 (0)	0 (0)
Dizziness	+ (%)	8 (0.9)	6 (0.8)	2 (1.0)	0 (0)	6 (1.1)	2 (1.0)	0 (0)	0 (0)
Warm feeling	+ (%)	36 (3.9)	32 (4.4)	4 (2.1)	1 (1.8)	25 (4.4)	8 (3.8)	2 (4.4)	0 (0)
Perspiration	+ (%)	156 (17.1)	133 * (18.5)	23 * (12.0)	7 (12.3)	107 (18.9)	33 (15.7)	5 (11.1)	4 (11.4)
Abdominal pain	+ (%)	53 (5.8)	49 * (6.8)	4 * (2.1)	1 (1.8)	41 (7.3)	10 (4.8)	1 (2.2)	0 (0)
Diarrhea	+ (%)	87 (9.5)	80 ** (11.1)	7 ** (3.6)	0 * (0)	65 * (11.5)	19 * (9.0)	3 * (6.7)	0 * (0)
Others	+ (%)	7 (0.8)	7 (1.0)	0 (0)	0 (0)	5 (0.9)	2 (1.0)	0 (0)	0 (0)

** : $p < 0.01$

* : $p < 0.05$

Table 4. Relation between the allergic diathesis, ataxic diathesis of digestive system, diarrhea, constipation and food poisoning, and occurrence of the symptoms

Symptoms \ Number of persons		Allergic diathesis		Ataxic diathesis		Diarrhea (in person)		Constipation (in person)		Food poisoning (in person)	
		+ 514	− 398	+ 685	− 227	+ 360	− 552	+ 635	− 277	+ 215	− 697
Stomach-ache	+ (%)	33 (6.4)	22 (5.5)	46 (6.7)	9 (4.0)	24 (6.7)	31 (5.6)	42 (6.6)	13 (4.7)	15 (7.0)	40 (5.7)
Oppresive feeling in the stomach	+ (%)	66 (12.8)	34 (8.5)	84 * (12.3)	16 * (7.0)	61 ** (16.9)	39 ** (7.1)	76 (12.0)	24 (8.7)	32 * (14.9)	68 * (9.8)
Heavy sitting on the stomach	+ (%)	155 (30.2)	102 (25.6)	213 ** (31.1)	44 ** (19.4)	129 ** (35.8)	128 ** (23.2)	199 ** (31.3)	58 ** (20.9)	74 * (34.4)	183 * (26.3)
Nausea	+ (%)	35 (6.8)	24 (6.0)	49 (7.2)	10 (4.4)	33 (9.7)	24 (4.3)	46 (7.2)	13 (4.7)	20 (9.3)	39 (5.6)
Thirst	+ (%)	195 ** (37.9)	118 ** (29.6)	258 ** (37.7)	55 ** (24.2)	150 ** (41.7)	163 ** (29.5)	241 ** (38.0)	72 ** (26.0)	78 (36.3)	235 (33.7)
Palpitation	+ (%)	2 (0.4)	2 (0.5)	4 (0.6)	0 (0)	3 (0.8)	1 (0.2)	3 (0.5)	1 (0.4)	0 (0)	4 (0.6)
Headache	+ (%)	1 (0.2)	4 (1.0)	4 (0.6)	1 (0.4)	3 (0.8)	2 (0.4)	3 (0.5)	2 (0.7)	1 (0.5)	4 (0.6)
Dizziness	+ (%)	6 (1.2)	2 (0.5)	7 (1.0)	1 (4.4)	6 (1.7)	2 (0.4)	6 (0.9)	2 (0.7)	2 (0.9)	6 (0.9)
Warm feeling	+ (%)	18 (3.5)	18 (4.5)	26 (3.8)	10 (4.4)	18 (5.0)	18 (3.3)	24 (3.8)	12 (4.3)	5 (2.3)	31 (4.4)
Perspiration	+ (%)	100 * (19.5)	56 * (14.1)	126 (18.4)	30 (13.2)	77 (21.4)	79 (14.3)	115 (18.1)	41 (14.8)	39 (18.1)	117 (16.8)
Abdominal pain	+ (%)	34 (6.6)	19 (4.8)	48 ** (7.0)	5 ** (2.2)	25 (6.9)	28 (5.1)	43 (6.8)	10 (3.6)	18 (8.4)	35 (5.0)
Diarrhea	+ (%)	53 (10.3)	34 (8.5)	74 * (10.8)	13 * (5.7)	32 (8.9)	55 (10.0)	70 * (11.0)	17 * (6.1)	30 (14.0)	57 (8.2)
Others	+ (%)	1 (0.2)	6 (1.5)	4 (0.6)	3 (1.3)	0 (0)	7 (1.3)	4 (0.6)	3 (1.1)	1 (0.5)	6 (0.9)

** : $p < 0.01$

* : $p < 0.05$

Table 5. Relation between the blood group, alcohol tolerance, digestive anamnesis and circulatory abnormalities and occurrence of symptoms

Symptoms / Number of persons		Blood Group					Alcohol Tolerance				Digestive Anamnesis		Circulatory Abnormalities	
		A 343	B 203	AB 98	O 246	Unclear 22	Heavy Drinker 297	Small Drinker 375	Constitutional Hypersensitivity 52	Unclear 188	+ 136	− 776	+ 164	− 748
Stomach-ache	+ (%)	23 (6.7)	10 (4.9)	5 (5.1)	16 (6.5)	1 (4.5)	16 (5.4)	18 (4.8)	2 (3.8)	19 (10.1)	10 (7.4)	45 (5.8)	13 (7.9)	44 (5.9)
Oppressive feeling in the stomach	+ (%)	36 (10.5)	23 (11.3)	10 (10.2)	28 (11.4)	3 (13.6)	26 (8.8)	41 (10.9)	6 (11.5)	27 (14.4)	20 (14.7)	80 (10.3)	25 (15.2)	75 (10.0)
Heavy sitting in the stomach	+ (%)	104 (30.3)	46 (22.7)	25 (25.5)	74 (30.1)	8 (36.4)	81 (27.3)	104 (27.7)	13 (25.0)	59 (31.4)	45 (33.1)	212 (27.3)	53 (32.3)	204 (27.3)
Nausea	+ (%)	26 (7.6)	10 (4.9)	6 (6.1)	14 (5.7)	3 (13.6)	21 (7.1)	20 (5.3)	3 (5.8)	15 (8.0)	9 (6.6)	50 (6.4)	9 (5.5)	50 (6.7)
Thirst	+ (%)	106 (30.9)	74 (36.5)	36 (36.7)	87 (35.4)	10 (45.5)	99 (33.3)	135 (36.0)	12 (23.1)	67 (35.6)	38 (27.9)	275 (35.4)	66 (40.2)	247 (33.0)
Palpitation	+ (%)	0 (0)	1 (0.5)	0 (0)	3 (1.2)	0 (0)	3 (1.0)	0 (0)	0 (0)	1 (0.5)	1 (0.7)	3 (0.4)	1 (10.0)	3 (0.4)
Headache	+ (%)	1 (0.3)	1 (0.5)	1 (1.0)	2 (0.8)	0 (0)	2 (1.0)	2 (0.5)	0 (0)	1 (0.5)	2 (1.5)	3 (0.4)	0 (0)	5 (0.7)
Dizziness	+ (%)	2 (0.6)	2 (1.0)	2 (2.0)	1 (0.4)	1 (4.5)	3 (1.0)	2 (0.5)	0 (0)	2 (1.1)	1 (0.7)	7 (0.9)	2 (1.2)	6 (0.8)
Warm feeling	+ (%)	14 (4.1)	8 (3.9)	4 (4.1)	7 (2.8)	3 (13.6)	14 (4.7)	13 (3.5)	2 (3.8)	7 (3.7)	7 (5.1)	29 (3.7)	5 (3.0)	31 (4.1)
Perspiration	+ (%)	55 (16.0)	35 (17.2)	13 (13.3)	49 (19.9)	4 (18.2)	56 (18.9)	61 (16.3)	4 (7.7)	35 (18.6)	25 (18.4)	131 (16.9)	30 (18.3)	126 (16.8)
Abdominal pain	+ (%)	19 (5.5)	14 (6.9)	8 (8.2)	12 (4.9)	0 (0)	19 (6.4)	21 (5.6)	3 (5.8)	10 (5.3)	9 (6.6)	44 (5.7)	8 (4.9)	45 (6.0)
Diarrhea	+ (%)	30 (8.7)	21 (10.3)	11 (11.2)	24 (9.8)	1 (4.5)	29 (9.8)	34 (9.1)	5 (9.6)	19 (10.1)	12 (8.8)	75 (9.7)	16 (9.8)	71 (9.5)
Others	+ (%)	1 (0.3)	2 (1.0)	2 (2.0)	2 (0.8)	0 (0)	1 (0.3)	3 (0.8)	0 (0)	3 (1.6)	1 (0.7)	6 (0.8)	2 (1.2)	5 (0.7)

Table 6. Relation between the occurrence of symptoms and the mean amount per month, at a time and the condition of taking Instant Ramen and the quantity of soup taken with Instant Ramen

		Mean amount taken per month (plate/month)				Amount taken at a time (plate/a time)				Quantity of soup taken with Instant Ramen			Condition of taking			
													on quasi full stomach		on empty stomach	
Symptoms \ Number of persons		≦ 2.0 [illegible]	2.1-6.0 354	6.1-10.0 83	10.1< 29	< 1.0 84	1.0 745	1.5 45	2≦ 38	little take 104	take a half 487	take all 321	after meal 12	during meal 489	before meal 52	in place of meal 349
Stomach-ache	+	29	20	5	1	4	47	2	2	7	30	18	0	26	4	15
	(%)	(6.5)	(5.3)	(6.0)	(3.4)	(4.8)	(6.3)	(4.4)	(5.3)	(6.7)	(6.2)	(5.6)	(0)	(5.3)	(7.7)	(4.3)
Oppresive feeling in the stomach	+	52	36	11	1	8	84	5	3	10	57	33	1	52	5	30
	(%)	(11.7)	(10.2)	(13.3)	(3.4)	(9.5)	(11.3)	(11.1)	(7.9)	(9.6)	(11.7)	(10.3)	(8.3)	(10.6)	(9.6)	(8.6)
Heavy sitting on the stomach	+	125	110	17	5	* 18	* 225	* 9	* 5	* 21	* 153	* 83	4	153	12	82
	(%)	(28.0)	(31.1)	(20.5)	(17.2)	(21.4)	(30.2)	(20.0)	(13.2)	(20.2)	(31.4)	(25.9)	(33.3)	(31.3)	(23.1)	(23.5)
Nausea	+	29	24	5	1	5	48	3	3	5	38	16	0	32	4	13
	(%)	(6.5)	(6.8)	(6.0)	(3.4)	(6.0)	(6.4)	(6.7)	(7.9)	(4.8)	(7.8)	(5.0)	(0)	(6.5)	(7.7)	(3.7)
Thirst	+	** 123	** 146	** 30	** 14	* 16	* 265	* 17	* 15	** 23	** 162	** 128	5	191	17	121
	(%)	(27.6)	(41.2)	(36.1)	(48.3)	(19.0)	(35.6)	(37.8)	(39.5)	(22.1)	(33.3)	(39.9)	(41.7)	(39.1)	(32.7)	(34.7)
Palpitation	+	4	0	0	0	1	3	0	0	0	2	2	0	0	0	2
	(%)	(0.9)	(0)	(0)	(0)	(1.2)	(0.4)	(0)	(0)	(0)	(0.4)	(0.6)	(0)	(0)	(0)	(0.6)
Headache	+	4	0	1	0	0	4	1	0	1	1	3	0	1	1	1
	(%)	(0.9)	(0)	(1.2)	(0)	(0)	(0.5)	(2.2)	(0)	(1.0)	(0.2)	(0.9)	(0)	(0.2)	(1.9)	(0.3)
Dizziness	+	5	2	1	0	1	7	0	0	1	4	3	0	3	0	3
	(%)	(1.1)	(0.6)	(1.2)	(0)	(1.2)	(0.9)	(0)	(0)	(1.0)	(0.8)	(0.9)	(0)	(0.6)	(0)	(0.9)
Warm feeling	+	* 18	* 12	* 2	* 4	1	29	4	2	2	18	16	0	24	3	12
	(%)	(4.0)	(3.4)	(2.4)	(13.8)	(1.2)	(3.9)	(8.9)	(5.3)	(1.9)	(3.7)	(5.0)	(0)	(4.9)	(5.8)	(3.4)
Perspiration	+	** 56	** 74	** 17	** 9	* 6	* 132	* 11	* 7	** 8	** 81	** 67	1	94	11	63
	(%)	(12.6)	(20.9)	(20.5)	(31.0)	(7.1)	(17.7)	(24.4)	(18.4)	(7.7)	(16.6)	(20.9)	(8.3)	(19.2)	(21.2)	(18.1)
Abdominal pain	+	25	23	3	2	4	45	2	2	5	30	18	* 1	* 33	* 2	* 8
	(%)	(5.6)	(6.5)	(3.6)	(6.9)	(4.8)	(6.0)	(4.4)	(5.3)	(4.8)	(6.2)	(5.6)	(8.3)	(6.7)	(3.8)	(2.3)
Diarrhea	+	* 39	* 42	* 2	* 4	5	75	4	3	9	47	31	0	54	5	24
	(%)	(8.7)	(11.9)	(2.4)	(13.8)	(6.0)	(10.1)	(8.9)	(7.9)	(8.7)	(9.7)	(9.7)	(0)	(11.0)	(9.6)	(6.9)
Others	+	5	1	0	1	0	7	0	0	1	2	4	0	3	1	3
	(%)	(1.1)	(0.3)	(0)	(3.4)	(0)	(0.9)	(0)	(0)	(1.0)	(0.4)	(1.2)	(0)	(0.6)	(1.9)	(0.9)

** : $p < 0.01$

* : $p < 0.05$

Table 7. Relation between occurrence of the symptoms and the MSG content in the soup of Instant Ramen, the addition of MSG to Instant Ramen at the time of taking and the choice in the seasoning of Instant Ramen.

		MSG content in the soup (g/plate)			Addition of MSG at the time of taking			Choice in the seasoning (especially the taste of MSG)	
Symptoms \ Number of persons		< 0.8 178	0.8-1.1 433	1.1 < 182	no adding 313	often adding 165	adding 434	Had better not make strong 448	Had better make strong 464
Stomach-ache	+ (%)	11 (6.2)	23 (5.3)	12 (6.6)	13 (4.2)	11 (6.7)	31 (7.1)	26 (5.8)	29 (6.3)
Oppresive feeling in the stomach	+ (%)	19 (10.7)	46 (10.6)	25 (13.7)	40 (12.8)	19 (11.5)	41 (9.4)	56 (12.5)	44 (9.5)
Heavy sitting on the stomach	+ (%)	47 (26.4)	121 (27.9)	56 (30.8)	92 (29.4)	48 (29.1)	117 (27.0)	140 * (31.3)	117 * (25.2)
Nausea	+ (%)	11 (6.2)	27 (6.2)	10 (5.5)	21 (6.7)	10 (6.1)	28 (6.5)	32 (7.1)	27 (5.8)
Thirst	+ (%)	66 (37.1)	159 (36.7)	61 (33.5)	91* (29.1)	64 * (38.8)	158* (36.4)	148 (33.0)	165 (35.6)
Palpitation	+ (%)	1 (0.6)	3 (0.7)	1 (0.5)	0 (0)	0 (0)	4 (0.9)	2 (0.4)	2 (0.4)
Headache	+ (%)	2 (1.1)	4 (0.9)	1 (0.5)	1 (0.3)	0 (0)	4 (0.9)	4 (0.9)	1 (0.2)
Hot head	+ (%)	1 (0.6)	6 (1.4)	2 (1.1)	3 (1.0)	1 (0.6)	4 (0.9)	6 (1.3)	2 (0.4)
Warm feeling	+ (%)	5 (2.8)	23 (5.3)	12 (6.6)	13 (4.2)	5 (3.0)	18 (4.1)	19 (4.2)	17 (3.7)
Perspiration	+ (%)	34 (19.1)	85 (19.6)	28 (15.4)	46 (14.7)	30 (18.2)	80 (18.4)	75 (16.7)	81 (17.5)
Abdominal pain	+ (%)	12 (6.7)	22 (5.1)	9 (4.9)	21 (6.7)	6 (3.6)	26 (6.0)	27 (6.0)	26 (5.6)
Diarrhea	+ (%)	15 (8.4)	44 (10.2)	15 (8.2)	36 (11.5)	12 (7.3)	39 (9.0)	44 (9.8)	43 (9.3)
Others	+ (%)	1 (0.6)	1 (0.2)	2 (1.1)	4 (1.3)	1 (0.6)	2 (0.5)	2 (0.4)	5 (1.1)

* : $p < 0.05$

Table 8. Relation between occurrence of the symptoms and the quantity of usual MSG intake

Symptoms \ Number of persons		Quantity of usual MSG intake (g/person/month)			
		≦30.0 497	30.1-60.0 208	60.1-90.0 125	90.1< 82
Stomach-ache	+ (%)	37 (7.4)	11 (5.3)	6 (4.8)	1 (1.2)
Oppresive feeling in the stomach	+ (%)	65 (13.1)	16 (7.7)	12 (9.6)	7 (8.5)
Heavy sitting on the stomach	+ (%)	151 (30.4)	53 (25.5)	28 (22.4)	25 (30.5)
Nausea	+ (%)	36 (7.2)	8 (3.8)	10 (8.0)	5 (6.1)
Thirst	+ (%)	169 (34.0)	68 (32.7)	44 (35.2)	32 (39.0)
Palpitation	+ (%)	2 (0.4)	0 (0)	2 (1.6)	0 (0)
Headache	+ (%)	3 (0.6)	1 (0.5)	1 (0.8)	0 (0)
Dizziness	+ (%)	3 (0.6)	2 (1.0)	1 (0.8)	2 (2.4)
Warm feeling	+ (%)	21 (4.2)	8 (3.8)	1 (0.8)	6 (7.3)
Perspiration	+ (%)	85 (17.1)	38 (18.3)	15 (12.0)	18 (22.0)
Abdominal pain	+ (%)	32 (6.4)	12 (5.8)	6 (4.8)	3 (3.7)
Diarrhea	+ (%)	53 (10.7)	14 (6.7)	11 (8.8)	9 (11.0)
Others	+ (%)	5 (1.0)	1 (0.5)	1 (0.8)	0 (0)

Table 9 Relation Between The Appearance Of Symptoms And The Average Monthly MSG-intake, The Allergic Diathesis And The Ataxic Diathesis of Digestive System

(9-1) Appearance of pressure sensation in stomach

			Average monthly MSG-intake (g)								
			≦ 30.0		30.1-60.0		60.1-90.0		90.1 ≦		
pressure sensation in stomach		Allergy	+	-	+	-	+	-	+	-	
Ataxic diathesis of digestive system	+	+	40 (25.1)	12 (20.1)	12(11.6)	3 (7.4)	5 (5.6)	5 (5.7)	4 (4.5)	2 (0.0)	**
		-	202(187.2)	128(145.9)	94(83.8)	36(53.5)	51(40.9)	37(41.6)	38(32.3)	16(21.8)	
	-	+	4 (4.8)	9 (3.9)	1 (2.2)	0 (1.4)	0 (1.1)	1 (1.1)	0 (0.9)	1 (0.6)	
		-	30 (60.9)	72 (51.1)	20(29.4)	42(18.7)	6(14.3)	20(12.1)	7(10.3)	14 (7.9)	

(9-2) Appearance of palpitation

			Average monthly MSG-intake (g)								
			≦ 30.0		30.1-60.0		60.1-90.0		90.1 ≦		
Palpitation		Allergy	+	-	+	-	+	-	+	-	
Ataxic diathesis of digestive system	+	+	2	0	0	0	0	2	0	0	**
		-	240	140	106	39	56	40	42	18	
	-	+	0	0	0	0	0	0	0	0	
		-	34	81	21	42	6	21	7	15	

(9-3) Apperance of headache

			Average monthly MSG-intake (g)								
			≦ 30.0		30.1-60.0		60.1-90.0		90.1 ≦		
Headache		Allergy	+	-	+	-	+	-	+	-	
Ataxic diathesis of digestive system	+	+	1	1	0	1	0	1	0	0	**
		-	241	139	106	38	56	41	42	18	
	-	+	0	1	0	0	0	0	0	0	
		-	34	80	21	42	6	21	7	15	

Note ** : $P < 0.01$

(9-4) Appearance of hot head

		Average monthly MSG-intage (g)								
		≦ 30.0		30.1-60.0		60.1-90.0		90.1 ≦		
Hot head / Allergy		+	-	+	-	+	-	+	-	
Ataxic diathesis of digestive system +	+	2	0	2	0	0	1	2	0	**
	-	240	140	104	39	56	41	40	18	
Ataxic diathesis of digestive system -	+	0	1	0	0	0	0	0	0	
	-	34	80	21	42	6	1	7	15	

(9-5) Appearance of warm feeling

		Average monthly MSG-intake (g)								
		≦ 30.0		30.1-60.0		60.1-90.0		90.1 ≦		
Appearance of warm feeling / Allergy		+	-	+	-	+	-	+	-	
Ataxic diathesis of digestive system +	+	8	7	5	2	0	1	2	1	**
	-	234	133	101	37	56	41	40	17	
Ataxic diathesis of digestive system -	+	1	5	0	1	0	0	2	1	
	-	33	76	21	41	6	21	5	14	

(9-6) Appearance of perspitation

		Average monthly MSG-intake (g)								
		≦ 30.0		30.1-60.0		60.1-90.0		90.1 ≦		
Palpitation / Allergy		+	-	+	-	+	-	+	-	
Ataxic diathesis of digestive system +	+	48	21	23	6	8	6	9	5	**
	-	194	119	83	33	48	36	33	13	
Ataxic diathesis of digestive system -	+	6	10	4	5	0	1	2	2	
	-	28	71	17	37	6	20	5	13	

Table 10 Relation Between The Allergic Diathesis And Ataxic Diathesis Of Digestive System

		Allergic Diathesis		
		+	-	
Ataxic diathesis of digestive system	+	446 (387)	239 (298)	**
	-	68 (128)	159 (90)	

Note ** : $p < 0.01$

(9-4) Appearance of hot head

		Average monthly MSG-intake (g)								
		≦ 30.0		30.1-60.0		60.1-90.0		90.1≦		
Hot head	Allergy	+	−	+	−	+	−	+	−	
Ataxic diathesis of digestive system +	+	2	0	2	0	0	1	2	0	**
	−	240	140	104	39	56	41	40	18	
Ataxic diathesis of digestive system −	+	0	1	0	0	0	0	0	0	
	−	34	80	21	42	6	1	7	15	

(9-5) Appearance of warm feeling

		Average monthly MSG-intake (g)								
		≦ 30.0		30.1-60.0		60.1-90.0		90.1≦		
Appearance of warm feeling	Allergy	+	−	+	−	+	−	+	−	
Ataxic diathesis of digestive system +	+	8	7	5	2	0	1	2	1	**
	−	234	133	101	37	56	41	40	17	
Ataxic diathesis of digestive system −	+	1	5	0	1	0	0	2	1	
	−	33	76	21	41	6	21	5	14	

(9-6) Appearance of perspitation

		Average monthly MSG-intake (g)								
		≦ 30.0		30.1-60.0		60.1-90.0		90.1≦		
Palpitation	Allergy	+	−	+	−	+	−	+	−	
Ataxic diathesis of digestive system +	+	48	21	23	6	8	6	9	5	**
	−	194	119	83	33	48	36	33	13	
Ataxic diathesis of digestive system −	+	6	10	4	5	0	1	2	2	
	−	28	71	17	37	6	20	5	13	

Table 10 Relation Between The Allergic Diathesis And Ataxic Diathesis Of Digestive System

		Allergic Diathesis		
		+	−	
Ataxic diathesis of digestive system	+	446 (387)	239 (298)	**
	−	68 (1[illegible]8)	159 (90)	

Note ** : $P < 0.01$

Table 11 Relation between The Facters And The Symptoms By Single Regression Analysis

(11-1) Pressure sensation in stomach

Facter	Coefficient	$\sqrt{\text{Variance of coefficient}}$	Multiple correlation coefficient	t level
MSG-intake	-0.0279	±0.0246	3.76	1.136
Allergy	0.0455	0.0208	7.25	2.194*
Ataxic diathesis of digestive system	0.0507	0.0238	7.05	2.130*
Allergy X Ataxic diathesis of digestive system	0.0311	0.028	4.72	1.425
MSG-intake X Allergy	-0.0524	0.0206	8.38	2.536*
MSG-intake X Ataxic diathesis of digestive system	-0.0305	0.0214	4.73	1.429

(11-2) Perspiration

Facter	Coefficient	$\sqrt{\text{Variance of coefficient}}$	Multiple correlation coefficient	t level
MSG-intake	-0.0150	±0.0299	1.67	0.505
Allergy	0.0538	0.0189	7.02	2.142*
Ataxic diathesis of digestive system	0.0518	0.0289	5.95	1.797
Allergy X Ataxic diathesis of digestive system	0.0123	0.0265	1.55	0.467
MSG-intake X Allergy	-0.0427	0.0255	5.64	1.705
MSG-intake X Ataxic diathesis of digestive system	-0.0211	0.0259	2.70	0.815

Note * : $P < 0.05$

Table 12 Multiple Regression Analysis To Symptoms

(12-1) Pressure sensation in stomach

Facter	MSG-intake	Allergy	Ataxic diathesis of digestive system	Allergy X ataxic diathesis of digestive system	MSG-intake X allergy	MSG-intake X ataxic diathesis of digestive system
Coefficient	-0.0351	-0.0008	0.0564	0.0222	-0.0434	0.0222
Varience of coefficient	±0.0290	0.0296	0.0310	0.0258	0.0260	0.0303
t Level	1.216	0.027	1.826	0.861	1.677	0.737
Multiple correlation coefficient	- 11.40% -					

(12-2) Perspiration

Factor	MSG-intake	Allergy	Ataxic diathesis of digestive system	Allergy X ataxic diathesis of digestive system	MSG-intake X allergy	MSG-intake X ataxic diathesis of digestive system
Coefficient	-0.0237	0.0368	0.0466	-0.0110	-0.0237	0.0227
Variance of coefficient	±0.0352	0.0360	0.0377	0.0314	0.0315	0.0368
t Level	0.678	1.027	1.242	0.354	0.755	0.619
Multiple correlation coefficient						

Table 13-1 Allergic Diathesis - MSG-intake - Pressure Sensation In Stomach

Allergy \ Pressure sensation in stomach \ MSG-intake		≦ 30.0	30.1-60.0	60.1-90.0	90.0 ~	Total
+	+	44 (36.0)	13 (15.0)	5 (9.0)	4 (5.9)	66
	-	232(244.1)	114(102.2)	57(61.4)	45(40.3)	448
-	+	21 (18.0)	3 (7.5)	6 (4.5)	3 (3.0)	33
	-	200(199.0)	78 (83.2)	57(50.0)	30(32.8)	365
Total		497	208	125	82	912

$\chi^2 = 12.51 < 18.31$ (ϕ = 16-3-1-1-1 = 10)

Table 13-2 Relation Between MSG-intake, Constitution and Symptoms

Factor	χ^2
Allergic diathesis-MSG-intake-Pressure sensation in stomach	12.51
Ataxic diathesis of digestive system - MSG-intake - Pressure sensation in stomach	12.96
Allergic diathesis - MSG-intake - Perspiration	8.63
Ataxic diathesis of digestive system - MSG-intake - Perspiration	9.86

Table 14 Relation Between Symptoms And Constitution (Allergy And Ataxic Diathesis of Digestive System)

Symptom	χ^2
Pressure sensation in stomach	7.5*
Palpitation	2.0
Headache	3.73
Dizziness	3.4
Warm sensation	0.8
Perspiration	5.94

Table 15 Relation Between Pressure Sensation In Stomach And Constitution (Allergic diathesis and ataxic diathesis of digestive system)

Ataxic diathesis of digestive system		+		-	
Allergic diathesis		+	-	+	-
Pressure sensation in stomach	+	61 (48.4)	22 (26.0)	5 (6.7)	11 (15.8)
	-	385 (396.5)	217 (213.0)	63 (60.6)	148 (141.5)

References

1) H. H. Kuok, New Engl. J. Med. 278, 796 (1968)

2) H. H. Schaumburg and R. Byck, New Eng. J. Med. 279, 105 (1968)

3) H. H. Schaumburg, R. Byck, R. Gerstl and J. H. Mashman, Science 163, 826 (1969)

Remarks --- Explanation on Instant Ramen

Instant Ramen (Prepared Oriental Type Noodle or Instant Noodle) is one type of noodle, which is mainly composed of noodle of α-starch, with separately packed dried-soup, and is generally taken after short boiling.

The manufacturing process of the noodle is as follows:

1. Materials and Manufacturing

1) Example of manufacturing process[1) 2)]

Egg, Lard, Sesame Oil
Wheat flour
Table salt, "Kansui" * → (Mixing) → (Kneading)
Food improver, Pigment

→ (Forming of Noodle) → (Steaming) → (Cooling)

→ (Molding) → (Frying) → (Draining the excess oil)

→ (Drying) → Packaging

* Kansui : Salty water including one or more of K_2CO_3, Na_2CO_3, $NaHCO_3$ and Phosphate of Potassium or Sodium.

2) Table of contents of separately packed soup

Table salt	45 - 60%
Sugar	3 - 10%
Flavor enhancer (Chemically processed)	10 - 20%
Flavor enhancer (Natural)	5 - 10%
Oil and Fat	6 - 15%
Spices *	3 - 5%

* Spices : onion, garlic, pepper, red pepper etc.

2. Composition

Instant Ramen per serving is composed of 90 - 100g of the noodle and 7 - 8g of separately packed soup.

Table of the composition of Instant Ramen [3]

Water	4.0(g)	Ashes	6.0(g)
Protein	19.0	Patassium	0.024
Fat	24.0	Sodium	2.0
Starch	46.0	Phosphorus	0.080
Cellulose	0.3	Ferrous	0.0022

3. Production and Consumption of Instant Ramen in Japan.

Production : 3,100,000,000 servings in 1967
: 3,300,000,000 servings in 1968 [4]

Consumption : 3,000,000,000 servings in 1967

Reference

1) Resources Bureau, Science and Technology Agency:

Report of Resources Bureau, Science and Technology Agency. No.49. "Report of Investigation on Food Additives" page 165 (1969).

2) Society for the Study of Food Stuffs:

"Technic of Noodle" page 76 (1967)

3) Resources Bureau, Science and Technology Agency:

"standard Tables of Food Composition in Japan" (third revised edition) page 18 (1963).

4) Nippon Shokuryo Shimbunsha:

"1969th Yearbook of Provisions. Part of Statistics and Data" page 166 (1969).

第一四八〇五號　第七十七類

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特許　明治四十一年七月二十五日

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「グルタミン」酸鹽ヲ主要成分トセル調味料製造法

本發明ハ鹽酸若クハ硫酸ノ如キ强酸ノ作用ニヨリ蛋白質若クハ蛋白質含有物質ヲ加水分解セシメタル果成物ヨリ「グルタミン」酸鹽ヲ主要成分トシテ含メル調味料ヲ製造スル方法ナリ「グルタミン」酸鹽類カ最モ濃厚ナル快美ノ味ヲ呈シ調味品タルニ適スルコト及ヒ此物カ調味用昆布ノ主要ナル有效成分タルコトハ本發明者カ始テ發見シタル所ニシテ此鹽ハ又實ニ普通醬油ノ有效成中主要ノモノナルコト疑ヲ容レス故ニ複雜ニシテ物資及ヒ時間ヲ費スルコト多キ從來ノ醬油醸造法ニ代ルニ簡易捷徑ノ方法ヲ以テシ且ツ有效成分ヲ含ムコト比較的ニ僅少ナル鰹節、昆布、肉「エキス」等ニ代ルヘキ濃厚純潔ニシテ而モ廉價ナル調味料ヲ製造スルコト是レ本發明ノ目的ナリ

本發明ヲ實行スルニ用フル蛋白質ハ其植物性タルト動物性タルトニ論ナク加水分解ニヨリテ比較的多量ノ「グルタミン」酸ヲ生スルモノヲ好トス例ヘハ小麥粉ヨリ製取スル麩質、牛乳ヨリ分離セル「カセイン」ノ如キハ原料トシテ適當ナルモノナリ然レトモ大豆ノ蛋白質ノ如ク分離ノ困難ナルモノニ在リテハ原料ヲ其儘若クハ單ニ脂油ヲ除去スル如キ一部分離ノ操作ヲ施シタルノミニテ使用スルヲ妨ケス加水分解ヲ行フニハ三乃至十規定濃度ノ鹽酸若クハ硫酸ヲ用ヒ之ニ重量八分一乃至三分一ノ蛋白質ヲ投入シ徐々ニ熱シテ溶解セシメ而シテ後溫度ヲ昇シ加熱ヲ繼續シテ加水分解ヲ完結セシム此際酸及ヒ水分ノ逃逸ヲ防ク爲ニ容器ノ上部ヲ密閉シ若クハ適當ノ冷却裝置ヲ備フルヲ要ス

硫酸ヲ用ヒタル場合ニハ石灰ヲ用ヒテ之ヲ中和シ生シタル石膏ヲ濾シ去リ尙ホ適量ノ炭酸曹達ヲ加ヘテ「カルシウム」分ヲ炭酸鹽トシテ沈澱セシム斯クスレハ開揚色ニシテ好良ノ味ヲ有スル液ヲ生スルヲ以テ之ヲ蒸發乾燥シテ一割乃至四割ノ「グルタミン」酸「ナトリウム」ヲ含メル調味料ヲ製ス

最モ純濃ノ調味料ヲ製スルニハ右ニ記載シタル濃褐色液ヲ蒸發シテ適度ノ濃ト爲シ而シテ之ニ適量ノ硫酸ヲ加ヘテ放置シ游離「グルタミン」酸ヲ結晶トシテ析出セシメ斯クシテ得タル「グルタミン」酸ヲ常法ニヨリテ脫色シ再結晶法ニヨリテ精製シタル後之ヲ曹達ニテ中和シテ純粹ノ「グルタミン」酸「ナトリウム」ヲ製ス此鹽ハ極メテ水ニ溶ケ易ケレトモ潮解性ナク白色無臭ニシテ最モ純潔濃厚ノ調味料タリ又右ノ如ク「ナトリウム」鹽ヲ造ル代ニ「グルタミン」酸七分ト重炭酸曹達四分トノ混合物ヲ造リテ之ニ代用スルモ可ナリ

鹽酸ヲ用ヒテ蛋白質ヲ加水分解シタル場合ニハ腐蝕質狀ノ黑色物ヲ生スルヲ以テ之ヲ濾シ去リタル濾液ヲ蒸溜シテ鹽酸ノ一部分ヲ回收シ濃稠ノ液ヲ殘留セシム此液ヲ曹達ニテ中和シタルモノ及ヒ之ヲ蒸發乾涸シタルモノハ調味料トシテ使用スルヲ得ヘシ

一層濃厚ノ調味料ヲ製スルニハ前記蒸溜殘液ヲ冷却放置シテ鹽化水素「グルタミン」酸ヲ結晶トシテ充分ニ析出セシメ之ヲ母液ヨリ別チ更ニ濃鹽酸ヨリ再結晶セシメテ純粹ノ鹽化水素「グルタミン」酸ト爲シ之ヲ曹達ニテ中和シ「グルタミン」酸「ナトリウム」ト食鹽トノ混合物ヲ製ス

食鹽分ヲ混有セサル純濃ノ調味料ヲ造ルニハ前記ノ方法ニヨリテ得タル鹽化水素「グルタミン」酸ヲ水ニ溶シ之ニ適量ノ曹達ヲ加ヘテ鹽化水素ヲ中和スレハ「グルタミン」酸ハ結晶トナリテ析出スルヲ以テ之ヲ用ヒテ「グルタミン」酸「ナトリウム」ヲ製スルコト上文ニ記シタルカ如シ

閣リ「ナトリウム」鹽ノミナラス「グルタミン」酸鹽ハ皆同樣ノ味ヲ呈スルモノニシテ「カルシウム」鹽「カリウム」鹽ノ如キハ其味殊ニ純粹ナリサレハ營養ノ目的上特殊ノ金屬鹽ノ供給スル必要アル場合ニハ其金屬ノ「グルタミン」酸鹽ヲ調味料トシテ使用スルヲ便トスルコトアルヘシ

强酸ノ作用ニヨリ蛋白質ヲ加水分解シテ製シ「グルタミン」酸鹽ヲ主要成分トセル調味料ハ昆布、鰹節、肉「エキス」等ト同樣ノ效用アルノミナラス茶、菓子、酒、酢ノ如キ固形若クハ液狀ノ飲食物及ヒ嗜好品ニ加ヘテ大ニ其味ヲ增進セシムルヲ得ヘシ特ニ此調味料ト食鹽トヲ適當ノ割合ニ水ニ溶シ之ニ適當ノ色素及ヒ香料ヲ加フレハ甚タ好良ノ醬油ヲ得ヘシ是レ此調味料ノ用途カ甚タ廣キヲ示スモノナリ

「グルタミン」酸ハ化學上既ニ久シク知ラレタルモノナレトモ何等實用ニ供セラレタルコトナク其價値ハ全ク學術研究ノ上ニ限ラレタリサレハ極メテ稀ニ學術用標品トシテ之ヲ販賣スル者アレトモ其價甚々貴ク獨逸國伯林ナル著名ノ化學藥品製造業者「カールバウム」ノ千九百七年度定價表ニ據レハ一瓦ノ價一・二「マルク」十瓦ノ價十「マルク」ナリ「グルタミン」酸「ナトリウム」ニ至リテハ其諸性質化學上ニ於テモ殆ト未知ニ屬シ固ヨリ之ヲ實用ニ供シタルモノナシ然ルニ前記ノ方法ニヨリテ多量ニ之ヲ製造スルトキハ其價一斤數圓ニ過キサレハ廣ク調味料トシテ使用スルニ適スルノミナラス其效力卓絶セルヲ以テ之ヲ昆布、鰹節等ニ比スレハ遙ニ廉價ノ調味料タリ之ヲ要スルニ强酸ヲ用ヒ蛋白質ヲ加水分解シ其成生物ノ全部若クハ一部ヲ中和シテ好良ナル調味料ヲ製造スル方法ハ從來未タ曾テ有ラサル所ニシテ本發明ヲ以テ嚆矢トス

特許法ニヨリ本發明ノ特許ヲ請求スル範圍ハ次ノ如シ

一、本文所載ノ目的ニ於テ本文ニ詳記シタル如ク强酸ヲ用ヒテ蛋白質若クハ蛋白質含有物ヲ加水分解セシメテ生スル果成物ノ全部若クハ一部ヲ鹽基ニテ中和シ因テ以テ固形若クハ液狀ノ飲食物及ヒ嗜好品ニ特異ノ好味ヲ附スヘキ「グルミタン」酸鹽ヲ主要成分トセル調味料ヲ製造スル方法

J. Tokyo Chem. Soc., 30 (1909) 820-836

薩摩菊油の起源を案するに藩主島津齊彬公同國甑島に產する野生菊よりして油を製することを同藩御藥園方に下命せること之が製造の濫觴なりと云ふ此菊油は同地方にありては興奮霍亂腹痛火傷外傷的防腐劑等內外用民間藥として貴重し猥りに之を他國に輸出することなし予は同地知人に囑し原植物標本と原油少量とを得たり就て檢するに原植物は Pyrethrum indicum にして原油は其色黃褐透明にして菊の香氣を有し其の物理的性質は

比重　（一五度）　〇・九四四八

比旋光度　右旋　一二度一〇分

原油の量は僅少にして化學的試驗を施すこと能はざりしも冷却するも毫も結晶を析出せず又樟腦樣香氣を認めず且つ游離の酸及アルデヒード類なし薩摩菊油は要するに前記リウノウギク油と全く異れるものなり。

新調味料に就て

理學博士　池田菊苗

吾人の諸種の感覺と我が化學との關係は種々の方面より見て最も興味ある問題であります。諸種の感覺の內臭覺と味覺とは特に化學的の感覺と稱へらるゝものであ

りまして物質の化學的構造と其の臭及び味との間に最も密接の關係あるべきことは明白でありますが實際學術的に究め明らめられた所は遺憾ながら尙ほ甚だ少いと言はねばなりません。光の感覺に於ては其の刺戟が全く物理的であるに關らず感覺の生ずる所以を說明するには化學變化を假定する學者がありますが光化學的作用が如何に普遍の現象であるかを思へば此の說明法は頗る根據あるものと認めねばなりません。諸物質の化學的性質と其の發し若くは吸收する光の性質との關係に就きては既に研究せられたる所が多くありまして一方に於てはスペクトロスコピーと爲り一方に於ては色素の構造論となつて居ります。併ながら概括して申せば物質が吾人に喚起する感覺と化學的構造との關係は尙ほ殆ど全く不明でありまして吾々が進んで之を開明するを俟つて居るものといはねばなりません。殊に味覺に關する諸問題中には吾々化學者の側より進歩を試み得べき準備の多少整つて居るものが少からぬやに見受けられます。

右に述べた所は純科學的なる化學物性論の方面でありますが如何にして吾人の感覺の快樂を滿足せしむべきかといふ應用科學の方面に於ては化學が既に成就したる所は決して鮮少でありません。吾人の眼を喜ばすべき數多の顏料染料は斯學によりて供給せられました。又種々の香料が人爲的に製造せられ近來頗に其の數を增加

したるは御承知の如くであります。吾人の味覺を滿足せしむる一段に至りましては其の進歩は前二者の如く顯著なるものではありません。從つて此の方面に於ては吾人が開拓すべき原野の極めて廣きを信ずるのであります。斯樣な次第で味覺に關する化學的研究は非常に有望なものと認めましたから自分が數年來從事して居りました理論化學上の研究とは稍縁の遠い仕事ではありますが之に著手する決心を致しました。

抑も味覺には古來酸甘鹹苦辛の五つを數へましたが此の內辛は一般の皮膚感覺に外ならぬとあつて現今の學者は之を味とは認めません。又金屬性の味、アルカリの味及澁味の如きは蛋白質の凝結若くは溶解に伴ふ感覺と離るべからざるものである、といふ點から之を味(少くとも純粹の味)と認めぬのであります。されば今日生理學者は皆此等の味の種々に混合したものであると說かれて居ります。併ながら自分は此心理學者によつて一般に認められて居るのは酸甘鹹苦の四味に過ぎませぬ。其の他の外に少くとも一種の區別し得べき味があると信じて居りました。それは魚類肉類等に於て吾人が「うまい」と感ずる一種の味でありまして鰹節、昆布などの煮出汁に於て其の味が最も明瞭に感せらるゝものであります。是は主觀的の事柄ではありますが幾多の人に就いて問ひ試みるに即時に若くは少時沈吟の後に同感なりと答ふるが

常であります。されば前に述べた四味已外に更に一種の味覺あることは殆ど疑を容れぬ次第であります。今或人の發議に從つて說明の便利の爲めに此の味を「うま」味と名づけて置きます。

次に起る問題は此の「うま」味を喚起する物質は果して如何なる化合物であるかといふことであります。複雜なる動植物體より未知の而も微量に存在すと假定すべき呈味成分を分離するは容易の業でないと信じましたが第一に調査する材料として昆布を選みました。昆布は充分に乾燥を經た植物であつて蛋白質は既に凝固して居りますから其の浸出液の組成が比較的簡單であらうと信じたから之を選んだ次第であります。昆布の分析の結果の公表されて居たものに據つてマンニットが如何に多く含まれて居るか鹽分は如何なるものにして其の量は幾何といふことは略分つて居りましたが呈味成分は所謂エキス分中に含まれてあるのであつて之に就ては何の消息も傳へて居りませなんだ。

昆布の呈味成分は浸出液中に在ること勿論でありますが此の液を蒸發して結晶點に達したとき之を放冷すれば多量にマンニットの結晶を析出致します。好良なる「だし」昆布を原料とし一瓩より一回の結晶によつて粗マンニット二百瓦許を得たことがあります。されば我邦にてはマンニットの原料としては昆布が最も適當なものであらう

と思ひます。他日マンニットを工業的に需要する場合には非常に廉價に之を昆布より採ることが出來るでありませう。マンニットの結晶を除き去つた母液を再び蒸發すれば食鹽の結晶を生じます而して其の液を結晶より傾瀉して之を冷却する時は復マンニットの結晶を得ることが出家ます。斯樣に煮詰めては結晶せしめ又其の母液を煮詰める此の操作を反覆すればマンニット、食鹽、鹽化カリウムなどを大部分除去することが出來ますが最後に殘る母液は眞空乾燥器中に於て乾燥せしむるも結晶を生じません。母液に無水アルコールを加ふれば沈澱を生せずして二液相を生じます。されば此の母液中に含まるゝ物質が如何に水に溶け易き物であるかを推知することが出來ます。昆布の呈味成分は總て此の母液中に集りまして、結晶となつて析出するものを精製しても就れも求むる所の味を呈するものではありませんでした。母液は中性の反應を呈して居るが之を乾燥して灼熱したものを水に溶せば強きアルカリ性の反應を呈しますから呈味物質が或有機酸の鹽であることは略推量することが出來ました。そこで其の酸を沈澱する爲めに種々の方法を試みましたが夾雜して居る鹽素、沃素、硫酸等は沈澱しましたが求むる所の酸はいつかな沈澱せぬのでありました。最後に硝酸鉛の飽和溶液を加へて始めて沈澱せしむることを得ました。此の沈澱は稍水に溶くるものでありますから之を生ずるには硝酸鉛を過量に加へなければなり

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ませなんだ。義に鉛糖及び鉛醋を用ひて試みたる場合に好果を得なかつたは蓋し此等の鉛鹽が錯鹽を造る傾向あると其の解離度の比較的小なるとに因るのでありませう。硝酸鉛を用ひて得た沈澱は鹽化物、硫酸鹽等を含むばかりでなく硝酸鉛をも幾分か含んで居りまして沸湯中に於ては半熔融し樹脂の如き物となりますが之を冷却すれば甚だ脆き塊となり容易に碎いて粉と爲すことが出來ます。此の粉を炭酸カルシウムと共に水中に投じ硫化水素を通じますれば總ての酸のカルシウム鹽を得ることが出來ます。濾過して硫化鉛及び過剩の炭酸カルシウムを除いた液を蒸發乾固して粉末と爲しソクスレット裝置を用ひ九八%の酒精にて長時間浸出を試みましたが苦味を呈する物質が微量に溶け來つた外何物をも抽出し得ませなんだ。此の操作を行ふた目的は鹽化カルシウム及び硝酸カルシウムを溶し出すに在つたのですが此等の鹽が少しも熱アルコールに溶け來らなんだは實に解すべからざる所であつて多分呈味鹽と複鹽を生じたのであらうと思はれます。此の點は尙ほ他日研究を遂げねばなりません。右の方法に由る鹽化物及び硝酸鹽を除去する目的が外れましたから硫酸銀を以て鹽素、沃素を除去し重土を以て硫酸を去り棒酸を以てカルシウムを除きまして未知の酸と少許の硝酸との混合溶液を得ました。此の液は尙ほ他の物質を含んで居つたかも知れませんが其の量は比較的少量に過ぎませなんだ。稍水

に溶け難き硫酸銀を用ひました爲めに最後に得た溶液の體積は頗る大なるものでありましたが減壓蒸發によつて濃縮して得た液を乾燥器中に於て硫酸上に放置しましたが數日の後に結晶を析出し始めましたが其の成長は甚だ遲々たるものであつて一週間位を經て米粒の大さに達しました。此の物は稍不透明であつて半面結晶を爲して居ります。硬度は有機化合物としては硬い方であつて水には少しより溶解しません而して其の溶液は酸性の反應を呈しました。又其の熔融點を測りましたが當初は一九二度でありましたが再三結晶して二〇〇度近くに昇りました。其の結晶を嚼むに砂の如く少く酸味と昆布「だし」に特有の味を呈して居りました。此の「うま」味は酸味の消滅した後に殊に分明に感知することを得ました。次に其の結晶をアルカリ等にて中和して鹽を作り之を嘗むるに最も濃厚なる「うま」味を呈して居りました。右の如くして昆布の呈味成分が一種の有機酸の鹽であつて其の酸は純粹に製取し得べきものである事が明瞭になりましたから更に多量の原料を用ひて上記の製法を行ひました。唯炭酸カルシウムの代りに炭酸バリウムを用ひた位が重なる相違であります。斯くして得たる結晶酸を分析し且つ其の水溶液の沸點より分子量を測り又濃度既知のアルカリを以て中和して其の當量を定めて $C_5H_9NO_4$ の分子式を得ました而して其の諸性質はグルタミン酸のそれと著く一致して居りますから愛に得

た酸がグルタミン酸であることは一點の疑なきに至りました。

此の研究によつて二つの事實を發見し得ました、即ち「だし」昆布がグルタミン酸鹽を含むこと及びグルタミン酸鹽は「うま」味の感覺を與ふるものであると云ふことであります。

グルタミン酸の味に就きましては E. Fischer は特異の「まうあ」(fader) 味と弱き酸味とを呈すといはれて居ます。(Untersuchungen über Aminosäuren, Polypeptide und Proteine, 69) 併ながらグルタミン酸鹽類の味に就いては未だ何等の記載にも接しません。且つ此の酸の鹽類はまだ多く研究せられて居りませんから其の性質の不分明なも無理ならぬ次第であります。例へば一價グルタミン酸のナトリウム鹽は潮解性物質であるかの如く記載せられて居りますが實際は甚だ結晶し易く常温に於ては食鹽と同程度の溶解度を有し梅雨時季の空氣に曝しても潮解することはありません。バリウム鹽は珪琺狀の物質と記載されて居りますが其實絹絲狀の結晶を爲すものであります。カルシウム鹽も不分明の結晶と爲すことが出來ますが唯カリウム鹽のみは未だ結晶せしむるを得ません。是等の鹽類の水溶液は孰も純粹なる「うま」味を呈して居ります。又アムモニウム、マグネシウム、亞鉛、カドミウム、アルミニウム、銅、第一鐵の一價グルタミン酸鹽溶液を此等の金屬の硫酸鹽とグルタミン酸バリウムとの複分解によつて

造りまして其の味を試みますに金屬樣の特異の味を別とすれば孰れも「うま」味を呈して居ります。斯樣の次第でありますから「うま」味は一價グルタミン酸イオンの味であることは疑なき所であります。恰も總ての中性硫酸鹽の呈する苦味が二價の硫酸イオンの味であると同樣であります。

グルタミン酸鹽の呈味性は頗る強いものでありまして其の threshold value を求めますにグルタミン酸ナトリウムを用ひまして大約三千分一であります。即ち三リットルの水溶液中に一瓦已上のグルタミン酸ナトリウムを含んで居れば其の味を感知することが出來ますが一瓦已下の場合には味を呈さぬのであります。アルカロイドや甘精などの味の極めて強いものに比較すれば劣つて居りますが之を普通に調味料として使用せらるゝ食鹽及び砂糖などに比ぶれば呈味性の強きものと言はねばなりません。今二三の物質の threshold value を擧げて見ませうならば

蔗糖	二百分一
食鹽	四百分一
グルタミン酸ナトリウム	三千分一
鹽酸	一萬分一

等でありますが之を稀釋度に改算しますれば

蔗糖	七〇・リットル
食鹽	二三・リットル
グルタミン酸ナトリウム	五〇〇・リットル
鹽酸	三七〇・リットル

でありましてグルタミン酸イオンは五百分一モルの濃度に於ても既に感知し得べく殆ど水素イオンの呈する酸味と其の強さを同うするものといふことが出來ます。食鹽が二十三分一モル已下の濃度に於て味を呈せざるは一寸不思議のやうでありますが元來食鹽の鹹味は(Na)といふ化合物の味でありまして鹽素イオン若くはナトリウム＝イオンの味でありません。二十三分一モル位の濃度に於ては九割ばかり解離して居りますから化合物の濃度は二百分一モル位でありまして水素イオン及びグルタミン酸イオンの價と略同位のものであります。

グルタミン酸イオンの濃度を増せば其の「うまみ」も強さを増すことは勿論でありますが此の二者が相比例した如くには感じません。詳しく言へば味の増加はイオン濃度の増加よりも少く感ずるのであります。恰も色の中で黄色は其の濃度を大に増加しても眼にはそれほど強く感じないのと同樣の關係であります。之に反して食鹽の味は濃度に比例するよりも強く増す如く感せらるゝものでありますが其の鹹味が

化合物の味であるとすれば解離說より見て當然の事といはねばなりません。斯樣な次第でありますから食鹽とグルタミン酸鹽との混和して存在する場合には双方共濃厚なる間は食鹽の味は強く感せられグルタミン酸イオンの味は之に壓せられる氣味でありますが水を加へて稀釋するに從ひグルタミン酸イオンの「うまみ」が段々明瞭になります。而して大に稀釋して食鹽の味の消え失せた後にもグルタミン酸イオンの味は尙ほ分明に感知することが出來ます。醬油の鑑定には現に此の關係を利用して居るといふことであります。

混合味の關係に於ても多少興味ある事實があります。グルタミン酸鹽の溶液に鹽の如き酸を加ふれば著しくグルタミン酸イオンの味を弱めますが是は恐くは反發味の現象ではありますまい。グルタミン酸は甚だ弱い酸でありますから水素イオンが他より加はる場合にはグルタミン酸イオンは之と化合して解離せざるグルタミン酸水素となる爲めに其の陰イオンが減少し從つて味の減退を來すのであつて眞に當然の事といはねばなりません。此の事實は「うまみ」がグルタミン酸イオンの性質であるとの斷定を確むるものであります。游離グルタミン酸が初め微酸味を呈するは固有の味であつて後に呈する「うま」味は其の中和によつて生ずるイオンに在るものと考へねばなりません。

少量の食鹽の存在する場合にグルタミン酸イオンの快味が著しく增進せらるゝ如き感あるは數多の人の經驗の一致する所でありますが此の事實は恐くは吾人の食餌が常に食鹽を含んで居るが爲めに吾人は「うまみ」と鹹味とを同時に味ふ習慣がある爲めでありませう。弱き鹹味が「うまみ」の感じを强くするといふ譯ではなからうと思ひます。併ながら鹽化物とグルタミン酸鹽との間に特殊の關係あることは總ての理論を離れて注目し置くべき事實であると思ひます。前にも御話し致した如くグルタミン酸カルシウムに混和したる鹽化カルシウムがアルコールに溶解せぬといふ特異の現象がありますし、又鹽化物の存在する場合にはグルタミン酸が硝酸銀によつて容易に沈澱するが鹽化物の無い場合には其の沈澱が非常に不十分であるといふ事實がありますし。是等の現象は混合結晶或は複鹽の成生によつて說明される事柄であるかも知れませんが又鹽素イオンとグルタミン酸イオンとの間に於ける特殊の作用に基くものであるかも知れません。斯樣に鹹味が「うま」味に對して特別の關係あるは味覺の研究上にも多少興味ある問題でありませう。

甘味と「うま」味との關係は如何にといふに砂糖の甘味はグルタミン酸イオンの味の爲めに殆ど影響せらるゝ事はない樣でありますが「うまみ」は稍强き甘味の爲めに屢〻消されて辨別し難くなります。グルタミン酸イオンの味と甘味との間には少しく

類した點があつてグルタミン酸イオンの濃度が threshold value に近い場合には甘味と誤認することがあります。併ながらグルタミン酸イオンの味は前にも述べました如く其の濃度を增加するも甚しく強くなるものではありませんが甘味の方は砂糖の濃さを增せば甚だ強くなるものでありまして兩味が全く別物であることは疑を容れません。之を色に譬へて「うま」味を黃色に比すれば甘味は赤の如きものであります。

グルタミン酸イオンの味が其の化學的構造と密接の關係を持つて居ることは勿論でありますが此の點に關しては何等明確の考を立てることが出來ません。Fischer が觀察されました所は大に參考となるものでありますから左に抄出致して置きます。(Untersuchungen über Aminosäuren, etc., (Berl), Fussnote より)

アミノ酸類の味は多少其の構造と關係を有するものにして此等の極めて相類似せる物質を區別するに味を利用し得べきが故に此の性質に關する余の經驗を總括し記載し置くは無用に非ずと思はる。余が試驗せる脂肪屬の簡單なる α アミノ酸は皆甘味を呈せり。(W. Sternberg: Chem. Centralblatt, 1899, II, 38 參照) 固體のまゝ味ふ時は溶解度小なるもの〻味弱きは覩易き理なり。グリコール、アラニン、ロイシンの甘味は人の知る所なり。尙ほ余が自から試驗せる例として合成的に得たる α アミノ酪酸、α アミノ n ヴレリアン酸、α アミノイソヴレリアン酸及び α アミノ n カプロン酸を掲ぐ。

βアミノ酸に在つては甘味は減退す、例へばβアミノ酪酸は殆ど味なくβアミノイソヴレリアン酸は甘味甚だ弱くして苦き跡味あり。

余が試用し得たる唯一のγアミノ酸即ちγアミノ酪酸は全く甘味なく唯弱き「まづき」味あり。

オキシアミノ酸に於ても同様の關係あり。セリン（αアミノβオキシプロピオン酸）及びαアミノαオキシヴレリアン酸は頗る甘きに反しイリセリン（βアミノαオキシプロピオン酸）は全く此の性質なしαピロリヂンカルボン酸は脂肪屬化合物と同じく強き甘味を呈せり。

芳香屬に於ては其の關係を異にするなり。フェニルアミノ醋酸（$C_6H_5 \cdot CH[NH_2] \cdot CO_2H$）及びチロシン（$HO \cdot C_6H_4 \cdot CH_2 \cdot CH[NH_2] \cdot CO_2H$）は殆ど味なく恰も白堊の如く微に「まづき」味を帶ぶ。之に反してフェニルアラニン（$C_6H_5 \cdot CH_2 \cdot CH[NH_2] \cdot CO_2H$）は甘し。

二鹽基アミノ酸の間にも亦差異あり。例へばグルタミン酸が酸味弱くまづき跡味あるに反しアスパラギン酸は殆ど酒石酸に均しき強き酸味を呈せり。

アミノ酸の多數の快美なる味は此等の物質が蛋白質中に存する事實と多少關聯せること明かにして O. Emmerling (Berichte d. d. chem. Gesellsch., 35, 2289) が數種の絲狀菌に對する種々の構造のアミノ酸の作用に就きて爲せる觀察を想起せしむ。

斯様の次第でありますからグルタミン酸イオンの「うま」味は第一に其のアミノ酸であること、第二に其のアミド基が一方のカルボキシル基に對してはαの位置を有し

報　文

他のカルボキシル基に對しては γ の位置を有する事實に關聯して居ることは明であります。アスパラギン酸イオンが毫も「うま」味を呈せぬのは注目すべき事柄であつて諸基の位置が味と關係あることを推想せしめます。又立體化學的構造が味と關係を有して居ることも申すまでもありません。グルタミン酸と同樣の構造を有して其のイオンが同樣の「うま」味を呈するものが尙ほ幾種か蛋白質の加水分解成生物中に存在することは想像するに難からざる所であります。又此の種の性質を呈する化合物を人工的に合成することも出來得るでありませう。

一定の構造と一種の味との關係を科學的に說明するは中々困難な事業であります。之に反して如何にして吾々が砂糖類の甘味若くはグルタミン酸イオンの「うま」味を嗜好するやうになつたかといふ事は目的論より一通り解釋することが出來る次第であると思ひます。

抑も嗜好は生物進化の結果主として生命の保續及び繁殖に必要な本能的作用を最適當な徑路に導く如く次第に發達し來つたものであつて食物に對する嗜好は成るべく營養價値多きものを選擇せしめる樣になつて居るべきは疑を容れぬ所であります。好良なる植物性食物の諸成分中味の最も著きは糖分の甘味であります又澱粉の如く其自身味なきものも口に入つては幾分か甘味を呈します。されば人類が植物

性食物を取る必要がある已上甘味の嗜好が發達して居ることは傾解し易き所であります。「うま」味の嗜好も同樣に說明することが出來ます。蛋白質の加水分解成生物の少量が常に動物體の諸部分に存在すべきことは新陳代謝の現象に伴ふ必然の結果でありまして現に肉汁を蒸發して造つた肉エキス中には少からざるグルタミン酸が他のアミノ酸と共に發見されて居ります。此等の諸成分中グルタミン酸鹽の味は最も顯著なるものでありまして極めて微量に存在する場合にも明に感知すること が出來ます。されば グルタミン酸イオンの味は動物性食物と離るべからざるものであつて營養價値多き此の種の食物を攝取せしむるには此の「うま」味の嗜好が自然に發達せねばならぬ道理であります。

既に此の嗜好ある已上之を合理的に滿足せしむる方法を考へねばなりませんが强酸類を用ひて蛋白質殊に植物性蛋白質を加水分解してグルタミン酸鹽を製し或は之を含んだ調味料を製造するが最も適當な方法でありまして此の製造法に關しては既に昨年の夏特許を受けました。グルタミン酸鹽中最も調味料として使用に便利なはナトリウム鹽でありますが併ながら純粹な鹽を製する時は廉價に出來ませんから他の成分を夾雜したまゝ調味料として使用することが却て適當であらうと思ひます。斯樣な混合物の方が營養價値は大なるべき筈であります。爰に述べました調味

料は沃度製造者として知られて居る鈴木三郎助氏が既に其の製造を開始し販賣して居ります。

醬油の味も一部分はグルタミン酸イオンの「うま」味に歸すべきこと疑なき所でありまして此の重要な調味料の製造法にも一大改革を加ふべき時期が既に到來したものと信じます。

蛋白質の加水分解成生物は學術上に於ては近來盛に研究せられつゝあるに係らず其の工業的應用は閑却せられて居る樣でありますが右に說きました調味料の製造などが導火となつて此の方面の化學工業の勃興せんことを切に希望する次第であります。

此の研究を爲すに當りまして栗原喜賈氏は助手として大に力を盡されましたから爰に一言して同氏の勞を謝します。

抄　錄

理論及物理化學

水素と鹽素との化合速度に酸素の減速作用

D. L. Chapman & P. S. MacMahon: Journ. Chem. Soc., 1909, 95, 959—964.

鹽酸の電解を久しく繼續すれば少しも空氣を混せざる鹽素と水素との混合氣體を得、而して其が光に感する度は空氣の減するに從ひ增加するも酸が消費せられて其濃度が減するときは減少す、此作用は酸素が水素と鹽素との化合の速度を減却するが爲にして、酸の濃度の減少により化合の遲緩なるものを生ずるは陽極に於て酸素の發生が著大となるが爲なり。

著者等は今回此酸素の減速作用を研究し[O]とSの乘積を算出せるに次の如き數を得たり、但し[O]は單位容積例へば一〇〇瓩の混合氣體中に存する酸素の容積にしてSは感度なり。

100 瓩中の酸素	感度	[O] × S
—	2·58	—
0·0858	0·2507	0·02151
0·1004	0·2172	0·02181

平均

Effect of L(+) Glutamic Acid on Physical Growth and Development

(A Study of 101 Cases)

DOROTHY S. JAEGER-LEE, M.D., CATHERINE I. KNOP, M.D., and MARY K. SARTWELL, M.D.
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Glutamic acid has been used experimentally in the treatment of mental deficiency since the report of Price, Waelsch and Putnam in 1943. Although large amounts of this material have been fed to patients, there have been no systematic studies of its effect upon growth and development when used over long periods of time. A project for the study of the effect of glutamic acid on mental growth initiated in the Child Welfare Clinic of Children's Hospital, Washington, D.C., in 1946 afforded an opportunity for a long range series of observations.

Mild symptoms of toxicity were reported by Zimmermann.[3] The authors investigated the possibility that prolonged feeding of glutamic acid, which plays such a significant role in metabolism, might have subtle and even harmful effects on physiologic functions, detectable as alterations in growth trends, body chemistry, or the hematologic picture. On the other hand it seemed equally possible that the reported favorable effects on mental growth might be due to an improvement in physical status.

Procedure of Study:

Detailed family, prenatal, birth and postnatal histories were taken by a pediatrician. Complete physical examination, including neurologic and endocrinologic consultations, were performed on each child. X-ray films were made of the carpal bones to check skeletal maturation. A complete blood count, Kahn and Mazzini tests, blood cholesterol determination, liver function tests, and urinalysis, including a test for the presence of phenylpyruvic acid were included in the preliminary study. Cephalin flocculation tests were used as liver function tests at first, but were replaced during the second year by thymol turbidity tests. When indicated, additional data were obtained through eye consultations, audiometer tests and encephalograms. The clinic secretary contacted the parents or guardians every month for a report of changes noted by them, difficulties in giving L(+) glutamic acid and other pertinent problems. Physical examinations and neurologic consultations and laboratory tests were repeated every six months. Endocrinologic consultations were repeated only in indicated cases. Wetzel grids were kept on all patients except a few for whom data could not be plotted on either the infant or childhood grids.

Congenital Stigmata:

There was a high percentage of congenital stigmata present in these children (66.3%). Although we realize this has no direct bearing on the effect of glutamic acid on physical growth, it is of value in helping the reader to understand better the type of child with whom we were working.

The congenital stigmata were classified as follows:

Pronated feet	31
Eye conditions	26
Strabismus	22
Microphthalmia	1
Cataract	1
Nystagmus	1
Myopia	1
Congenital heart disease	5
Hernia	4
Deformity of fingers or toes	4
Malocclusion	2
Deafness	2
Birth mark	1
Genu valgus	1
Cloacal anlage	1
Harelip	1
Undescended testicles	1

Of these 101 children, ranging in age from two weeks to thirteen years three months, the length of time of administration of L(+) glutamic acid was as follows:

35 children	6 months
29 children	12 months
21 children	18 months
16 children	24 months

History—prenatal, birth, and postnatal:

There were positive family histories (mental retardation, epilepsy or neuropsychoses) in 24 instances (24.2%), positive pregnancy factors (multiple pregnancy, toxic symptoms, illness or accidents during pregnancy, Rh negative blood of mother, or threatened abortion) in 45 instances (45.4%), and birth factors (prematurity, difficult delivery or anoxia) in 31 instances (31.3%).

Physical Examinations:

The general appearance and state of nutrition were classified as good (G), satisfactory (S), and poor (P) regardless of the presence or absence of acute infection such as nasopharyngitis or otitis media. On this basis no child regressed during his period of observation and 29.7 per cent showed improvement.

For more objective evaluation, the Wetzel grids were employed. Individual heights, weights, and ages of these children as measured at each physical examination at six month intervals throughout the study were plotted on the grids, and all measurements obtained from previous hospital records, schools, clinics, and family physicians were plotted for the time prior to this study. Because 16 individuals fell in the group between the infant and childhood grids, only 85 children could be included in this portion of the study—23 on the infant grids and 62 on the childhood grids.

These children were divided into two groups. The first group was composed of those on whom previous height and weight determinations were available so that their growth and development prior to our study could be plotted. The second group included those children on whom no previous records could be obtained.

There were ten infants and 28 children in the first group. The results in channel trends of the 28 patients plotted on the Wetzel grids for children are shown in Table 1.

TABLE 1

Age	Upward (toward more obese A4 Channel)	No Change	Down (toward poor B4 Channel)
½-1 yr.	(1) 33.3%	(2) 66.6%	
1-2 yrs.	(2) 28.5%	(3) 42.8%	(2) 28.5%
2-5 yrs.	(2) 50.0%		(2) 50.0%
5-8 yrs.	(4) 26.7%	(6) 40.0%	(5) 33.3%
8+ yrs.	(2) 21.1%	(6) 66.6%	(1) 11.1%
Totals	(11) 29.0%	(17) 43.7%	(10) 26.3%

These same children were divided into three groups labelled Stocky, Medium, and Slender, according to their position on the Wetzel grid channels. The following chart demonstrates the changes in body builds which occurred between the examination prior to the use of glutamic acid and that made at the termination of the course of glutamic acid.

TABLE 2

	Stocky	Medium	Slender	Totals	No.
More Stocky		41.1%	25.0%	28.5%	8
No Change	57.1%	41.1%	25.0%	42.8%	12
More Slender	42.8%	17.6%	50.0%	28.5%	8

For the children in Group I maturation was determined just prior to the use of glutamic acid and again at the culmination of the course of glutamic acid.

TABLE 3

Age	Prior to G.A.			After G.A.		
	A	N	R	A	N	R
½-1 yr.	2		1	2	1	
1-2 yrs.	2	2	3	1	3	3
2-5 yrs.	4			3	1	
5-8 yrs.	7	3	5	8	2	5
8+ yrs.	3	2	4	4	1	4
Totals	18	7	13	18	8	12
%	47.3	18.4	34.2	47.3	21.0	31.6

"A" indicates the child was advanced above the 67% line or standard development.

"N" indicates the child followed the normal auxodrome of its age.

"R" indicates the child was retarded behind the 67% line or standard development.

Group II comprised 47 children on whom no height and weight determinations had been made prior to our study. For this group maturation attained prior to the use of glutamic acid was compared with that at the termination of a course of glutamic acid.

TABLE 4

	Prior to G.A.	After Course of G.A.
Totals:	73.5% A	76.4% A
	8.8% N	5.8% N
	17.6% R	17.6% R

See explanation on Table 3.

In an attempt to correlate the size of the average dose of L(+) glutamic acid received by each individual with any change in physical growth and development the following chart was developed using maturation as the means of comparison. The letters "A," "N," and "R" have the same meaning as in pre-

vious charts. The dosage is measured in Grams/10 pounds of body weight/day.

TABLE 5

Dosage	0-2.0			2.1-4.0			4.1-6.0			6.1+		
	A	N	R	A	N	R	A	N	R	A	N	R
Children:												
Before G.A.	11	3	4	21	4	9	3	1		4		2
After G.A.	12	2	4	22	3	9	4	1		3	1	2
Infants:												
Before G.A.	4	1	1	1	3	2	2	1	4			4
After G.A.	2	3	1	2	2	2	2	1	4	1	1	2
Totals:												
Before G.A.	15	4	5	22	7	11	5	2	4	4		6
After G.A.	14	5	5	24	5	11	6	1	4	4	2	4

The data in these charts indicates that children receiving large doses of glutamic acid were not affected differently from those receiving smaller doses.

Laboratory Data

Blood counts were done every six months. The results of the initial and final counts were compared in order to determine the effect of L(+) glutamic acid on the hematopoietic system.

Summary of Laboratory Data

	0	—	+	
	No Change	Decrease	Increase	Not Done
RBC	46	18	23	14
Hemoglobin	42	17	28	14
WBC	63	9	14	15
Thymol T.	83		3	15

Red blood cells were considered to have increased (+) if they showed an increase of 500,000 cells/mm³ or more, and decreased (—) if they decreased 500,000 cells/mm³ or more. There was no change in the red cell count in 52.9 per cent of the children, while according to our criteria 20.7 per cent decreased and 26.4 per cent increased. The mean initial red blood cell count was 4,060,000; after receiving 1+ glutamic acid it was 4,080,000.

Changes in the number of grams of hemoglobin per cu. mm. were considered to be significant if there was a deviation of 1 gm more or less from the original hemoglobin level. There was no change in 48.3 per cent of the children. The hemoglobin decreased in 19.5 per cent of the children and increased in 32.2 per cent. The average initial hemoglobin estimation was 12.39 gms; after receiving L(+) glutamic acid it was 12.62 gms.

Iron was presscribed in addition to the L(+) glutamic acid in seven cases because of hypochromic anaemia. Three of these children showed an increase in hemoglobin, three showed no improvement, and one had a decrease in hemoglobin.

For white cells, a range of 5,000 WBC per cu. m.m. to 10,000 WBC per cu. m.m. was considered to be normal. The white blood cell count stayed within normal limits in 62.7 per cent, was unchanged in 73.1 per cent, decreased below normal range in 3.5 per cent, and increased above normal range in 4.7 per cent. The average initial white blood cell count was 7,708 WBC; after receiving 1+ glutamic acid it was 7,780 WBC.

All urinalyses were within normal limits except in two cases (siblings) in which tests showed the presence of phenylpyruvic acid.

Cephalin flocculation tests and thymol turbidity tests were done to detect the presence of liver damage. All initial tests were within normal limits. One child had a positive cephalin flocculation test and three children had thymol turbidity tests of six or above (although only one was over ten, which is considered by some workers to be the upper limit of normal). The thymol turbidity test was normal in the one child with a positive cephalin flocculation test. One of the children with an abnormally high thymol turbidity test gave a normal cephalin flocculation test. The other two abnormally high thymol turbidity tests were not checked with cephalin flocculation tests.

The effect of L(+) glutamic acid on certain clinical types of mental retardation:

Mongolism: These children offered an especially interesting group to observe because of their altered rate of growth. There were 11 Mongoloid idiots in our study, ranging in age from two weeks to 12 years. When their heights and weights were plotted on Wetzel grids, three children gained in physique channels, two lost in physique channels and six stayed in the same channel. On Wetzel's maturation chart five showed gains in their development levels, four showed losses in developmental levels, and two stayed at the same developmental level.

Hypothyroidism: Four mentally retarded children suffering from hypothyroidism are

included in our group. Their ages varied from four to 11 years. Prior to this experiment, each had been treated with thyroid extract in adequate amounts resulting in satisfactory physical growth. During the experiment the administration of thyroid extract was continued in a routine manner. On Wetzel grids two children showed a loss of one or more physique channels and two children showed a gain in physique channels. On the maturation chart three children showed a gain in developmental level. At the close of the study two children were in the retarded group and two children were in the advanced group of developmental levels.

Toxic Symptoms: Toxic symptoms due to L(+) glutamic acid occurred in 24.8 per cent of the cases. These were determined by a careful review of the notes made of the parents' monthly reports and clinic visits. No symptoms were noted as being present unless they occurred constantly during the period the child received L(+) glutamic acid. In many instances they would disappear after maintaining the same dose over a period of time. None of the symptoms were of a serious nature nor did they have a harmful effect. As noted above, L(+) glutamic acid causes no deleterious effect on the liver, kidneys or hematopoietic system. The most common symptoms were nausea and vomiting which occurred fairly constantly in 13 patients. In many instances the children disliked L(+) glutamic acid intensely because of its taste and consistency, in spite of ingenious methods employed by the mother to disguise the unpleasant qualities of the medication. These 13 instances of nausea and vomiting, however, seemed to result from a toxic effect of the drug rather than a psychological reaction to it. This agrees with evidence that glutamic acid administered intravenously causes nausea and vomiting. In three children who had atopic eczema, an increase in intensity of the rash could be correlated with the increase of dosage of L(+) glutamic acid. Nocturia and frequency occurred in two patients. Two of the children were restless and hyper-irritable when receiving a relatively small dosage of L(+) glutamic acid. Diarrhea was present in two children. Single instances of headache, abdominal pain and diaper rash were reported.

Summary

The absence of changes in the physical status of children fed glutamic acid is worthy of comment. Some of these children were receiving over thirty percent of their nitrogen intake in the form of glutamic acid. This would represent an increase of over five fold in the normal glutamic acid content of the diet. Since this medication was usually taken with meals a highly unbalanced mixture of amino acids must have been absorbed. It appears that supplementation of the diet with non-essential amino acid has no effect at least so long as some essential amino acids are ingested, and so long as the total nitrogen requirement is completed with an easily utilized source of nitrogen, in this case glutamic acid.

Our results with Mongoloid children do not agree with those reported by Zimmermann[6] who noted physical gains when treating these children with glutamic acid. There is no explanation for this discrepancy. Our group of Mongoloid children was considerably smaller than his. The four hypothyroid children included in our study showed no consistent alteration in growth during the experimental period.

Conclusions

1. Administration of up to 1.75 grams of L(+) glutamic acid per kilogram of body weight per day to mentally deficient children has no effect upon the physical growth and development as measured with the Wetzel grid.

2. The medication has no effect upon the total or differential white blood count, the red blood count or hemoglobin, or the thymol turbidity test.

3. Glutamic acid, in dosage now being utilized in the experimental therapy of mental deficiency, has no discernible long range toxicity.

Authors' Note: This study was made possible through grants from the International Minerals and Chemical Corporation; from the Child Welfare Society of Children's Hospital; and from Walker Laboratories. The L+ Glutamic Acid was supplied by the International Minerals and Chemical Corporation, Chicago, Illinois.

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John A. Washington, as Endocrinological Consultant, is gratefully acknowledged. We also wish to thank Miss Caroline Sweeney, Mrs. Nora Gross, and Mrs. Mary Hoover, without whose aid and cooperation this study would have been impossible. Thanks are also due to Dr. Samuel P. Bessman, present Director of the Glutamic Acid Research Project, for the thymol turbidities done in the latter part of this study.

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The Improbable and the Impossible

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> Man can believe the impossible, but man can never believe the improbable.
>
> *Oscar Wilde.*

The logical contradiction—belief in the impossible coupled with disbelief in the improbable—has long been noted, but no explanations have been forthcoming to account for the paradox. The paradox is merely restated in aphoristic terms, as, for example, in Montesquieu's allusion to pagan religions: "Man cannot make a worm, but makes gods by the dozen."

When the Frenchman, Leban, invented the gas lamp, nobody believed the "improbable" assertion that a lamp could burn without a wick. Leban's beloved Paris was not lit by gas until fourteen years after the inventor's death. When Edison demonstrated his first phonograph, a great French authority "exposed" the invention as "a typical American hoax," explaining that Edison had simply hired a ventriloquist. Nobody believed that the improbable, a "talking machine," could exist. The history of inventions is crowded with similar examples. The fact per se is known only too well.

On the other hand the history of superstitions proves exactly the opposite: the impossible is readily believed. It is quite conceivable that those who were skeptical of Leban's and Edison's inventions believed in —ghosts.

What is the meaning of this logically inexplicable dichotomy?

The riddle becomes less mysterious upon application of psychiatric experiences with the two systems: of consciousness and of the unconscious. *Conscious reasoning is used in judging the improbable; in "judging" the impossible, infantile megalomania and omnipotence emerge from the depths of the unconscious* and are applied to the problem. The two phenomena—the improbable and the impossible—are thus judged by *different yardsticks.*

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1 9 7 4

(3) **Sulfate** Proceed as directed under Purity (3) in Fumaric Acid. Monosodium Fumarate meets the requirement of Fumaric Acid.

(4) **Arsenic** Dissolve 0.5 of Monosodium Fumarate in 10 ml of water by heating, and cool. The solution meets the requirement of the test for Arsenic, as directed under Section 23 in General Tests, using 10 ml of stannous chloride T.S. and 3 g of sandy zinc. For the control, prepare in the same manner as the sample.

(5) **Heavy metals** Proceed as directed under Purity (4) in Fumaric Acid. Monosodium Fumarate meets the requirement of Fumaric Acid.

Loss on drying Monosodium Fumarate, when dried at 120° for 4 hours, loses not more than 0.5 per cent of its weight.

Residue on ignition Dry Monosodium Fumarate at 120° for 4 hours, and proceed as directed under Residue on Ignition of Section 9 in General Tests. The residue of Monosodium Fumarate is not less than 50.2 and not more than 52.5 per cent.

Assay Weigh accurately about 0.3 g of Monosodium Fumarate, previously dried at 120° for 4 hours, and dissolve in 30 ml of water. Titrate with 0.1 N sodium hydroxide solution, using 2 drops of phenolphthalein T.S. as the indicator.

1 ml of 0.1 N sodium hydroxide = 13.81 mg of $C_4H_3NaO_4$

Monosodium *L*-Glutamate

$$HOOC{\cdot}\underset{\underset{NH_2}{|}}{CH}{\cdot}CH_2{\cdot}CH_2{\cdot}COONa{\cdot}H_2O$$

$C_5H_8NNaO_4{\cdot}H_2O$ Mol. Wt. 187.13

Monosodium *L*-Glutamate, when dried at 100° for 5 hours, contains not less than 99 per cent of $C_5H_8NNaO_4{\cdot}H_2O$.

Description Monosodium *L*-Glutamate occurs as colorless to white, prismatic crystals, or as a white crystalline powder, having a characteristic teste.

Identification (1) Heat 5 ml of Monosodium *L*-Glutamate solution (1 → 1,000) with 1 ml of ninhydrin T.S. for 3 minutes. A violet color is produced.

(2) Monosodium *L*-Glutamate responds to the tests for Sodium Salt, as directed under Identification Tests of Section 4 in General Tests.

Purity (1) **Clarity and color of solution** Dissolve 1 g of Monosodium *L*-Glutamate in 10 ml of water. The solution is colorless and clear.

(2) **pH** Proceed as directed under pH Determination (3) Glass Electrode Method of Section 21 in General Tests. The pH of Monosodium *L*-Glutamate solution (1 → 10) is between 6.7 and 7.2.

(3) **Specific rotation** Weigh accurately about 5 g of Monosodium *L*-Glutamate, previously dried at 100° for 5 hours, and dissolve in 2.5 N hydrochloric acid to make 50 ml. Proceed as directed under Optical Rotation of Section 14 in General Tests. The specific rotation, $[\alpha]_D^{20}$ is between +24.8° and +25.3°.

(4) **Chloride** Perform the test for Chloride as directed under Section 1 in General Tests, using 0.1 g of Monosodium *L*-Glutamate. The solution corresponds to not more than 0.2 ml of 0.01 N hydrochloric acid.

(5) **Ammonium salt** Proceed as directed under Purity (5) in Monosodium *L*-Aspartate, using 2 ml of ammonia standard solution.

(6) **Arsenic** Dissolve 0.5 g of Monosodium *L*-Glutamate in 5 ml of water. The solution meets the requirement of the test for Arsenic, as directed under Section 23 in General Tests.

(7) **Heavy metals** Dissolve 1 g of Monosodium *L*-Glutamate in about 20 ml of water, and add 2 ml of diluted acetic acid. Proceed as directed under Heavy Metals Test of Section 11 in General Tests. The heavy metals limit for Monosodium *L*-Glutamate is not more than 0.001 per cent.

(8) **Other amino acid** Proceed as directed under Purity (8) in Monosodium *L*-Aspartate.

Loss on drying Monosodium *L*-Glutamate, when dried at 100° for 5 hours, loses not more than 0.5 per cent of its weight.

Assay Weigh accurately about 0.15 g of Monosodium *L*-Glutamate, previously dried at 100° for 5 hours, and proceed as directed under Assay in *DL*-Alanine.

1 ml of 0.1 N perchloric acid = 9.356 mg of $C_5H_8NNaO_4 \cdot H_2O$

at the giant synapse[109], analysis has shown[110] that while the equilibrium potential for the synaptic potential is +28 mV, the reversal potential for the glutamate induced postsynaptic response is —22 mV. Thus, glutamic acid does not appear to be the neurotransmitter at the giant synapse of the squid where there is a large amount of free amino acids (including glutamate), as was observed above for peripheral nerves of invertebrates suspected of possessing a glutamate neuromuscular transmitter system.

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Consistent with the vesicular or quantal hypothesis of neuromuscular trans-

mission derived from studies of the cholinergic neuromuscular synapse of vertebrates[22,11,15,51,73], the transmitter of the invertebrate neuromuscular junction is released in quantal packets[78,79,152,153] and numerous synaptic vesicles are found in the synaptic terminal[6,7]. Glutamic acid is recovered from the perfusate of the synaptic region only when the excitatory motoneuron is stimulated[76,155,156]; however, alanine, glycine and aspartate were also recovered[155,156]. Several points suggest that glutamic acid is the best transmitter suspect of the above 4 amino acids[155]: (1) an increase in external calcium increases glutamate and alanine release but has no effect on the release of glycine or aspartate, and alanine is inactive at this synapse; (2) the amount of glutamate release was quantitatively related to the stimulus; (3) the release of alanine, glycine, or aspartate was not increased as the presynaptic stimulus rate was increased. The specificity of this release phenomenon is further exemplified by studies of the inhibitory neuromuscular synapse in these same preparations, where GABA is the transmitter candidate[6,7,130,148,154]. GABA is specifically released by stimulation of the inhibitory motor fibers in a manner which is proportional to the stimulus rate and duration[122]. Conversely, no GABA at all was released into the perfusate during stimulation of the excitatory motor innervation.

The above findings suggest that there are two separate transmitter systems in close apposition at the invertebrate muscle, one which is excitatory (glutamate) and another which is inhibitory (GABA). Each transmitter must be acting upon its own unique receptor system at the postsynaptic membrane. This is evidenced by data[148] which have shown that the desensitization effect of prolonged glutamate iontophoresis upon the glutamate receptor system is not observed for the GABA receptor system. A decreased size of the EPSP was observed but the IPSP was not effected.

Thus, there is strong evidence establishing several of the transmitter criteria[53,107,121,165] for glutamic acid as the excitatory neuromuscular transmitter in several invertebrates. Although there is some disagreement as to just what *the* criteria are, there would appear to be 6 main points. The transmitter candidate must: (1) be present in the presynaptic terminal in a transmitter pool; (2) be released in a quantal fashion from the presynaptic terminal upon stimulation in amounts sufficient to cause the postsynaptic response; (3) have the same effect upon the postsynaptic membrane as the substance released from the presynaptic terminal; (4) interact with the postsynaptic receptor site in an identical manner as does the actual transmitter. In addition, there must be (5) a synthetic and degradative system presynaptically for maintaining transmitter levels of this candidate, and (6) a means by which released transmitter can be removed from the extracellular fluid. The possibility that glutamate is not the actual transmitter has been suggested[160], with glutamate indicated as being only similar to the actual transmitter substance. The fact that no other excitatory substance besides glutamate and aspartate is found in sufficient amounts in crustacean nerve[85], however, makes the above suggestion very unlikely. Specific gaps in our knowledge here are basically 3-fold: (1) the nature of the mechanism whereby the proposed glutamate transmitter system is maintained and regulated presynaptically; (2) the nature of the transmitter receptor and drug (glutamate) receptor interaction; (3) detailed comparison of the properties of the endplate potential and the glutamate

induced postsynaptic response. Work in the above three areas will do much to solidify our understanding of the proposed glutamate neuromuscular transmitter system in invertebrates. Interestingly, there is some evidence that there may be a similar glutamate–GABA system operative in the CNS of lower vertebrates such as the goldfish[46] where it has been shown that there is a system of excitatory glutamate and inhibitory GABA inputs specifically arranged on the Mauthner cell.

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Recent reviews[30,32,86,88,135] have discussed various aspects of the excitatory effects of glutamic acid in the CNS of mammals. Glutamate has been found to be a potent excitant in the spinal cord[36–38], primary sensory nuclei of the caudal medulla [57,145], reticular formation[24,35,67], thalamus[108], corpus striatum[52], caudate nucleus[20], hippocampus[19,65], and cerebral cortex[22,30,52,82,87,90,92,99].

The response of glutamate by these units is usually immediate, both for the on and for the off response, as the iontophoretic current is initiated and terminated, respectively. There is often a post-response depression, where, while the spontaneous activity is lessened, one is still able to elicit action potentials. Extensive analysis with several different substances has shown that glutamic acid is one of many excitants to the mammalian central nervous system. Just as aspartate, glutamate and cysteate are all potent excitants upon spinal neurons[38], their respective alpha decarboxylation products β-alanine, GABA, and taurine are potent inhibitors. L-Glutamate is a more potent excitant than is D-glutamate as is also the case for the respective aspartate enantiomorphs[31,39,40]. L-Glutamate is also a more potent excitant than L-aspartate, or D-aspartate[89]. The relative effectiveness of the L- and D-enantiomorphs of glutamate and aspartate appear to depend upon the neuronal population studied. While spinal neurons are strongly excited by the L and D forms[14] of these two amino acids, cortical neurons are much more sensitive to the L than the D form[89]. *N*-Methyl-D-aspartate and D-homocysteate, among other substances, are much more potent excitants than L-glutamate[31,39,40]. Thus L-glutamate, which is the substance considered as a possible transmitter candidate here, is neither the strongest nor the weakest excitant. It is important to keep in mind, however, that even though several pharmacological analogs of the naturally occurring amino acids may be very potent excitants, they are not as a rule found in sufficient concentrations in neural tissue to be considered as transmitters.

Curtis and coworkers have indicated on several occasions[30,31,35–37,39,40] that the effects of amino acids such as glutamic acid upon neurons may not be specifically related to any synaptic transmitter process. Here, amino acids such as glutamic acid would be binding to a specific region of the membrane important in permeability control, which is common to all neuronal membranes. Since this binding site is not a postsynaptic receptor for a specific synaptic transmitter, the permeability changes taking place are suggested as being independent of any specific synaptic process. Important in the above hypothesis indicating that glutamic acid is a poor transmitter candidate is the idea that it has a widespread non-specific effect on all neurons. However, several studies have indicated that not all units are responsive to glutamate. Tha-

Review Article

GLUTAMIC ACID AS A SYNAPTIC TRANSMITTER IN THE NERVOUS SYSTEM. A REVIEW

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INTRODUCTION

Besides its possible importance as a transmitter at certain synapses in the nervous system, glutamic acid has other functions including incorporation into proteins[21,27] and peptides[126], being involved in fatty acid synthesis[41], contributing, along with glutamine, to the regulation of ammonia levels[17,163], and control of osmotic or anionic balance[83,96,100,105]. It may also serve as a precursor for GABA and as a precursor for Krebs cycle intermediates (see later discussion for references). Therefore, there is presently a great need to correlate the neuroanatomical, neurochemical, and electrophysiological data on glutamic acid in the nervous system, in order that the possible transmitter role can be clarified. We are here faced with discerning any possible transmitter roles for a substance which appears to be so ubiquitously important in the nervous system.

ANALYSIS OF THE TRANSMITTER ROLE OF GLUTAMIC ACID IN THE INVERTEBRATE NERVOUS SYSTEM

Invertebrate nerves contain very high levels of free glutamate and other free amino acids[100,105,142], while the peripheral nerves of vertebrates contain lower free amino acid levels[19,105,137]. The reason for the high free amino acid levels of invertebrate nerve is not clear, although some association with the peripheral glutamate and GABA transmitter systems, metabolism, and osmolar or anionic balance have been suggested[83,96,100,105,162]. The high amounts of amino acids other than glutamate may thus be *partially* accounted for by the fact that the aminotransferases could have a role in the regulation of the glutamate and/or GABA transmitter pools in the synaptic terminals. Recent data, however, suggest that one must use caution when attempting to interpret the reasons for the high free amino acid content of invertebrate nerve. Although very high free amino acid levels are found in the squid giant axon and glutamate has been found to be excitatory at the postsynaptic membrane

at the giant synapse[109], analysis has shown[110] that while the equilibrium potential for the synaptic potential is +28 mV, the reversal potential for the glutamate induced postsynaptic response is —22 mV. Thus, glutamic acid does not appear to be the neurotransmitter at the giant synapse of the squid where there is a large amount of free amino acids (including glutamate), as was observed above for peripheral nerves of invertebrates suspected of possessing a glutamate neuromuscular transmitter system.

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lamic neurons vary markedly in their responsiveness to glutamate from region to region[108], while only very few of the units in the olfactory bulb are responsive to the drug applied iontophoretically[161]. The large pyramidal neurons of the cerebral cortex often showed a high threshold to iontophoretically applied glutamate[86], whereas cortical glial cells are unresponsive to this amino acid[91]. Also, glutamic acid must be iontophoresed onto the external portion of the cell membrane in order to be effective, intracellular application being ineffective[4,29,149]. It would thus appear that the excitatory effects of glutamic acid can be quite specific.

The excitation associated with glutamic acid involves an increased intracellular sodium, a decreased intracellular potassium, and an increased metabolic expenditure due to the stimulated active transport pump by the changed ionic picture[23,64,106]. Because of many technical difficulties[33,135] it has not been possible to definitely compare the postsynaptic neurotransmitter and glutamate responses in the mammalian CNS. Some work on the spinal cord[32], however, has suggested that the monosynaptic excitatory postsynaptic potential and the glutamate induced depolarization have different properties *as recorded from the perikaryon of the motoneuron*. The major problem here is that while glutamate application, and thus the glutamate induced depolarization, is near the intracellular recording electrode, the excitatory postsynaptic potentials are occurring at varying distances along the dendrites, away from the recording electrode. Since changes in equivalent membrane resistance (anomalous rectification) have been observed[111] at the site of the polarizing electrode as the membrane potential is altered, this would be influencing the glutamate induced depolarization and would have a weak, variable effect on the more distant monosynaptic EPSP during reversal potential determinations. Thus, a difference in the properties of the glutamate induced depolarization and the excitatory postsynaptic potentials as recorded above[32], would not prove that these two responses are different. The analysis of postsynaptic response equilibrium potentials, as well as other properties of these responses, poses one of the major problems in the study of the glutamate transmitter system in the CNS of mammals today.

Glutamate release was observed when specific projection systems in the brain were stimulated[69,70]. The release of this substance may be instrumental in the production of spreading depression[82,125,150], a process caused by mechanical, chemical, or electrical stimuli to the cortex, and which spreads slowly at a few mm/min across the cortical surface producing a depolarization and depression of electrical activity[116]. Since other excitatory substances may also produce this phenomenon[39], however, the exact mechanism of spreading depression remains to be elucidated. Arousal states are associated with high glutamate and low GABA levels in the cortical effluent, while sleep is associated with the converse[68]. Stimulation of the medial reticular formation produces an increased acetylcholine in the cortical extracellular fluid, and an even greater increase in glutamate[69,70]. Stimulation of the mesial thalamus produced an increased acetylcholine only. Thus, although glutamate appears to be released from nerve preparations such as the sciatic nerve[12,167] in a manner which may be common, to one degree or another, to many neuronal tissues, the above release phenomena[69,70] were observed during activity in specific projection systems. This suggests that the

work discussed above may be concerned with the release of glutamate as a neurotransmitter from the synapses of these projection systems although further work is needed in order to definitely show this.

Release studies of glutamic acid have not been explored to their fullest as yet, due to a number of difficulties. The main problem lies in the method of collection and *specific* stimulation, as well as associating any changes in glutamate release with the release of a transmitter substance from a specific synaptic system. With the possibilities of changes in nontransmitter release being present[5,42,96,167], a further complication is introduced. When a neuron is stimulated there will be an increased sodium influx and an increased potassium efflux, resulting, for a time during and briefly after stimulation in an increased extracellular potassium immediately surrounding the cell. This increased extracellular potassium has been shown to increase the efflux of glutamate from inside the cell[5]. Increased electrical activity may elevate the active transport of glutamate out of the cell[167], although an increased membrane permeability during such periods is also a possibility[42]. During activity there may also be subtle changes in cell volume, and it has been demonstrated that active amino acid transport mechanisms may be linked to isosmotic regulation of cell volume[96]. Active transport processes resulting in cells taking up the released glutamate transmitter[34] make it difficult in many neuronal systems for one to observe an increase due to stimulation, unless special precautions are taken. With a substance like acetylcholine, it would be most important to block the degradative enzyme acetylcholinesterase, while with glutamate one must block the active transport process by which the amino acid is taken into the cell, in order to maximize recovery in the collected fluid. If uptake of transmitter levels of the substance is closely linked to the postsynaptic receptor site, then a competitive inhibitor of the receptor–transmitter binding process may be useful also. An inability to respond at rates rapid enough to release sufficient amounts of glutamate to be effectively detected in any collected effluent make such studies extremely difficult for certain neuronal systems.

NEUROCHEMICAL ANALYSIS OF GLUTAMIC ACID AND FREE AMINO ACIDS IN THE MAMMALIAN CNS

The dorsal sensory neurons have a branching axon with a characteristic microstructure at the axonal bifurcation[62], where one branch is directed towards the periphery to terminate as a receptor where no synaptic vesicles are found[1]. In contrast, the terminals of the dorsal root fibers directed centrally are observed to be loaded with synaptic vesicles[127,128,139]. Free glutamic acid is higher in the dorsal root projecting toward the cord where a transmitter function is envisioned, than in the peripheral extension[19,71]. This is true for glutamic acid only, no other amino acid showing this difference. It is interesting to note that free glutamate is also quite low in the peripheral receptor terminal of the Pacinian corpuscle[137], this observation being consistent with the above data. Further, glutamic acid is higher in the dorsal root than in the ventral root[3,19,50,60,71,73], while the components of the cholinergic transmitter system are higher in the ventral root than in the dorsal root[101,169], these differences being consistent with the respective transmitter roles for glutamate and acetylcholine.

While the components of the acetylcholine system function in synaptic transmission only, the components of proposed glutamic acid system have general metabolic roles as well. In conjunction with this is the fact that the ventral/dorsal root ratio for acetylcholine and cholineacetyltransferase range from 50 to 300 and 82 to 123 respectively[101], and the dorsal/ventral root ratio for glutamate is 1.3–2.3 (ref. 71). This is consistent with the ubiquitous presence of the components of the glutamate transmitter system, and the restriction of the components of the acetylcholine system to the areas where it has a transmitter role. Free glutamate is also higher in the dorsal root ganglion than in the sympathetic ganglion[71], while monoamine oxidase is higher in the sympathetic ganglion than in the dorsal root ganglion[58], this again being consistent with the respective roles of the proposed glutamate and noradrenergic transmitter systems. Analysis of enzymes associated with glutamate metabolism has not as yet shown any significant differences in activity between the dorsal and ventral root tissues[59].

While free glutamate levels in the dorsal root and dorsal root ganglion appear to be similar[49,71], this amino acid is higher in the spinal cord grey[3,60,71,73] and in the primary sensory nuclei of the medulla[71], where the dorsal root fibers terminate. Thus, there would appear to be high glutamate levels associated with regions containing the primary afferent neuron system.

Total free amino acid levels in the dorsal root ganglion are much higher than the dorsal root levels[71], with many amino acids being elevated to account for this difference[49,71]. Although much of the high amino acid content of the ganglion may be accounted for by the use in protein synthesis in the perikarya, some of it may be useful in the formation and regulation of the transmitter levels of glutamate destined for the central terminals. Although this possibility has been explored in great detail in the cerebral cortex, as will be seen later, there have been no studies at all on the regulation of transmitter levels of the transmitter candidate glutamic acid in the dorsal sensory neuron. Since glutamine levels in the sensory ganglion are higher than glutamate levels[49,71], it would also be of interest to determine whether glutamine in the perikaryon of the dorsal sensory neuron may be instrumental as a storage form and/or precursor of elevated glutamate levels being directed centrally.

The above data on glutamic acid in the dorsal sensory neuron system suggest that there is a differential flow mechanism existent in the axonal bifurcation directing more free glutamate proximally towards the cord than distally towards the periphery. Virtually nothing is known about the gating process or the flow process for glutamic acid here. Tritiated glutamic acid has been shown[77] to flow proximo-distally in nerves of snails and frogs at rates of 720 mm/day and 120 mm/day respectively. The studies of axoplasmic flow in the dorsal sensory neuron system of mammals have been conducted with tritiated or ^{14}C labeled leucine because of its stability compared to other labeled amino acids. This permits a more accurate picture of the flow process to be obtained with a minimum of side reactions to complicate the observed distribution. Rate estimates for the flow of labeled leucine from the dorsal root ganglion down the axonal extensions have revealed a slow flow rate of 1.3 mm/day[97] and rapid flow rates of 401–410 mm/day[119,120] or 500 mm/day[97]. Recently, it was shown that this fast transport mechanism in the axon is (1) distinct from the slow flow mechanism[119],

(2) a local mechanism present along the whole axon[119], (3) dependent upon oxidative metabolism for continued functioning[118], and (4) in need of a *local* energy source (ATP) along the entire length of nerve[117]. Thus, although we know something of the general nature of slow and rapid axonal transport processes in the dorsal sensory neuron, there is little knowledge of the relationship of these processes to *different* free amino acids moving down the axon. For glutamic acid, which is very active metabolically and will not be a stable labeled substance, analysis of axoplasmic flow processes may necessitate extensive analysis of label transfer to other substances. There is a need, therefore, to clarify the mechanism whereby more glutamate is directed centrally than peripherally in the dorsal sensory neuron. This may then provide some clues concerning the roles of the perikaryon and the synaptic terminal in regulating the glutamate transmitter pool.

In addition to the spinal cord (above) free amino acid levels have been extensively investigated in several regions of the CNS representing a cross section of the various areas[10,16,48,66,67,71,72,144]. Although each particular region is characterized by a peculiar free amino distribution pattern, there does appear to be a pattern of distribution for certain amino acids between the different CNS areas. For glutamic acid there is a stepwise increase in content when progressing from lower to higher areas[72], with the levels being in μmoles/g: (1) sympathetic ganglion (2.98); (2) dorsal root ganglion (4.30); (3) grey of the lumbosacral spinal cord and primary sensory nuclei of the caudal medulla (6.92); (4) superior and inferior colliculi, and thalamus (8.38); (5) primary somatic sensory cortex, primary auditory cortex, cerebellum, and caudate nucleus (10.9); (6) caudate nucleus, pyriform lobe, and the lower junction of the sylvian and ectosylvian gyri (12.4). (The caudate nucleus was intermediate between the latter two groups.) This high level of glutamate in the cerebral cortex has been shown to be related to the functional integrity of the cortex. When the suprasylvian gyrus of cats was isolated from the surrounding cortex and underlying white matter[14] there was a decrease in glutamate, aspartate, and GABA and an increase in glutamine, all of which were maximal at one week after isolation. Even at its lower

TABLE I

FREE AMINO ACIDS IN THE NERVOUS SYSTEM

Levels of individual free amino acids are compared in the dorsal root ganglion (DRG), grey of the lumbosacral spinal cord (G-LSC), thalamus, cerebellum, and cerebral cortex. All values are given in μmoles/g.

	DRG[71]	*G-LSC*[166]	*Thalamus*[10]	*Cerebellum*[10]	*Cerebral cortex*[10]
Arginine	2.17	0.14	0.173	0.193	0.170
Alanine	2.39	0.62	0.591	0.895	0.853
Aspartate	2.33	2.14	2.71	2.85	3.09
Glycine	2.11	4.47	1.72	1.49	1.25
Serine threonine	2.20	0.62	1.69	1.87	1.70
Taurine	1.23	1.58[110]	1.06	3.12	1.89
Glutamate	4.30[72]	6.91[72]	8.94[72]	10.8[72]	12.9[72]

level at seven days after isolation, however, glutamate levels in the cortex are still almost twice as high as the levels observed in the dorsal root ganglion (above). Glutamic acid is unique in this fact, no other amino acid showing such a distribution, as is evident in Table I which shows the levels of several amino acids in addition to glutamate in representative regions of the nervous system. This glutamate gradient has been suggested for the cat[72] to be sigmoidally related to oxygen consumption, indicating that glutamic acid is closely tied to metabolic functions in the CNS.

Most of the work on the distribution of glutamic acid or its associated enzymes in various subcellular fractions obtained by ultracentrifugation and density gradient studies, have been carried out on cerebral cortical tissue. This has been the preferred choice since glutamic acid is very high in the cortex, and it may have a transmitter role here. Although most of the glutamate was observed to be in the soluble fraction[132], that in the particulate fraction was associated with the portion containing the nerve ending fragments[132,164], and the iontophoresis of isolated and specially prepared fractions has an excitatory effect due to the contained glutamate[92]. In fact, the association of glutamate and GABA with synaptic vesicles is greater than that for a substance such as lactate dehydrogenase[94] which is not specifically associated with any transmitter system. While glutamic acid seems to be mainly in the soluble fraction, tritiated glutamic acid, when applied to incubated cortical slices, concentrates in a synaptosomal fraction[93]. Although the above association of glutamic acid with a synaptosomal or synaptic vesicular fraction suggests that this amino acid is here functioning as a synaptic transmitter, there are other roles possible as well. Since glutamic acid is a precursor of the inhibitory transmitter candidate GABA, a portion of this glutamate may be serving as a GABA precursor[11,13,15,18]. This suggestion is partially supported by data[9,164] indicating that glutamate decarboxylase, which catalyzes the conversion of glutamate to GABA, is associated with a particulate fraction containing large amounts of nerve ending fragments. Recently[170], however, it has been shown that there is a synaptosomal fraction accumulating glutamate which is unique from the corresponding fractions accumulating GABA.

Several workers[103,133,168] suggest caution concerning the interpretation of the subcellular distribution of glutamate. Glutamate is suggested as being more of a soluble cytoplasmic marker and was not specifically associated with the synaptic vesicular fraction[133]. The glutamate transmitter system appears to be different from the cholinergic transmitter system and may not show a marked concentration in any synaptic vesicular component[103,168]. Part of the problem here is due, of course, to the many functions that glutamic acid has besides possibly being a synaptic transmitter. However, although it would seem that glutamic acid is widely distributed in the subcellular fractions of cerebral cortical tissue, recent data[93,170] have revealed what may be a specific glutamate transmitter pool, and further clarifying work is needed. Another major difficulty may be caused by the very high level of glutamate in the cerebral cortex not being related to any glutamate transmitter system at all, but, instead to a large amount of nontransmitter glutamate. Perhaps a better tissue for these studies would be the dorsal root terminals where there appears to be a relatively small metabolic pool of glutamate compared to cerebral cortex.

METABOLISM AND THE GLUTAMATE SYSTEM OF MAMMALIAN NEURONS

Glutamic acid is actively taken up into the brain against a concentration gradient[146, 147,151,155]. This uptake of glutamate appears to be greatest in brain tissue compared to other tissue[56,138], and glutamine may be instrumental here in that it crosses the blood-brain barrier more easily than glutamate[138,146], although the majority of glutamine molecules here are primarily used for the removal of ammonia from the brain. Glutamic acid is also actively transported into brain mitochondria[8], where there are specific mechanisms for handling this amino acid metabolically. The suggestion that there are changes in mitochondrial properties with CNS development[136], that there may be differences in mitochondria located in different portions of the neuron[63], and that there is a difference in properties of brain mitochondria compared to liver mitochondria for instance[61], introduces the possibility of associating some of the observed differences in the glutamate-glutamine system in the various CNS areas with specific mitochondrial differences.

It has long been known that with systemic injection of labeled glutamate, the specific radioactivity of glutamine very soon after injection exceeds that observed for glutamate in brain tissue[15,95,162], although this is not a unique property of brain tissue. In fact, Rose and Cory[131] have indicated that this does not occur at all in the octopus brain. This phenomenon is a property of the mammalian brain, however, and recent studies provide clues which may help to explain this observation. There is a great amount of evidence suggesting a compartmentation of glutamic acid in the nervous system[11-13,15,17,102,121,157,158]. The brain seems to be unique in this respect compared to a tissue such as liver[17]. There appears to be a large and a small pool or compartment of glutamate, with the large pool being relatively inaccessible to the small pool. The compartmentation of glutamic acid occurs as the brain matures towards adult form[157], and the time course for the development of compartmentation varies for different CNS regions[12].

The small pool of glutamate is associated with glutamic acid dehydrogenase and glutamine synthetase[121,158] and what has been called a 'synthetic tricarboxylic acid cycle', with the primary precursors being acetate, butyrate, phenylalanine, proline, tyrosine, bicarbonate, pyruvate, and glucose. This small glutamate pool is indicated as being the primary precursor of glutamine[11,13,15,102], thus explaining the higher specific radioactivity of brain glutamine compared to glutamate after systemic injection of labeled glutamate[15,95,162] since this pool has a rapid turnover.

In association with the above, it was noted[46] that very early after stimulation a decrease in glutamine but not glutamate was observed in the cat cerebral cortex, one interpretation being that glutamic acid was serving as an excitatory synaptic transmitter with glutamine being converted to glutamate as needed in order to maintain the transmitter pool of glutamate. It remains to be determined whether this latter conversion of glutamine to glutamate is taking place through the small or large glutamate pool.

The larger pool of glutamate is associated with transaminases[11,121] and a 'tricarboxylic acid energy cycle'. The feed-in of glutamate formed here to glutamate

in the small pool or to glutamine is minimal[11,13,15,102]. The transaminases would have the function of converting their respective amino acids to the corresponding oxo acids in order to keep the TCA cycle intermediates regulated at proper levels. The most important precursors are, besides certain amino acids, glucose, glycerol and pyruvate or lactate[121].

Basic to the concept of any proposed glutamate transmitter system is the idea of a metabolic pool of glutamate common to all neurons and a transmitter pool found only in those neurons where this substance is the synaptic transmitter. There is, therefore, the necessity of delineating and differentiating the mechanisms whereby metabolic and transmitter levels of glutamate are each regulated. Thus, it is imperative that a firm grasp of the general metabolism of glutamate is developed, with the concept of regulation of transmitter levels being built upon this. Much more work is needed before the roles of the two functionally separate tricarboxylic acid cycles and glutamate pools, discussed above, and their relation to any proposed glutamate transmitter systems can be clearly outlined. It would appear, however, that a dual system for metabolism of glutamate is present in brain tissue, and this may be related in some way to the independent regulation of the metabolic and transmitter pools of this amino acid.

Aspartic acid is intimately related to metabolism in the brain[2] with aspartate serving as a source of glutamate[11], and glutamate serving as a source of aspartate through the tricarboxylic acid cycle[110]. A large portion of the aspartate pool in brain is associated with the tricarboxylic acid 'energy cycle'[158] as is true also for a large fraction of the brain glutamate pool[11,121]. Aspartic acid, as well as the other amino acids through amino transferase activity, may thus have a significant role in control of transmitter and/or nontransmitter pools of glutamate in the brain.

Since glucose is important for its role in serving as a source for glutamic acid[101,121,157] as well as for production of metabolic energy, the importance of glycogen in glutamate metabolism must also be considered. Glycogen has been found to be present in many regions of the nervous system[28,43,54,84,112,113,141], and can be in several different forms. Electron microscopically the neuron usually is characterized by low glycogen levels[55], exceptions being in the hypothalamus and area postrema[111]. Pannese[123] observed tight glycogen particle-smooth endoplasmic reticulum complexes in the perikaryon of the dorsal sensory neuron. This is interesting, since it is here that there is fairly good evidence for a glutamate transmitter system being present, and this membrane-particle complex appears to be unique to the perikaryon of the dorsal sensory neuron. The possibility that these specialized glycogen stores in the dorsal sensory neuron are for the purpose of (1) metabolic need when glucose is low, or, (2) regulation of glutamic transmitter levels, must therefore be tested. Recent data[25] suggest that glycogen storage may be just one of several properties in which neurons differing functionally have characteristic differences. Also, since glycogen in the nervous system is such a labile substance, and serves as a source of metabolic substrate during increased activity[26,71,80,82,98,111,115,131], there is the problem of delineating, during increased activity, the specific role of glycogen relative to a possible glutamic transmitter pool in addition to its general metabolic importance.

However, (1) since glutamate is so closely related to oxidative metabolism and the tricarboxylic acid cycle, and (2) since glycogen and glucose have a key role in providing substrate for the tricarboxylic acid cycle, glycogen may very well have a role in maintaining the metabolic cycles in the dorsal sensory neuron needed in excess for regulation of transmitter levels of glutamate as well as for the usual function of supplying energy.

SUMMARY AND COMMENTS

In addition to the many functions which glutamic acid has in the nervous system, data from several disciplines has suggested that this substance is a good transmitter candidate for certain neuronal systems. (1) It is excitatory to neurons from many areas of the nervous system, and seems to be discriminative *to a certain degree* in the ability to do this. (2) It has been observed to be released when specific projection systems are stimulated. (3) Although it is common component of all neurons, it has a specific localization in a synaptic vesicular fraction as well. (4) Quantitatively, its distribution in certain neuronal systems is consistent with a transmitter function. (5) There is more than one pool of glutamate present in brain, with each pool being related to functionally different tricarboxylic acid cycles — this at least being consistent with the roles of glutamic acid in general neuronal functioning as well as in chemical transmission at certain synapses.

Certain problems, however, have arisen, particularly with respect to the mammalian CNS, and suggest caution and further study. (1) Analysis of postsynaptic responses have not as yet been sufficient to prove the identity of the excitatory postsynaptic potential and the glutamate induced response. (2) None of the release studies definitely prove that glutamate is being released from a specific synaptic system as a synaptic transmitter, but merely suggest that this may be possible. For the proposed invertebrate glutamate transmitter system, however, the glutamate release studies can be related rather specifically to a particular synapse. (3) The association of a glutamate transmitter pool with a synaptic vesicular or synaptosomal fraction is at present unclear — this partially being due to the other roles of glutamate in the CNS, and to the synaptic multiplicity of most CNS regions. (4) Besides the role of glutamate in the CNS as a synaptic transmitter at certain synapses, there is the suggestion of a rather unique involvement, in the CNS compared to other tissues, in ammonia metabolism, transamination, and oxidative metabolism. This fact has introduced complications into the interpretation of neurochemical analysis of the glutamate transmitter system. (5) Also, most of the glutamate transmitter studies, whether neurochemical or electrophysiological, have not dealt with a particular neuronal network in the mammalian CNS. We have not concentrated our efforts on a particular neuronal system in the mammalian nervous system as has been done so excellently in the invertebrate neuromuscular junction for glutamic acid and GABA, or even in the vertebrate cholinergic neuromuscular junction. It is necessary now to determine whether glutamic acid fulfills *all* of the transmitter criteria for a *single neuronal system* where it is a transmitter candidate. This could then provide a model of

approach for the analysis of the transmitter role of glutamate in the rest of the mammalian CNS.

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The Use of Monosodium Glutamate[a] in Frozen Pork Sausage[b]

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The effect of MSG on seasoned and unseasoned frozen pork sausage was studied at two levels of concentration. MSG increased the desirability of seasoned sausage after 4 months' storage. Despite some quality deterioration all groups treated with MSG were very acceptable after a year's storage.

The storage of frozen pork sausage for use from 6 months to a year later is quite prevalent among freezer locker patrons and home freezer owners. In many instances, however, the product becomes very unsatisfactory during that time. Butler *(1)* and Dubois and Tressler *(3)* show that salt in frozen sausage speeds the development of rancidity, whereas spices seem to have an opposite effect. Unseasoned ground pork, although it retains its color and original properties for a longer period in storage, is undesirable from the housewife's standpoint because of the labor involved and the difficulty of obtaining a properly seasoned product when only small amounts are thawed. Therefore, the addition of a substance to increase the freezer storage life of seasoned pork sausage is desirable.

Use of monosodium glutamate to enhance the flavor of certain foods has been widely accepted *(2, 4, 5, 8)*. Its favorable use in frozen vegetables has been reported by Sluey and Eichenberger *(7)*. Norton, Tressler and Farkas *(6)* have shown that its use in many frozen meats, including pork sausage, greatly improves the acceptability of the frozen product. These workers also stated that MSG demonstrated, organoleptically, some properties as an antioxidant.

This study was designed to test the effect of MSG on seasoned and unseasoned frozen pork sausage when added at two levels of concentration and when the sausage was kept in freezer storage for as long as a year.

EXPERIMENTAL PROCEDURE

The pork used in this study was produced from shoulders and trimmings taken from Hampshire barrows and gilts weighing about 200 pounds each. The hogs were slaughtered in the conventional manner, and their carcasses were chilled at 34-38° F. (1.1-3.3° C.) for approximately 48 hours. The meat was cut into chunks and was mixed so as to have an estimated fat content of 25-35%. In order to check the estimation, after grinding samples were obtained for moisture and ether extract determinations. Ether extraction was done by the continuous process for 6 hours, using ethyl ether. Moisture was determined by the use of a vacuum oven at 70° C. for 24 hours. Analysis revealed the mixture to contain 31.2% ether extract and 48.0% moisture.

Six groups of sausage were treated as follows:

1. Control-- not seasoned.
2. Seasoned.
3. Not seasoned plus 0.1% MSG.
4. Seasoned plus 0.1% MSG.
5. Not seasoned plus 0.3% MSG.
6. Seasoned plus 0.3% MSG.

The following seasoning was added to each 6 pounds of meat:

1½ ounces salt
½ ounce black pepper
¼ ounce sage

The seasoning was thoroughly mixed with the pieces of meat before they were ground and then the mixture ground once through a 3 mm. plate. The MSG was mixed with the seasoning when seasoning was used and was mixed with the meat before grinding when seasoning was not used.

After being ground, the sausage was tightly wrapped into approximately 1 pound packages, using a heavy commercial locker paper. The weight of each package was recorded on the paper to the nearest gram. The sausage was then frozen in an air blast at 0° F. (−17.8° C.) and stored at 0° F.

After 2 months of storage, monthly taste tests were conducted. The sausage was removed from the freezer, weighed, and allowed to thaw for approximately 24 hours in a 40° F. (4.4° C.) cooler. The packages were then unwrapped and the sausage removed and placed in containers so that it could be mixed. Four pounds of sausage from each group was usually used in the taste test. Before being cooked the unseasoned sausage was seasoned at the same rate as that which had been seasoned before being frozen. All the sausage was kneaded alike in order to insure a uniform texture. The sausage was made into patties and was pan broiled in open skillets over a gas flame. The grease was poured off as it accumulated. For the first five taste tests the control group, the seasoned group containing no MSG, and the two groups containing 0.1% MSG were used. Beginning with the sixth taste test all six groups were used.

The taste panel of 8 to 14 members included about equal numbers of men and women.

The judges ranked the samples, using a score card with the following classifications: *excellent, very good, good, fair, slightly rancid, moderately rancid, extremely rancid,* and *inedible*. The rankings were given a numerical rating of from 7 for *excellent* to 0 for *inedible*. The samples were coded in each test. The results were analyzed statistically by analysis of variance.

RESULTS AND DISCUSSION

The loss of weight in freezer storage was negligible, averaging less than 1% even after a year's storage. Visual appearance indicated that the seasoned sausage had darkened with age whereas all unseasoned samples were still bright after a year. The seasoned sausage without MSG appeared to have a slightly stronger odor than the seasoned with MSG. The unseasoned sausage appeared to have a much fresher odor after a year's storage. Table 1 gives the results of the palatability panel.

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[a] Supplied by Huron Milling Company, New York, N. Y.

[b] The investigation reported in this paper relates to a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

[c] The author acknowledges the services rendered by Miss Florence Imlay, Field Agent in Foods and Nutrition, and Mrs. Pearl J. Haak, Field Agent in Foods, in preparing the sausage for the palatability tests.

In all cases, after 4 months' storage the seasoned sausage without MSG was less desirable than either the seasoned plus MSG, the unseasoned control or the unseasoned plus MSG. These differences were significant or highly significant at all periods except at the eleventh month. This perhaps can be explained because some of the veteran taste panel members could not participate; consequently, there was more variability among tasters.

TABLE 1

Palatability scores on frozen sausage

Month after freezing	Sample treatment					
	Control	Seasoned	Not seasoned plus 0.1% MSG	Seasoned plus 0.1% MSG	Not seasoned plus 0.3% MSG	Seasoned plus 0.3% MSG
2	5.9	5.1	5.8	5.1		
3	5.4	5.6	5.9	5.9		
4	5.6	4.1**	5.9	6.2		
5	5.7	3.5**	6.5	6.0		
6	5.6	3.6**	5.8	4.2		
7	4.5	3.1**	5.5	4.9	5.3	5.5
8	4.5	2.0**	4.8	5.8	5.8	5.5
9	5.3	3.0**	5.2	5.6	5.1	5.9
10	5.1	3.5**	5.8	4.7	4.7	5.0
11	4.8	3.9	4.1	4.9	4.5	4.8
12	4.8	3.2**	4.9	5.6	5.3	5.8

* Significant at 0.05 level.
** Significant at 0.01 level.

Data were further analyzed without using group 2 (the seasoned sausage without MSG). There were no significant differences among the groups except at the sixth month when the seasoned plus 0.1% MSG group was significantly less desirable. As this occurred only once little significance is indicated. Therefore, these data show that unseasoned sausage, unseasoned sausage with MSG, or seasoned sausage plus MSG are similar in acceptability for as long as a year in freezer storage. Although the control and the MSG treated samples were less desirable after several months' storage they were still very acceptable after a year, whereas the seasoned group without MSG was unsatisfactory after 4 to 5 months.

The addition of 0.3% MSG instead of the 0.1% had little effect upon the desirability.

SUMMARY AND CONCLUSIONS

Sausage was prepared in six ways. One group was an unseasoned control; one group was seasoned; two groups were not seasoned but contained 0.1% and 0.3% respectively of monosodium glutamate, and two groups were seasoned and also contained 0.1% and 0.3% respectively, of monosodium glutamate. The sausage was frozen and then subjected to a taste panel each month after 2 months' storage, all sausage being seasoned and kneaded alike before being cooked.

The addition of monosodium glutamate to seasoned sausage significantly increased the desirability of the sausage after 4 months' storage. Little difference was detected between the unseasoned control and the seasoned and unseasoned groups when monosodium glutamate was added. The addition of 0.3% instead of 0.1% monosodium glutamate was of no additional advantage.

Although there seemed to be some deterioration in quality, all groups treated with monosodium glutamate were very acceptable after a year of storage, whereas the seasoned sausage without monosodium glutamate was much less desirable even after 4 months of storage.

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A Research Note

EFFECT OF CONCURRENT CALORIC INTAKE ON THE RESPONSE TO ORAL MONOSODIUM L-GLUTAMATE IN SUSCEPTIBLE SUBJECTS

INTRODUCTION

IN A PREVIOUS paper (Kenney and Tidball, 1972) we reported a placebo controlled study of the susceptibility of a group of volunteers to a 5g oral dose of monosodium L-glutamate (MSG) served in tomato juice. Twenty five of the total of 77 subjects tested reported symptoms following the dose of MSG. members of the reacting group revealed a dose-response relationship in the dosage range of 1–5g. In both the initial survey and in the subsequent study, the MSG was administered at least 2 hr after the previous snack (breakfast) and an analysis of the symptom occurrence in relation to the quantity or nature of food taken revealed no effect of previous feeding.

Both culinary and commercial practice in the use of MSG would make the ingestion of 5g unlikely in a single dose of fluid and in fact any ingestion would normally be as part of a meal of significant calorie content. Natural L-glutamic acid moreover, as a constituent of protein, is frequently ingested in quantities in excess of 5g at a single meal.

A study was therefore undertaken to assess the susceptibility of a population of proven reactors to a standard dose (5g) of MSG given as part of a lunch-type snack. In addition the study was designed to test the response to naturally occurring L-glutamic acid in amounts comparable with the test dose of MSG.

MATERIAL & METHODS

THE SUBJECTS for the study were 13 members of the reactor group who had previously been studied in depth. They were acquainted with the objectives of the study to which they had given their informed consent. On each test occasion subjects were provided with a packaged lunch accompanied by a glass of tomato juice. Each subject was given a questionnaire which she was required to answer in the 2 hr following the snack.

Four lunch-type snacks were used in the study. The compositions of the snacks were selected to provide 400–470 calories with in one case a high fat low protein mixture; in another a high carbohydrate, low protein mixture, and in the two other cases high protein content with differing contents of natural L-glutamic acid. The compositions of the snacks were as follows:

Snack A: 50g cream cheese, 2 rye crisp crackers, 10g margarine and 150 ml tomato juice.

Snack B: 75g Wensleydale cheese, 2 rye crisp crackers, 150 ml tomato juice (margarine if desired).

Snack C: 75g Gruyere cheese, 2 rye crisp crackers, 150 ml tomato juice (margarine if desired).

Snack D: 4 oatmeal cookies, 20g jelly or marmalade, 2 saltine crackers, 150 ml tomato juice (margarine if desired).

These snacks provided calories, protein and natural L-glutamic acid in the following amounts:

	Calories (Approx)	Protein (g)	L-glutamic acid (g)
Snack A:	400	4	0.15
Snack B:	420	28	5.5
Snack C:	470	35	7.8
Snack D	440	6	0.2

Each snack was administered to each subject four times: on two occasions the tomato juice contained 5g of MSG and on the other occasions the juice was lightly salted (0.8g NaCl per 150 ml) to provide an equivalent-taste placebo.

RESULTS & DISCUSSION

RESPONSES RECORDED following the ingestion of either placebo juice or the juice containing MSG differed in no qualitative way from those reported in the earlier study and included sensations of warmth or burning, stiffness or tightness (usually of face or neck), weakness of the limbs, pressure (usually of the head or chest) and tingling. In addition there were reports of more general malaise or headache. The frequency of symptom occurrence is recorded in Table 1. Snack designations including the letter P indicate a snack in which the juice contained no added MSG. Snacks accompanied by the test dose of MSG have the designation M. The latency of symptom onset in this series is consistently longer than when MSG is administered in juice unaccompanied by a snack (Kenney and Tidball, 1972, Table 13). However, the wide variability clouds the possible significance of this observation.

Snack A:	35 min ± 16
Snack B:	32 min ± 12
Snack C:	27 min ± 15
Snack D:	34 min ± 14
Previous series:	22 min ± 7

In the previous study (Kenney and Tidball, 1972) of the dose-reaction relationship of these subjects a placebo response rate of 20% was obtained. It is apparent therefore that the ingestion, along with the placebo juice, of as much as 5g of natural L-glutamic acid in the form of protein produces no additional response in this susceptible population. The slightly higher response seen with snack CP is not significantly different from the other placebo series. However, the response rate to snacks containing 5g of added MSG is significantly lower ($P < 0.01$) in cases B, C and D than in the case of Snack A or the previously determined response to this dose of MSG given without a snack. This protection appears to be afforded equally well by high protein (and high natural L-glutamic acid) snacks (B and C) and high carbohydrate snacks (Snack D). However, the high fat snack A (which is also of low bulk) affords no protection.

Table 1—Frequency of symptom occurrence

Snack[a]	No. of administrations	No. of positive responses	Percentage of positive responses
AP	24	4	16.6%
AM	24	19	79.1
BP	22	3	13.6
BM	23	10	43.4
CP	23	6	26.1
CM	23	10	43.4
DP	24	5	20.8
DM	24	11	45.8

[a] P indicates juice contained no added MSG; M indicates juice contained test dose of MSG.

In practical terms this outcome would suggest that untoward symptoms will be provoked by MSG in a general unselected population even less frequently than was earlier suggested when the condiment is used in the culinary setting of rather small amounts in dishes with a significant calorie content. Of the total population studied earlier 32.5% reacted at the 5g level. In the present study giving a snack with the juice halved the frequency of symptom occurrence at the 5g level (Snacks B, C and D). Furthermore the dose-response characteristics of these susceptible subjects indicates that at a more realistic level of perhaps 1g or 2g, symptom occurrence following the ingestion of MSG in a snack would be no more frequent than in response to an equivalent flavor placebo.

REFERENCE

Kenney, R.A. and Tidball, C.S. 1972. Human susceptibility to oral monosodium L-glutamate. Am. J. Clin. Nutr. 25: 140.

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3,003,925

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M. glutamicus

3,003,925
METHOD OF PRODUCING L-GLUTAMIC ACID BY FERMENTATION

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This invention relates to a method of producing l-glutamic acid by fermentation. Particularly, it relates to a method of producing and accumulating l-glutamic acid in a substantial amount by culturing a strain which belongs to a new species of Micrococcus, named *Micrococcus glutamicus*, in a particular culturing medium under adequate fermentation conditions, and of recovering the same.

It is well known that microorganisms can produce in a suitable medium various kinds of amino acids. The amount produced, however, has been extremely small and it has never been reported that any particular kind of amino acid was accumulated in large quantities in a medium by a fermentation process.

The reason for extreme difficulty in the accumulation of any amino acids in a fermenting medium is that amino acids are the components of proteins, and any amino acid once produced in a medium is apt to be easily resynthesized to proteins, polypeptides or the like, or is apt to be converted or decomposed to other substances by various biochemical reactions. In other words, an amino acid can hardly be accumulated in a culturing medium in the monomeric state. The word "monomeric state" is used in the present specification to mean monomolecular state whether it be a free acid or a salt.

This is the reason why a fermentation process, a direct use of biochemical activities of a living population of microorganism, has never been proposed heretofore for the biosynthesis and accumulation of l-glutamic acid. The known biosynthesis is effected in some enzymatic system, that is, a particular kind of enzyme is extracted from microorganisms, or from some animal or plant tissues, and the enzyme is reacted with suitable substrates e.g. α-ketoglutaric acid and ammonium compounds under a very limited reaction condition.

An object of the present invention is to provide a method for producing directly a substantial amount of l-glutamic acid by culturing a certain microorganism in a liquid medium.

A further object of the present invention is to provide a method for accumulating a large amount of amino acid, particularly l-glutamic acid, in the form of monomeric state by culturing a certain microorganism in a liquid medium.

A further object of the present invention is to provide a method for inhibiting any side reactions such as polymerizing or decomposing of l-glutamic acid that would occur during the cultivation of a certain microorganism in a liquid medium.

A still further object of the present invention is to provide a method of accumulating l-glutamic acid in a liquid medium in the monomeric state, that is, a readily recoverable form.

According to the present invention, a new strain of Micrococcus is cultured in a suitable medium under adequate fermentation conditions, and a substantial amount of l-glutamic acid is accumulated in the medium. A one-step aerobic fermentation, in the form of a reductive amination, produces l-glutamic acid from carbohydrates. The process is industrially and economically feasible. No enzymes are employed.

The inventors have found new strains which are suitable for the present fermentation process. Said strains are actually strains of a new species of a Micrococcus designated *M. glutamicus* and include a strain designated *M. glutamicus* No. 534 (also called Micrococcus No. 534) which was isolated from sewage, and other several strains which were isolated from air or soil. Their microbiological characteristics are stated below. We inventors of the present invention, could not find in Bergey's Manual of Determinative Bacteriology (5th and 6th editions) a species that could be strictly identified with said new species which we have named "*Micrococcus glutamicus*." By ultraviolet irradiation of the strain No. 534, color mutants (No. 613, No. 614) and auxotrophic mutants (No. 601, No. 602) were also obtained.

M. glutamicus No. 534 is compared with other micrococci in Table I. In this table we followed to the key of the "Bergey's Manual of Determinative Bacteriology, 6th ed."

As shown in Table I, the species of No. 18 to No. 22 are fundamentally different from *M. glutamicus* No. 534 in their anaerobic character. The species of No. 13 to No. 17 are definitely differentiated from *M. glutamicus* No. 534 in their red pigment production. The species of No. 1 to No. 5 are clearly different from *M. glutamicus* No. 534 in their inability to produce nitrites from nitrates. The species of No. 8 to No. 10 and No. 6 are different in gelatin liquefying character and acid production in milk from *M. glutamicus* No. 534. The species of No. 7, No. 11 and No. 12 are different in acid production in milk from *M. glutamicus* No. 534. The species of No. 8, No. 9*a*, No. 9*b* and No. 11 are also different in pigment production.

In addition, the species of No. 6 to No. 8 are differentiated in their ability to utilize ammonium salt as a sole source of nitrogen in Hucker's medium.

TABLE I

Comparison of M. glutamicus *No. 534 with other micrococci*

	A	B	C	D	E	F	G	H
1. *M. luteus*	+	−	−*					
2. *M. [illegible]*	+	−	−*					
3. *M. [illegible]*	+	−	−*					
4. *M. [illegible]*	+	−	−*					
5. *M. [illegible]*	+	−	−*					
6. *M. [illegible]*	+	−	−*					
7. *M. candidus*	+	−	−*					

See footnote at end of table.

TABLE I—Continued

	A	B	C	D	E	F	G	H
6. *M. conglomeratus*	+	−	+	+*	+	+*	+*	+*
7. *M. flavus*	+	−	+	+*	+	+*	−	+*
8. *M. cascolyticus***	+	−	+	+*	−*	+*	+*	+*
9. *M. pyogenes* var. *aureus*	+	−	+	−	Orange*	+*	+*	+*
10. *M. pyogenes* var. *albus*	+	−	+	−	−*	+*	+*	+*
11. *M. citreus*	+	−	+	−	+	+*	+*	+*
12. *M. sodonensis*	+	−	+	−	Orange*	+	−	+
13. *M. epidermidis*	+	−	+	−	−*	+*	−	+*
14. *M. lysodeikticus*	+	+*	------	------	------	------	------	------
15. *M. cinnabareus*	+	+*	------	------	------	------	------	------
16. *M. roseus*	+	+*	------	------	------	------	------	------
17. *M. roseochrous*	+	+*	------	------	------	------	------	------
18. *M. aurantiacus*	+	+*	------	------	------	------	------	------
19. *M. aerogenes*	−*	------	------	------	------	------	------	------
20. *M. saccharolyticus*	−*	------	------	------	------	------	------	------
21. *M. nigrescens*	−*	------	------	------	------	------	------	------
22. *M. grigoroffi*	−*	------	------	------	------	------	------	------
23. *M. anaerobius*	−*	------	------	------	------	------	------	------
M. glutamicus No. 534	+	−	+	−	+	−	−	−

A—Relation to free oxygen (+: aerobic to facultatively anaerobic, −: anaerobic).
B—Reddish pigment production on agar.
C—Nitrate reduction to nitrite.
D—Ammonium salt utilization as a sole source of nitrogen.
E—Yellow pigment production on agar.
F—Acid production in milk.
G—Gelatin liquefaction.
H—Acid production from lactose.
* Different from *M. glutamicus* No. 534.
** Two strains of *M. cascolyticus* are found in Bergey's Manual of Determinative Bacteriology, 6th edition.
Each strain is compared in the Table I. (In the absence of other definition the + and the − indicate the presence and absence, respectively, of the stated characteristic.)

A comparison of l-glutamic acid dehydrogenase activity among various species of genus Micrococcus is given in Table II. A description of experimental methods follows:

(A) *Preparation of l-glutamic acid dehydrogenase from micrococci cells.*—The composition of the culture medium was as follows: meat extract 0.5%, peptone 1.0%, glucose 2.0%, NaCl 0.25%, K_2HPO_4 0.1%, $MgSO_4.7H_2O$ 0.25% and yeast extract 0.2%. The pH of the medium was adjusted at 7.0. Organism tested was inoculated in the above medium, and cultured for 20 to 24 hours at 28° C. on a shaking-culture device. After the culture, cells were harvested by centrifugation and washed twice with biological saline water. Washed cells were homogenized by grinding with about twice the amount of fine quartz sands. The homogenate thus obtained was centrifuged for 10 minutes at 10,000 r.p.m. The supernatant was then dialized for 24 hours against M/15 phosphate buffer (pH 7.8) at 0° C. This dializate was used as l-glutamic acid dehydrogenase preparation.

(B) *Assay method for the determination of l-glutamic acid dehydrogenase activity.*—In the assay of l-glutamic acid dehydrogenase activity, method of Olson and Anfinsen (J. Biol. Chem. 197, 67 (1952)), was modified. The composition of the test solution was as follows:

0.5 ml. of M/10 l-glutamate solution, 1.0 ml. of coenzyme solution (each 1 μM of TPN and DPN were contained in 1.0 ml. of this solution), and 1.0 ml. of M/15 phosphate buffer, pH 78. At zero time, the above test solution and 1.0 ml. of enzyme preparation were mixed in a Beckmann spectrophotometric cuvette, and the absorbancy at 340 mμ was measured every minute for 6 minutes using Beckmann model DU spectrophotometer. The increase of absorbancy during five minutes after the first one minute was defined as ΔE. ΔE was proportional to the concentration of the enzyme if the increment of absorbancy was lower than 0.070. Specific activity of the enzyme was defined as ΔE/gram of protein in enzyme solution. All the assays were carried out at 30° C.

Results.—The results are shown in Table II. As seen in the table, *Micrococcus glutamicus* has considerably higher activity of l-glutamic acid dehydrogenase than other known species of Micrococcus, therefore *M. glutamicus* is easily distinguishable from other species.

TABLE II

Comparative activities of l-glutamic acid dehydrogenase in various species of genus Micrococcus

Strain:	Specific activity (ΔE/gr. of protein)
M. epidermidis ATCC 155	1.4
M. lysodeikticus ATCC 4698	Trace
M. varians ATCC 399	6.0
M. citreus ATCC 4012	3.0
M. cascolyticus ATCC 8460	Trace
M. sodonensis Aaronson	Trace
M. conglomeratus Migula	28.0
M. pyogenes var. *albus* Schroeter	Trace
M. flavus ATCC 10240	75.0
M. glutamicus No. 534	580.0
M. glutamicus No. 541	551.0
M. glutamicus No. 560	434.0
M. glutamicus No. 588	570.0
M. glutamicus No. 582	560.0

The characteristics of *Micrococcus glutamicus*, represented by *Micrococcus glutamicus* No. 534 (ATCC No. 13032), will now be described in detail. The experimental tests were done according to the methods described in the Descriptive Chart of "Manual of Methods for Pure Culture Study of Bacteria," published by the Society of American Bacteriologists (9th edition), 1950.

Its morphological and physiological characters are as follows:

Slightly ellipsoidal spheres, usually in pairs, but also in single and irregular masses. Size of majority: 0.6-1.0 microns. Elongated forms are seen in some conditions. Not motile, spores are not formed. Gram-positive.

Agar slant: Moderate growth, filiform, dull, pale yellow.

Agar colonies: Circular, smooth, perfect, slightly elevated, pale yellow.

Broth: Slightly turbid, rings are seen sometimes along tube. Flocculent sediment, no odor.

Litmus milk: No change or slightly alkaline.

Gelatin stab: No liquefaction or very faint liquefaction.

Hydrogen sulfide not formed.

Indole not produced.

Starch not hydrolyzed.

Nitrites produced from nitrates.

Catalase-positive.

Urease produced.

Phosphatase-negative.

Voges-Proskauer reaction: Negative to very weak.

$NH_4H_2PO_4$ is not utilized as a sole source of nitrogen in Tucker's medium.

Citrate utilization in Koser's medium is negative.

Casein dissimilation: Negative or very weak.

Reduction of dyes: Methylene-blue, 2,6-dichlorophenol-indophenol, Janus green and 2,3,5-triphenyltetrazolium chloride are reduced.

Methyl-red test: Weakly acidic.

Temperature relations: No growth at 47° C., very slight growth at 42° C., good growth at 28–37° C. Optimum temperature is about 30° C.

Optimum pH, between 7 and 8. Growth occurs between 6 and 9.

Acid from glucose, fructose, mannose, sucrose and maltose. No acid from lactose. (See Table III.)

L-glutamic acid is accumulated in a large quantity aerobically in the presence of carbohydrates, ammonium ion and inorganic salts.

Production of organic acid: α-ketoglutaric acid and lactic acid are produced in glucose media.

Aerobic.

Habitat: Air, soil and sewage.

Reference is made to the paper entitled "Taxonomical Study of Glutamic Acid Accumulating Bacteria, *Micrococcus glutamicus* nov. sp." by S. Kinoshita, K. Nakayama and S. Akita. This manuscript was received by the editorial board of "Bulletin of the Agricultural Chemical Society of Japan," December 1957 (Faculty of Agriculture, University of Tokyo).

TABLE III

Acid production from carbohydrates by M. glutamicus *No. 534*

Carbohydrates	Agar medium	Liquid medium**	Carbohydrates	Agar medium	Liquid medium**
Inositol	−	±	Melezitose	−	−
Mannitol	−	±	Starch	−	−
Rhamnose	−	−	Inulin	−	−
Xylose	−	±	Dextrin	−	−
Glucose	+	+	Glycogen	−	−
Fructose	+	+	Glycerol	−	−
Galactose	−	−	Erythritol	−	−
Mannose	+	+	Adonitol	−	−
Lactose	−	−	Mannitol	−*	−*
Sucrose	+	+	Sorbitol	−	−
Maltose	+	+	Dulcitol	−	−
Trehalose	±*	−	Salicin	−*	−
Melibiose	−	−	Aesculin	−	−
Cellobiose	−	−	α-Methylglucoside	−	−
Raffinose	−	−			

+: Acid is produced; −: Acid is not produced; ±: Acid production is doubtful.

* Acid is produced by some other strains of *M. glutamicus*.

** The composition of liquid medium is as follows: peptone 2%, carbohydrate to be tested 1%, distilled water. To 100 ml. of this solution, 1 ml. of 1.2% bromcresol purple alcohol (95%) solution was added before sterilization. pH of the medium was adjusted to neutrality using NaOH solution.

In view of the above properties and observations, the inventors recognized that the present strain belonged to a new species of the genus of Micrococcus, because there was none which could be identified with any one in the aforementioned Bergey's Manual.

The reasons will now be described in detail. On comparing with Bergey's Manual of Determinative Bacteriology (6th edition) our new species, *Micrococcus glutamicus*, seems most closely related to *Micrococcus aurantiacus* and *Micrococcus epidermidis*, but differs from said known species as set forth in Table IV.

TABLE IV

Comparison of M. glutamicus *No. 534 with* M. aurantiacus *and* M. epidermidis

Media	*M. aurantiacus*	*M. epidermidis*	[illegible]
Milk	Weakly acidic	Produces acid	[illegible]
Lactose	Produces acid	[illegible]	[illegible]
Nutrient broth	Turbid with pellicle.	[illegible] and sediment.	[illegible]
Nutrient agar	Buff to scant orange-yellow beaded growth, raised, glistening.	White	[illegible] with, milky white to pale yellow.
Habitat	Usually isolated from infections but also found in milk, cheese and dust.	Skin and mucous membranes.	Air, soil, sewage.

As for l-glutamic acid productivity, *Micrococcus glutamicus* has a remarkable productivity on the culturing medium specified in the examples which will be described later, while *Micrococcus epidermidis* has no such l-glutamic acid productivity. In serological nature also, precipitation test reveals that our new species is different from other species.

There is found no species which is identical with *Micrococcus glutamicus* in Bergey's Manual of Determinative Bacteriology (6th edition).

Strains of our new species other than *Micrococcus glutamicus* No. 534 also have the same properties as the latter.

Any culturing medium as shown in the examples, nitrogen sources, inorganic substances, etc., may be used, regardless of whether synthetic or organic.

The sugar and nitrogen source may be of any kind, provided that they are utilizable for the strain employed. As for the sugar, glucose, fructose, mannose, sucrose, maltose, starch acid-hydrolyzate, molasses etc., as enumerated hereinabove, may be used separately or in combinations of two or more.

As for the nitrogen source, ammonia, urea, ammonium chloride, ammonium acetate or other inorganic or organic ammonium salts, peptone, NZ amine (enzymatic hydrolyzate of casein manufactured by Sheffield Chemical Co), meat extract, corn steep liquor, casein hydrolyzate, fish meal or its digested product, soybean meal or its digested product etc., may be used.

The culturing temperature may be 28° C. to 33° C. Either shaking culture or submerged culture with aeration and agitation is effected at pH 6 to 9 for a period of 2 to 5 days.

After the complete fermentation the cells are removed and the fermentation filtrate is concentrated under reduced pressure. The concentrate thus obtained is adjusted to pH 3.2 with 5 N HCl and then left standing in a cold room to obtain crude crystals of l-glutamic acid.

As anyone skilled in the art will readily understand, to use mutants of the present strain is also within the scope of the present invention.

The l-glutamic acid or its salt accumulated in the medium is recovered by a known suitable process, such as ion exchange resin method, such a process per se being no significant part of the present invention.

The present invention will be illustrated by the following examples, which are given merely for illustration.

EXAMPLE 1.—CULTURING BY SYNTHETIC MEDIUM

Micrococcus glutamicus No. 534 was cultured for 24 hours in glucose bouillon by shaking culture at 28° C. and used as inoculum. 30 ml. of fermentation medium

was distributed in a 250 ml. flask and inoculated with above inoculum and shaken by a rotary shaker at 220 r.p.m. The composition of the fermentation medium was as follows: 50 gr. glucose, 8 gr. urea, 0.5 gr. K_2HPO_4, 0.1 gr. $MgSO_4.7H_2O$ and 5 mgr. $FeCl_2.6H_2O$ per 1 liter (the unaccounted for portion of the liter consisted of water); pH being about 7.5. Submerged cultures with aeration and agitation were effected at both 33° C. and 28° C.

The pH was controlled during the fermentation by the addition of urea.

The analytical results during fermentation are given in Table V.

TABLE V

Culturing temp., ° C.	Culturing duration, days	pH	Residual glucose, gr./100 ml.	l-Glutamic acid, mgr./100 ml.	Yield based on consumed glucose, percent
33	1	8.0	2.8	660	30.0
	2	7.3	1.0	700	22.0
28	2	8.2	2.3	610	22.6
	3	0.8	1.2	810	21.3
	4	8.7	0.2	1,130	23.5

After two-day culture at 33° C. the cells were removed from the fermentation broth and 1 l. of the filtrate was concentrated under reduced pressure.

This concentrate was adjusted to pH 3.2 with 5 N HCl and left standing in a cold room, whereby 6.3 g. of l-glutamic acid crude crystals were obtained. By four-day culture at 28° C., 10.4 gr. of l-glutamic acid crude crystals were obtained from 1 l. of the fermentation filtrate in a similar manner.

EXAMPLE 2.—CULTURING BY ORGANIC MEDIUM

Inoculum and procedures were same as in Example 1. The fermentation medium was as follows: 100 gr. glucose, 5 gr. meat extract, 5 gr. NZ amine, 15 gr. urea, 1 gr. K_2HPO_4 and 0.25 gr. $MgSO_4 \cdot 7H_2O$ per 1 liter (the unaccounted for portion of the liter consisting of water); pH being about 7.0. Submerged culture with aeration and agitation was effected at 28° C.

The analytical results during fermentation are given in Table VI.

TABLE VI

Culturing duration, days	pH	Residual sugar, gr./100 ml.	l-Glutamic acid, mgr./100 ml.	Yield based on consumed sugar, percent
1	8.4	7.2	370	13.0
2	8.2	5.6	1,200	28.4
3	7.8	3.5	1,700	30.1
4	0.2	2.0	2,400	34.9

The above examples are only illustrative and various other saccharine materials, for example fructose, sucrose, maltose, xylose and starch acid-hydrolyzate, and various other organic and inorganic nitrogenous sources such, for example, as ammonia, ammonium salts such as ammonium chloride, ammonium sulphate, ammonium carbonate, ammonium acetate and the like, peptone, digested soybean meal, digested fish meal, casein hydrolyzate, and corn steep liquor etc. are usable in place of the nutrient materials in the fermentation medium mentioned in Example 2.

As will be clear from the above descriptions, in a synthetic medium as well as in an organic medium, an amount as high as 20–30% of consumed sugar can be converted to l-glutamic acid.

In order to control the pH value of the culturing medium within a range from 6 to 9, neutralizing agents such as ammonia, urea, compounds containing basic nitrogenous radical [NH_4OH, $(NH_2)_2CO$, $(NH_4)_2CO_3$, etc.], and caustic alkalis [NaOH, etc.] may be used.

Various combinations of saccharine materials, nitrogen sources and pH control agents are contemplated for the culturing in both synthetic and organic media and the specific examples are merely illustrative and are not to be considered limitative with respect to particular materials employed except for the presence of *Micrococcus glutamicus*.

Further illustrative of the instant invention are the following examples:

EXAMPLE 3

Fermentation medium was prepared as follows: 50 gr. of glucose, 8 gr. of urea, 0.5 gr. of K_2HPO_4, 0.1 gr. of $MgSO_4 \cdot 7H_2O$ and 5 mgr. of $FeCl_2 \cdot 6H_2O$ were dissolved in tap water and made up to 1 liter. The pH of the medium was approximately 7.5. Each 30 ml. of this medium was distributed in 250 ml. Erlenmeyer flasks and autoclaved at 110° C. for 10 minutes. To one flask of this sterilized medium, 3 ml. of inoculum of *Micrococcus glutamicus* No. 534, which was grown in glucose-bouillon medium on shaking culture device at 28° C. for 24 hours, were added. Fermentation was carried out on a shaking culture device at 28° C. During the fermentation, the pH of the medium was maintained at from 6 to 9 by adding 0.5 to 1.0 ml. of 10% NH_4OH at each 4 to 6 hour interval. After 4 days, the culture medium was centrifuged and bacterial cells were removed. l-Glutamic acid content of the supernatant was 11.3 mg. per ml. according to the enzymatic determination using l-glutamic acid decarboxylase. The supernatant was concentrated in vacuo to 1/8 of the original volume and the pH of this concentrate was adjusted to 3.2 with 5 N HCl. This solution was left to stand in cold-room until a substantial amount of crude l-glutamic acid crystallized. From 1 liter of the fermented medium, 10.4 gr. of crude l-glutamic acid was obtained.

EXAMPLE 4

The process described in Example 3 was repeated using urea as a neutralizing agent. During the fermentation, the pH of the medium was maintained at from 6 to 9 by adding 0.5 ml. of 10% urea solution at 4 to 6 hour intervals. After 4 days, the l-glutamic acid content in the culture medium reached 13.2 mgr./ml. From 1 liter of this culture medium, 11.5 gr. of crude l-glutamic acid was obtained.

EXAMPLE 5

The process described in Example 3 was repeated using $(NH_4)_2CO_3$ as a neutralizing agent. During the culture, the pH of the medium was adjusted at 6 to 9 by adding 0.5 ml. of 10% $(NH_4)_2CO_3$ at 4 to 6 hour intervals. After 4 days' culture, the culture medium was found to contain 12.0 mgr. of l-glutamic acid per ml.

EXAMPLE 6

The process described in Example 3 was repeated using 50 gr. of fructose in place of glucose. As a neutralizing agent, 10% urea solution was used as described in Example 4. After 5 days' culture, the culture medium was found to contain 7.2 mgr. of l-glutamic acid per ml.

EXAMPLE 7

The process described in Example 3 was repeated using 50 gr. of sucrose in place of glucose. As a neutralizing agent, 10% urea solution was used as described in Example 4. After 5 days' culture, the culture medium was found to contain 8.5 mgr. of l-glutamic acid per ml.

EXAMPLE 8

The process described in Example 3 was repeated using 50 gr. of maltose in place of glucose. After 4 days' culture, the culture medium was found to contain 10.8 mgr. of l-glutamic acid per ml.

EXAMPLE 9

The process described in Example 3 was repeated

using starch acid-hydrolyzate which was equivalent to 50 gr. of glucose, and 10% $(NH_4)_2CO_3$ solution was used as a neutralizing agent. After 3 days' culture, the culture medium was found to contain 12.8 mgr. of l-glutamic acid per ml.

EXAMPLE 10

The process described in Example 3 was repeated using the following medium: 100 gr. of glucose, 5 gr. of meat extract, 5 gr. of NZ amine, 5 gr. of urea, 1 gr. of K_2HPO_4, and 0.24 gr. of $MgSO_4 \cdot 7H_2O$ were dissolved in tap water and made up to 1 liter. The pH of the medium was around 7.0. After sterilization and cooling, the medium was inoculated with the same organism and the submerged culture with aeration and agitation was effected at 28° C. During the culture, the pH of the medium was adjusted at from 6 to 9 with 10% urea solution as described in Example 4. After 4 days' culture, the culture medium was found to contain 24.8 mgr. of l-glutamic acid per ml.

EXAMPLE 11

The process described in Example 10 was repeated using 15 gr. of $(NH_4)_2SO_4$ in place of 5 gr. of urea and employing 4% NaOH solution as a neutralizing agent. After 3 days' culture, the culture medium was found to contain 19.0 mgr. of l-glutamic acid per ml.

EXAMPLE 12

The process described in Example 10 was repeated at temperature of 32° C. instead of 28° C. After 4 days' culture, the culture medium was found to contain 22.4 mgr. of l-glutamic acid per ml.

EXAMPLE 13

The process described in Example 4 was repeated using *Micrococcus glutamicus* No. 613 (ATCC No. 13059, a white color mutant of *M. glutamicus* No. 534). After 4 days' culture, the medium was found to contain 9.5 mgr. of l-glutamic acid per ml.

EXAMPLE 14

The process described in Example 4 was repeated using *Micrococcus glutamicus* No. 614 (ATCC No. 13060, a yellow mutant of *M. glutamicus* No. 534). After 4 days' culture, the medium was found to contain 9.2 mgr. of l-glutamic acid per ml.

Mutant strains can be obtained by the irradiation of ultraviolet light on *M. glutamicus* No. 534. Two colored mutants (white and yellow types), and several auxotrophic mutants were obtained. From natural source many strains which conformed to the same species of *M. glutamicus* were isolated. The characters of some of those strains are tabulated in the Table VII. Each of the listed strains such as *M. glutamicus* No. 541, No. 560, No. 582, No. 588, No. 613 (ATCC No. 13059), No. 614 (ATCC No. 13060), No. 601 and No. 602 may be cultured in place of *M. glutamicus* No. 534 in each of the examples (1 to 14, inclusive), to produce l-glutamic acid in a culturing medium. The saccharides, the nutrient materials and pH controlling agents may vary as set forth in columns 7 and 8.

TABLE VII

Micrococcus glutamicus and its mutants

Strain	Source of isolation	Comparative characters			
		Specific activity, Δ E/gr.	Color	Optimum temperature (° C.)	Auxotrophic requirement
M. glutamicus:					
No. 534	Sewage	560	Normal	28-30	None.
No. 541	Monocolony isolation of No. 534.	531	do	28-30	Do.
No. 561	Air	434	do	30-33	Do.
No. 582	Soil	250	do	30-33	Do.
No. 588	Animal feces	870	do	30-33	Do.
No. 613	U.V. mutant of No. 534		White	28-30	Do.
No. 614	do		Yellow	28-30	Do.
No. 601	do		Normal	26-34	Methionine.
No. 602	do		do	28-30	Histidine.

EXAMPLE 15.—RECOVERY OF GLUTAMIC ACID FROM GLUTAMIC ACID WHOLE BROTH

To 2 liters of glutamic acid broth produced by the method of Example 3 and having an initial pH of 8.1, 10 gms. of aluminum sulfate contained in 5% solution was added. The pH of the solution dropped to 7.65. The pH was adjusted to 3.4 by adding 28.5 ml. of concentrated sulfuric acid. At this point an assay was done which showed 50.74 g. of glutamic acid per liter of broth.

The broth was then coagulated at 87° C. for a period of 5 hours and 20 minutes. 40 g. of Super-Cel were then added as admix, and the slurry containing Super-Cel and broth was then filtered through No. 4A porcelain funnel having 40 g. of Super-Cel precoat on it. The filtered cake was washed with three 125 ml. portions of water, the water being at a temperature of 80° C. The filtration was rapid. The volume of the filtered broth was 2,380 ml. The filtered broth contained 36.95 g. of glutamic acid per liter. The filtration yield was 86.6%. The filtered broth was then concentrated under reduced pressure to 650 ml. thereby causing the glutamic acid concentration to be 150 grams per liter. The concentrated filtered broth was then aged at 5° C. for a period of approximately 20 hours. During this aging period the broth was agitated. At the end of the aging period, the broth was filtered and the precipitate was washed with four 54 ml. portions of water, the water being at a temperature of between 0 and 5° C. The washed precipitate was then air dried. The weight of washed precipitate was 65.5 gm. This was equivalent to 61.67 grams of pure glutamic acid, since the purity of the washed precipitate was 94.6%.

Micrococcus glutamicus No. 534 (ATCC No. 13032) is a strain of a new species belonging to the genus Micrococcus. This is shown below with reference to the "Key to the species of genus Micrococcus" as set forth in Bergey's Manual of Determinative Bacteriology.

I. "Key to the species of genus Micrococcus" from Bergey's Manual, 6th Edition:

(1) Aerobic to facultatively anaerobic species.

M. glutamicus No. 534 is aerobic.

B. Nitrites produced from nitrates.

M. glutamicus No. 534 produces nitrites from nitrates.

(2) Can not utilize $NH_4H_2PO_4$ as a sole source of nitrogen.

M. glutamicus No. 534 conforms to this description.

(a) Gelatin not liquefied or very slowly liquefied.

M. glutamicus No. 534 conforms to this description.

In this section, 2 species namely *M. aurantiacus* and *M. epidermidis* are included.

M. glutamicus is compared with and differentiated from *M. aurantiacus* and *M. epidermidis* in Table IV. As shown in said table, *M. glutamicus* No. 534 is different from those two species in pigment on agar, acid production in milk and acid production from lactose.

In addition, in *M. epidermidis*, Voges-Proskauer reaction is positive although in *M. glutamicus* No. 534 this reaction is negative. This reaction is an important character according to C. Shaw, J. M. Stil and S. T. Cowan (J. Gen. Microbiol. 5, 1010, 1951).

II. "Key to the genera of family Micrococcaceae" from Bergey's Manual, 7th edition, by Breed, Robert S.; Murray, E.G.D.; and Smith, Nathan R.; published by The Williams & Wilkins Company in Baltimore, Maryland; October, 1957:

(1) Aerobic to facultatively anaerobic species. Also includes some obligate anaerobes that occur in packets (Sarcina).

M. glutamicus No. 534 is aerobic.

A. Cells are generally found in irregular masses; occasionally they are single or in pairs.

M. glutamicus No. 534 conforms to this description.

(1) Action on glucose, if any, is oxidative. Aerobic. *M. glutamicus* No. 534 is aerobic. Therefore, *M. glutamicus* No. 534 belongs to genus *I. Micrococcus.*

According to the "Key to the species of genus Micrococcus" in Bergey's Manual, 7th ed:

(1) May or may not reduce nitrates to nitrites No free nitrogen or nitrous oxide gas produced from nitrates.

M. glutamicus No. 534 conforms to this key.

A. No pink or red pigment produced on agar media in young cultures. *M. glutamicus* No. 534 conforms to this key.

(2) Nitrites produced from nitrates. *M. glutamicus* No. 534 produces nitrites from nitrates.

In this section, no species is described which does not utilize $NH_4H_2PO_4$ as a sole source of nitrogen. *M. glutamicus* No. 534 does not utilize $NH_4H_2PO_4$ as a sole source of nitrogen. So, it is clear that *M. glutamicus* No. 534 is different from any species listed in Bergey's Manual, 7th ed. That means *M. glutamicus* is a new species.

The classification of *Micrococcus glutamicus* No. 534 is dependent upon the conditions under which the culture is grown, the criteria considered dominant in establishing the genus, and the classification scheme accepted by the taxonomist. Other investigators may classify the same organism as either a Micrococcus, a Brevibacterium, a Corynebacterium or a Bacterium.

It is thought that the invention and its advantages will be understood from the foregoing description, and it is apparent that various changes may be made in the process without departing from the spirit and scope of the invention or sacrificing its material advantages, the process hereinbefore described being merely illustrative of preferred embodiments of the invention.

This application is a continuation-in-part of copending application Serial No. 714,068, filed February 10, 1958 and now abandoned, which is a continuation-in-part of Serial No. 624,198, filed November 26, 1956, and now abandoned.

Having thus disclosed the invention what is claimed is:

1. A method for producing l-glutamate selected from the group consisting of l-glutamic acid and a salt thereof which comprises aerobically culturing *Micrococcus glutamicus* in a culturing medium containing carbohydrate, nitrogen source and inorganic material, controlling the pH value of the culturing medium within the range of from about 6 to about 9 by the addition of neutralizing agent, whereby a substantial amount of l-glutamate is accumulated in the culturing medium, and recovering said l-glutamate.

2. A method of producing l-glutamate selected from the group consisting of l-glutamic acid and a salt thereof which comprises culturing *Micrococcus glutamicus* under aerobic conditions in a culturing medium containing a fermentable carbon source, a nitrogen source and inorganic material, and controlling the pH value of the culturing medium during said culturing within the range of from 6 to 9 by the addition thereto of neutralizing agent, whereby a substantial amount of l-glutamate is accumulated in the culturing medium.

3. A method of producing a compound having an l-glutamate radical which comprises culturing *Micrococcus glutamicus* under aerobic conditions in a culturing medium containing carbohydrate, nitrogen source and inorganic material, and controlling the pH value of the culturing medium during said culturing within the range of from about 6 to about 9 by the addition thereto of neutralizing agent, whereby a substantial amount of said compound having an l-glutamate radical is accumulated in the culturing medium.

4. A method of producing a compound having an l-glutamate radical which method comprises culturing *Micrococcus glutamicus* under aerobic conditions in a culturing medium containing (1) at least one carbohydrate selected from the group consisting of glucose, fructose, sucrose, maltose, xylose and starch acid-hydrolyzate, (2) nitrogen source, and (3) inorganic material; controlling the pH value of the culturing medium during said culturing within the range from about 6 to about 9 by the addition thereto of neutralizing agent, whereby a substantial amount of the compound having an l-glutamate radical is accumulated in the culturing medium; and recovering said compound.

5. A method of producing a compound having an l-glutamate radical which method comprises culturing *Micrococcus glutamicus* under aerobic conditions in a culturing medium containing (1) carbohydrate, (2) at least one nitrogen source selected from the group consisting of urea, ammonia, ammonium salt, peptone, corn steep liquor, hydrolyzed casein, fish meal, meat extract and digested soybean meal, and (3) inorganic material; controlling the pH value of the culturing medium during said culturing within the range from about 6 to about 9 by the addition thereto of neutralizing agent, whereby a substantial amount of the compound having an l-glutamate radical is accumulated in the culturing medium; and recovering said compound.

6. A method of producing a compound having an l-glutamate radical which method comprises culturing *Micrococcus glutamicus* under aerobic conditions in a culturing medium containing carbohydrate, nitrogen source and inorganic material; controlling the pH value of the culturing medium during said culturing within the range from about 6 to about 9 by the addition thereto of ammonia, whereby a substantial amount of the compound having an l-glutamate radical is accumulated in the culturing medium; and recovering said compound.

7. A method of producing a compound having an l-glutamate radical which method comprises culturing *Micrococcus glutamicus* under aerobic conditions in a culturing medium containing carbohydrate, nitrogen source and inorganic material; controlling the pH value of the culturing medium during said culturing within the range from about 6 to about 9 by the addition thereto of urea, whereby a substantial amount of the compound

[illegible] an l-glutamate radical is accumulated in the culturing medium; and recovering said compound.

8. A method of producing a compound having an l-glutamate radical which method comprises culturing *Micrococcus glutamicus* under aerobic conditions in a culturing medium containing carbohydrate, nitrogen source and inorganic material; controlling the pH value of the culturing medium during said culturing within the range from about 6 to about 9 by the addition thereto of neutralizing agent containing a basic nitrogenous radical, whereby a substantial amount of the compound having an l-glutamate radical is accumulated in the culturing medium; and recovering said compound.

9. A method of producing a compound having an l-glutamate radical which method comprises culturing *Micrococcus glutamicus* under aerobic conditions in a culturing medium containing carbohydrate, nitrogen source and inorganic material; controlling the pH value of the culturing medium during said culturing within the range from about 6 to about 9 by the addition thereto of caustic alkali, whereby a substantial amount of the compound having an l-glutamate radical is accumulated in the culturing medium; and recovering said compound.

10. A method of producing a compound having an l-glutamate radical which comprises culturing a mutant of *Micrococcus glutamicus* under aerobic conditions in a culturing medium containing carbohydrate, nitrogen source and inorganic material; controlling the pH value of the culturing medium during said culturing within the range of from about 6 to about 9 by the addition thereto of neutralizing agent, whereby a substantial amount of said compound having an l-glutamate radical is accumulated in the culturing medium; and recovering said compound.

11. A method of producing a compound having an l-glutamate radical which comprises culturing a microorganism corresponding to ATCC No. 13059 under aerobic conditions in a culturing medium containing carbohydrate, nitrogen source and inorganic material; controlling the pH value of the culturing medium during said culturing within the range of from about 6 to about 9 by the addition thereto of neutralizing agent, whereby a substantial amount of said compound having an l-glutamate radical is accumulated in the culturing medium; and recovering said compound.

12. A method of producing a compound having an l-glutamate radical which comprises culturing a microorganism corresponding to ATCC No. 13060 under aerobic conditions in a culturing medium containing carbohydrate, nitrogen source and inorganic material; controlling the pH value of the culturing medium during said culturing within the range of from about 6 to about 9 by the addition thereto of neutralizing agent, whereby a substantial amount of said compound having an l-glutamate radical is accumulated in the culturing medium; and recovering said compound.

13. A method of producing a compound having an l-glutamate radical which comprises culturing a microorganism corresponding to ATCC No. 13032 under aerobic conditions in a culturing medium containing carbohydrate, nitrogen source and inorganic material; controlling the pH value of the culturing medium during said culturing within the range of from about 6 to about 9 by the addition thereto of neutralizing agent, whereby a substantial amount of said compound having an l-glutamate radical is accumulated in the culturing medium; and recovering said compound.

14. A one-step method of directly producing, by reductive amination, l-glutamic acid from carbohydrate and a nitrogen source in a liquid culturing medium, said step consisting of culturing *Micrococcus* [illegible] under aerobic conditions in the culturing medium while maintaining said medium within a pH range of from 6 to 9.

15. A one-step method of directly producing, by reductive amination, a compound having an l-glutamate radical from carbohydrate and a nitrogen source in a liquid culturing medium, said step consisting of culturing a microorganism corresponding to ATCC No. 13032 under aerobic conditions in the culturing medium while maintaining said medium within a pH range of from 6 to 9.

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The Contribution of Peptides and Amino Acids to the Taste of Foodstuffs

Jiro Kirimura, Akira Shimizu, Akimitsu Kimizuka, Tsunchiko Ninomiya, and Noboru Katsuya

The tastes of individual amino acids were characterized as being sweet, salty, sour, bitter, or MSG-like. The contribution of amino acids to the taste of various foodstuffs is discussed. The tastes of various peptides were characterized as being sour, bitter, or practically tasteless. Peptides from sake, soy sauce, and partial hydrolyzates of soybean protein were isolated and their tastes, as well as other properties, described.

The importance of amino acids in contributing to the taste of foodstuffs was first recognized by Ikeda at Tokyo University in 1908 (Ikeda, 1908). He discovered that monosodium L-glutamate (MSG) was the essential component of the taste-imparting ingredients of traditional Japanese food seasoners, such as sea tangle (a type of seaweed). At that time the taste of traditional Japanese foods such as sake, miso, and soy sauce were presumed to be due to the amino acids which were released from the natural proteins during the course of fermentation. In addition, the taste of cheese had been known, in Europe and the United States, to be characterized by amino acids formed during the ripening process.

Since then, many studies on the taste of amino acids in foods and on their production by extraction, fermentation, or by chemical syntheses have resulted in mass production of various amino acids, and in a consequent reduction of their price. Today, amino acids are used in food processing not only to enhance the nutritive value of many processed foods such as cereals, but also to enhance or improve otherwise the taste of natural characteristics of many foodstuffs.

The taste of peptides and their relative contribution to the taste of foods have been in the past the topic of extensive investigations; studies have been undertaken on the distribution, isolation, and elucidation of the chemical structures of peptides and some of their properties in foods. This paper deals with the role that some specific amino acids and peptides play in determining the taste of certain foodstuffs.

TASTE OF INDIVIDUAL AMINO ACIDS

Free amino acids in natural food extractives are important taste contributors. The term "extractives" as used in this paper is defined as the mixtures that can be extracted from foodstuffs with warm or hot water. Food extractives are believed to consist mainly of such substances as free amino acids, peptides, nucleotides, and organic acids and their esters, bases, sugars, and salts. Although it is the combination of several factors, among them the complex of all the organoleptic components present, that determine the taste of a food, extractives are the principal contributor. It is well known that MSG and 5′-nucleotides are mutually synergistic and play an important role in taste.

The taste characteristics of individual amino acids have been studied by many investigators including the author's group (Yoshida *et al.*, 1966) and that of Solms (Solms *et al.*, 1965), in which quantitative evaluations were reported. In the author's laboratories, a panel consisting of 100 people was chosen by screening approximately 1000 employees of the Ajinomoto Co., Inc., by means of a sensitive test. The age of the panelists, which included both men and women, ranged from 19 to 40 years. The taste intensities of individual amino acids were estimated by comparing them to that of solutions of sodium chloride at concentrations of from 0.2000 to 5.123 grams per 100 ml.

The nature of the tastes were characterized as being sweet, salty, sour, bitter, MSG-like, or otherwise; and their intensities measured using a 0 to 10 scale. The results of the sensory tests showed good agreement among the panelists.

Figure 1 illustrates the nature and the intensity of the taste of arginine, serine, glutamic acid, and alanine. Arginine has a bitter taste accompanied by slight sweetness, whereas serine has a sweet taste accompanied by sourness and a MSG-like taste. Glutamic acid is a combination of the sour taste with a taste like MSG. In alanine the sweet taste is combined with a slight taste like MSG. In general, the tastes of the individual amino acids are complex and need to be described by more than one taste characteristic. A classification of amino acids according to their organoleptic characteristics and their threshold values is summarized in Table I.

Of the amino acids tested, the taste intensities of alanine, arginine, glutamic acid, serine, and threonine increased as

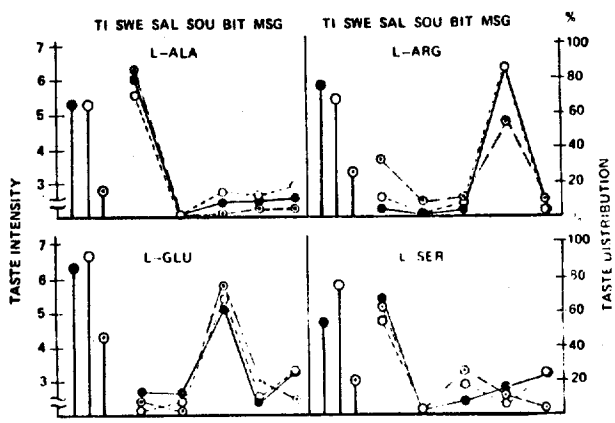

Figure 1. Taste characteristics of some amino acids

—● Solid; ○ High concentration (in water); - - Low concentration (in water)
TI: Relative taste intensity compared with 0.2 to 5.123% sodium chloride solution
SWE: Sweetness; SOU: Sourness; BIT: Bitterness; MSG: Taste like monosodium L-glutamate
Values of high and low concentration (g./dl.): Ala 0.5, 5.0; Arg 0.2, 1.0; Glu 0.025, 0.2; Ser 1.5, 15.0

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Table I. Threshold Values and Organoleptic Characteristics of Amino Acids

Amino Acids	T.V.,[a] Mg/Dl.	SWE[b]	SAL[b]	SOU[b]	BIT[b]	MSG[b]
Sweet Amino Acids						
Hyp	50	***			**	
Lys·HCl	50	**			**	
Ala	60	***				
Gly	130	***				*
Ser	150	***		*		*
Gln	250	*				
Thr	260	***		*	*	
Pro	300	***			***	
Sour and MSG-like Amino Acids						
Asp	3			***		*
Glu	5			***		**
His·HCl	5			***		
Asn	100			**	*	
Glu·Na	30	*	*			***
Asp·Na	100		**			**
Bitter Amino Acids						
His	20				**	
Arg·HCl	30				***	
Met	30				***	*
Val	40				***	
Arg	50				***	
Ile	90				***	
Phe	90				***	
Trp	90				***	
Leu	190				***	

[a] T.V.: threshold value.
[b] Asterisks denote relative taste intensities.

Table II. Free Amino Acid Content of Extractives (Ohara, 1966) (Mg% on Wet Basis)

Amino Acids	Mackerel	Uni[a]	Tricholoma Shimeji	Purple Laver	Green Tea[b]
Asp	9.8	4	53	230	136
Thr	9.6	68	21	78	61
Ser	6.9	130	51	53	81
Glu	20	103	68	640	668
Pro	5.4	26	13	62	18
Gly	54	842	12	125	47
Ala	37	261	33	1092	25
Val	14	154	9.1	21	6.1
Met	7.3	47	1.7	0	0.6
Ile	9.6	100	6.5	14	47
Leu	14	176	11	20	34
Tyr	6.6	158	8.6	0	4
Phe	9.2	79	19	15	10
Trp	2.2	39	2.6	0	12
His	563	54	25	0	6.7
Lys	22	215	119	24	7.4
Arg	6.1	316	41	90	142

[a] Unripe gonad of sea urchin, taurine 105 mg%.
[b] On moisture-free basis, theanine 1727 mg%.

their concentrations were increased. Taste analyses were performed also using solid amino acids, but little difference was observed between aqueous and solid samples except in the cases of aspartate, serine, and threonine. The taste intensification resulting from the addition of MSG to foods is a consequence of the flavor contributory effect of MSG by itself and also of the synergism between 5′-nucleotides existing in foods and added MSG. This synergism is also observed between 5′-inosinate and either C_7 monosodium monoamino dicarboxylates, homocysteic acid, tricholomic acid, ibotenic acid, or DL-threo-β-oxyglutamate.

Table III. Taste Threshold Values of Some Dipeptides and Amino Acids

Peptides	T.V., %	Amino Acids	T.V., %
γ-L-Glu-L-Glu	0.0025	L-Glu	0.005
L-Leu-L-Leu	0.10	L-Leu	0.19
Gly-Gly	[a]	Gly	0.11

[a] Little or no taste at 1%.

TASTE OF AMINO ACID MIXTURES

Recently, in connection with studies on the inherent taste of free amino acids and their distribution in foods, the taste of various combinations of amino acids has been studied. Not only does the distribution of free amino acids differ from food to food, but quantitative changes within each food may be seasonal. Table II shows qualitative and quantitative data on the free amino acid content of five different foods (Ohara, 1966). In mackerel, histidine accounts for about 70% of the total free amino acids. In uni, a very tasty Japanese product made from the unripe gonad of the sea urchin, glycine predominates, followed by arginine, lysine, alanine, serine, glutamic acid, and taurine. Similarly, the predominant amino acid in *Tricholoma shimeji* (edible fungi) is lysine; in purple laver (a type of seaweed) alanine and glutamic acid predominate; theanine and glutamic acid are the major amino acids of green tea. Some of these amino acids *per se* are essential for the taste of some foods. Thus, methionine is the most specific component of the taste of uni, and glycine is very important in contributing to the distinct taste of lobster and crab.

In beef extract, however, no specific amino acid characterizes its taste but, rather, all the free amino acids combine to give the complex taste sensation.

In our laboratories a hydrolyzed protein concentrate, Ajimate F-40, was developed as a replacement for beef extracts, on the basis of studies on the contribution of free amino acids to the taste of foodstuffs.

TASTE OF INDIVIDUAL PEPTIDES

The authors have previously observed that the taste intensities of peptides are generally weak, compared to those of the corresponding free amino acids (Kimizuka *et al.*, 1963). However, as shown in Table III, the threshold values of L-Leu-L-Leu and γ-L-Glu-L-Glu were approximately half those of L-Leu and L-Glu, respectively.

Sixty different peptides were tested in 0.2% aqueous solutions by 12 trained panel members to evaluate their taste characteristics, and the following are some generalizations resulting from this work.

The taste characteristics of peptides are complex, but can be classified into three groups: Compounds in Group 1 have a sour taste; those in Group 2 have a bitter taste; and those in Group 3 have little or no taste. As shown in Table IV, peptides such as L-Ala L-Asp, γ-L-Glu-L-Glu, and Gly-L-Asp-L-Ser-Gly are sour, peptides such as L-Leu-L-Leu, L-Arg-L-Pro, and L-Val-L-Val-L-Val are bitter, and peptides such as L-Lys-Glu, L-Phe-L-Phe, and Gly-Gly-Gly-Gly have little or no taste.

It appears that a relationship exists between the patterns of component amino acids and the taste of dipeptides. Dipeptides which tasted sour contained two acidic amino acids, acidic- and neutral-amino acids, or acidic- and aromatic-amino acids.

Table IV. Taste of Peptides in 0.2% Aqueous Solution

Group 1. Sourness

Gly-L-Asp, Gly-L-Glu
L-Ala-L-Asp, L-Ala-L-Glu
L-Ser-L-Asp, L-Ser-L-Glu
L-Val-L-Asp, L-Val-L-Glu
L-Asp-L-Ala, L-Asp-L-Asp
L-Glu-L-Ala, L-Glu-L-Asp, L-Glu-L-Glu
L-Glu-L-Phe,[a] L-Glu-L-Tyr[a]
γ-L-Glu-Gly,[b] γ-L-Glu-L-Ala,[b] γ-L-Glu-L-Asp[b]
γ-L-Glu-L-Glu[b]
L-Phe-L-Asp, L-Phe-L-Glu
L-Trp-L-Asp, L-Trp-L-Glu
Gly-L-Asp-L-Ser-Gly
L-Pro-Gly-Gly-L-Glu
L-Val-L-Val-L-Glu

Group 2. Bitterness

Gly-L-Ile, Gly-L-Met, Gly-L-Phe, Gly-L-Try
L-Ala-L-Phe
L-Val-L-Ala, L-Val-L-Val, L-Val-L-Leu
L-Leu-Gly, L-Leu-L-Leu, L-Leu-L-Tyr
L-Lys-Gly, L-Lys-L-Ala
L-His-L-His
L-Val-L-Val-L-Val
L-Arg-L-Pro

Group 3. Having Little or No Taste

Gly-Gly, Gly-L-Ala, Gly-L-Ser, Gly-L-Thr
Gly-L-Asp(NH_2), Gly-L-Pro
L-Ala-Gly, L-Ala-L-Ala
L-Val-Gly
L-Lys-L-Glu
L-Phe-L-Phe[c]
L-Pro-Gly, L-Pro-L-Ala
Gly-Gly-Gly-Gly

[a] Accompanied by bitterness and astringency.
[b] Accompanied by astringency.
[c] Taste in solid state.

It is presumed that the sour taste is caused by hydrogen ions arising from the dissociation of dipeptides. The order of the taste intensity is 1) >2) >3).

Dipeptides which tasted bitter contained neutral amino acids with either large alkyl groups ($\geq C_3$) or a combination of large ($\geq C_3$) and small ($\leq C_2$) alkyl groups, neutral and aromatic amino acids, or neutral and basic amino acids.

Dipeptides having little or no taste were composed of two amino acids with small alkyl groups, acidic- and basic-amino acids, or two aromatic amino acids (Figure 2).

Table V. Effect of Peptides on the Stability of Soybean oil

Peptides (0.02%)	Peroxide Value[a] 5 Hrs.	10 Hrs.
None	83.0	241.4
Gly-L-Leu	16.9	136.2
β-Ala-3-Methyl-L-His	25.3	145.4
Gly-Gly	51.7	319.7
γ-L-Glu-L-Glu	59.4	252.2
Gly	58.6	228.6
Butyl hydroxy anisole	22.9	141.5

[a] Official and Tentative Method of the American Oil Chemists' Society (1947).

It is interesting that dipeptides composed of sweet amino acids, Gly-Gly, Gly-L-Ala, and Gly-L-Pro, are almost tasteless. The disappearance of sweetness may be a result of chain lengthening. The combination of an acidic and a basic amino acid, such as L-Lys-L-Glu and L-Arg-L-Glu, is also tasteless. This may be due to interactions between the amino groups and the carboxyl groups in the dipeptide molecule.

Dipeptides composed of two aromatic amino acids, such as L-Phe-L-Phe, had essentially bitter tastes, but this was not thoroughly evaluated because of the low solubilities of these peptides in water. The taste characteristics and taste intensities of dipeptides are summarized in Figure 2.

Another interesting aspect of some of these peptides was that they showed rather strong antioxidant activity, Gly-L-Leu being the most effective. Antioxidant activity was determined by means of the active oxygen method, in which the stability of a vegetable oil was determined by measuring peroxide values after 5 and 10 hours. Dipeptides were found to be more effective stabilizers of vegetable oils than of animal fats (Table V).

PEPTIDES IN BREWED BEVERAGES AND FOODSTUFFS

It is important to correlate chemical structure and taste of peptides for better utilization of peptides in foodstuffs. The authors have also studied the relationship between

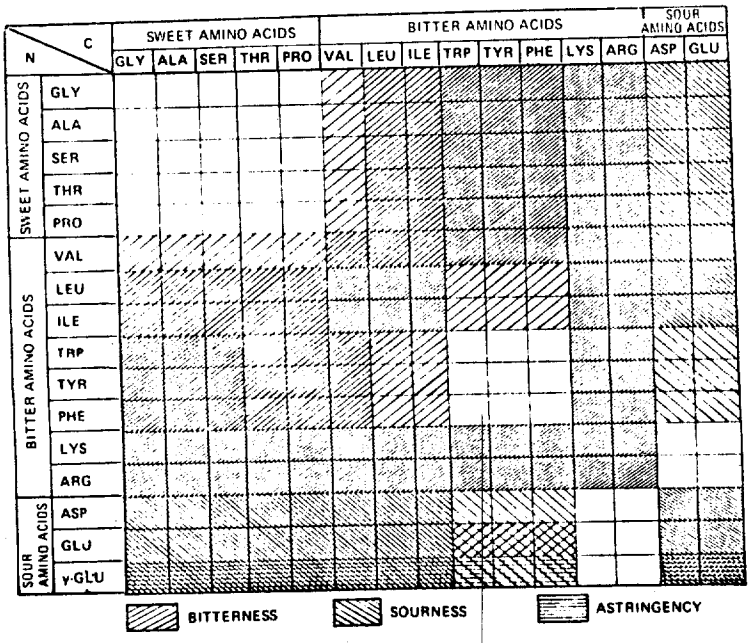

Figure 2. Taste of dipeptides

Upper left N/C
N = N-terminal amino acid
C = C-terminal amino acid

Table VI. Effect of Peptides on "Body" of Synthetic Sake (Tajima *et al.*, 1968)

Sample	Sensory Score
Control synthetic sake	5.0
+0.03% L-Val-L-Glu	6.8
+0.03% L-Pro-L-Glu	5.8
+0.03% L-Pro-L-Asp	5.8
+0.03% γ-L-Glu-L-Glu	5.2
+0.03% L-Val-L-Val	5.4
+0.03% Gly-L-Leu	7.0
Natural sake	10.0

amino acid composition and molecular weight, on one hand, and taste, on the other, especially in the case of peptides of higher molecular weight.

The distribution of peptides in natural foodstuffs, such as beef and whale extracts, cheese, seaweeds, etc., and the isolation of peptides therefrom have been studied by many investigators. Since peptides occur in relatively large amounts in some typical Japanese beverages and foodstuffs, such as sake, miso, and soy sauce, the peptides o these foods have been the objects of studies in Japan (Tajima and Sato, 1964; Takahashi and Nose, 1958; Takeuchi and Yoshii, 1967).

Sake. Sake is a traditional Japanese alcoholic beverage which is made from rice by mixed fermentation using fungi, yeast, and bacteria. The unique taste of sake is said to be derived mainly from components of rice, such as hydrolyzates of protein, starch, etc. The content of free amino acids in sake is approximately 0.3%, and they are very important contributors to the unique taste of sake. The peptides in sake amount to from 20 to 30% of the free amino acid content. Molecular sieve chromatography with Dowex-50 columns was used to separate groups of peptides in sake (Takahashi and Nose, 1958), and about 70% of the water-soluble nitrogen in sake consists of amino acids and di- or tripeptides and small amounts of higher molecular weight peptides (Figure 3). The only dipeptides isolated and crystallized were Val-Gln and Ile-Gln (Tajima and Sato, 1964). Artificial sake consumed in Japan is made by blending known ingredients found in natural sake, and thus it is similar to natural sake in taste. It is made by blending alcohol, glucose, succinic acid, lactic acid, inorganic phosphates, sodium chloride, MSG, glycine, and alanine. To this artificial sake, 0.03% of dipeptides was added by Tajima *et al.* (1968), and the effect of dipeptides on the taste was evaluated by a trained panel consisting of six members. All the dipeptides which were added to artificial sake improved the body, compared with the control, and caused the taste of artificial sake to come closer to that of natural sake (Table VI). It appears that peptides are not the principal tasty substances in sake, but rather that they contribute to the complexity and balance of the taste of natural sake. Addition of dipeptides to artificial sake also resulted in an increased buffering capacity of the product. As shown in Figure 4, Gly-L-Leu and L-Pro-L-Glu increased the buffer action at pH > 9, and L-Val-L-Glu showed strong buffer action at pH 7 to 8. In general, peptides have complex dissociation constants, compared to those of amino acids, and this property may result in the fine regulation of pH and may be related to the ability exhibited by peptides to improve the body of artificial sake.

Miso. Miso is a brewed foodstuff made from soybean

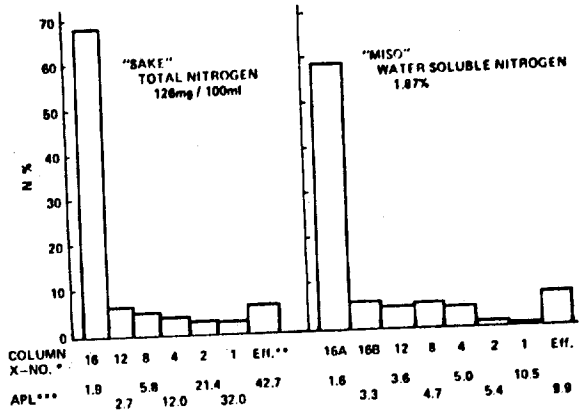

Figure 3. Distribution patterns of peptides in "sake" and "miso" (Takeuchi and Nose, 1958)

* : Cross-linking number of Dowex-50 column
** : Final effluent through molecular sieve chromatography
*** : Average peptide length

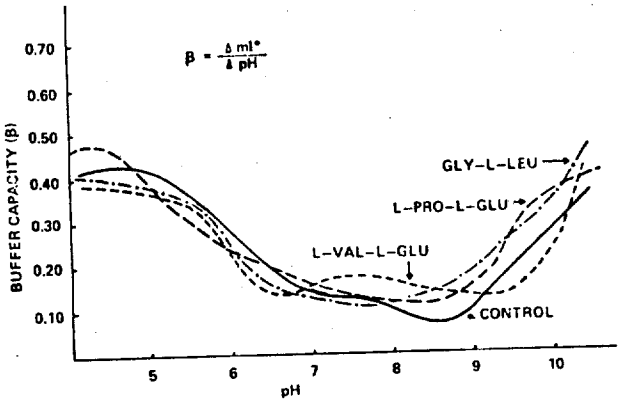

Figure 4. Effect of peptides on the buffer capacity on synthetic sake

*Δ ml. = Quantity of 1/10*N* NaOH or 1/10*N* HCl added (Lehmann, 1922)

and/or rice. There are three main types of miso with respect to the composition of the raw materials—i.e., soybean miso (soybean 100%), sendai miso (rice/soybean *ca.* 5.5), and shiro miso (rice/soybean *ca.* 22). In all three types of ripened miso, approximately the same distribution pattern of peptides was observed, regardless of the difference in raw materials. As shown in Figure 3, the average peptide length (APL) is less than 5.0 (Takeuchi and Yoshii, 1967). The amino acid composition of the major peptides of the hydrolyzate was determined, and it was found that they were acidic peptides containing aspartic acid, glutamic acid, and glycine.

Soy Sauce. Peptide Content of Soy Sauce. The total nitrogen content of Japanese soy sauce is generally 1.5%, one-half of which is in the form of free amino acids and about 12 to 15% in peptide form. The APL of peptides in soy sauce is from 3.5 to 3.8 (Table VII).

Amino Acid Composition and APL of Peptides. The amino acid content of the peptides was determined, and the results showed that in soy sauce larger amounts of aspartic acid, leucine, and glycine, and smaller amounts of glutamic acid and valine were present in peptide form than were present as free amino acids (Figure 5).

The nitrogen distribution of dialyzed soy sauce was examined by molecular sieve chromatography using six different cross-linked Dowex-50 resins, and most of the

Table VII. N-Distribution and APL of Soy Sauce A

Components	ND[a] (%)	APL[b]
Free amino acids	53	1.3
Peptides	13	3.8
Pyroglutamic acid	3	1.0
NH_3-N and unknown	31	0
Soy sauce A	100	1.75

[a] ND: Nitrogen distribution.
[b] APL: Average peptide length $= \frac{\text{(N of amino acids and/or peptides)}}{\text{amino-N}}$

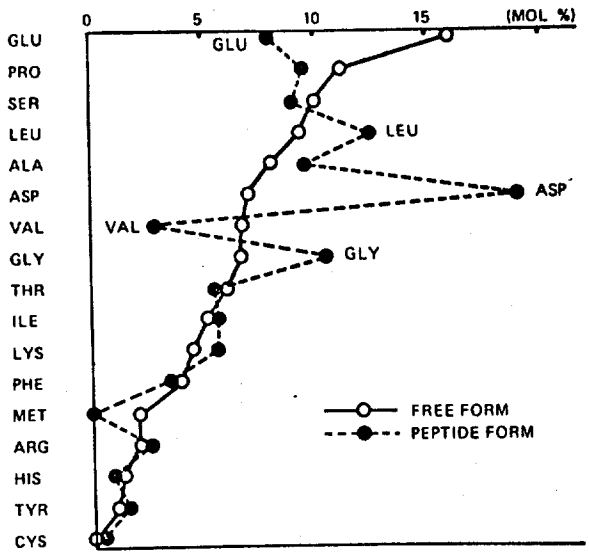

Figure 5. Amino acid composition of soy sauce A

nitrogen was in the form of low molecular weight peptides (Figure 6).

PREPARATION OF SOY SAUCE PEPTIDES. In studies on the contribution of peptides to the taste of soy sauce, peptide fractions were separated from soy sauce by dialysis and molecular sieve chromatography (Figure 7).

Ten liters of soy sauce which had been naturally brewed for months were dialyzed in Visking's cellulose acetate tubing ($1^7/_8$-inch inflated diameter) against distilled water at 20° C. for 12 hours. The diffusate was then passed through three Dowex-50 columns (X-16, X-4, and X-1), and two peptides having APL's of 3.8 and 4.1 were isolated from the X-4 and X-1 columns, respectively. Two different peptides were isolated, in a similar manner, from 8 liters of a soybean protein solution (2.5%) that was hydrolyzed

Table VIII. Analysis of Peptide Fractions from Soy Sauce and Protein Hydrolyzate

Origin	Name	APL	T-N, %[a]	NH_4-Cl, %[a]	Ash, %[a]	Sp. Visc. (η/η)[b]
Soy sauce A	Peptide A	3.8	11.5	2.3	0.2	1.4
	Peptide B	4.1	14.9	1.5	0.4	1.45
Pronase hydrolyzate	Peptide C	4.4	16.5	7.5	0.4	1.4
	Peptide D	6.2	16.1	2.6	0.4	1.35

[a] Dry basis.
[b] 9% aqueous solution.

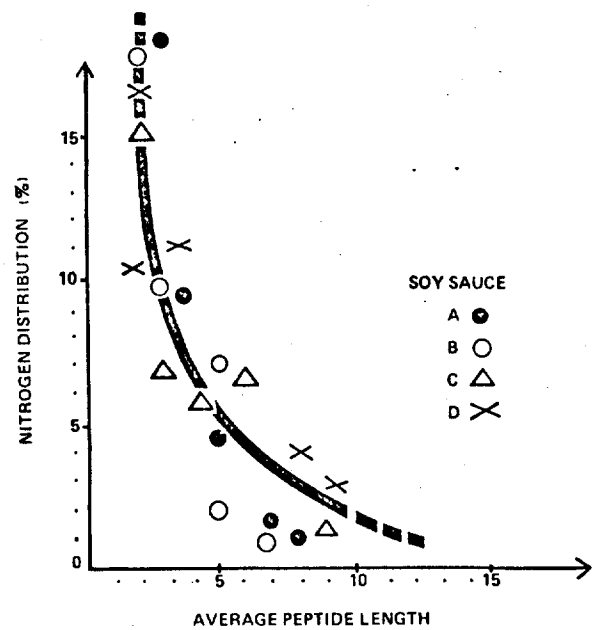

Figure 6. N-distribution of dialyzed soy sauce

by 4 grams of Pronase AF (a neutral proteinase from *Streptomyces griceus*) at pH 8.0, 55° C., and for 15 hours. These conditions of enzymic hydrolysis are quite different from those employed in the commercial preparation of soy sauce in which raw materials—soybean and wheat—are hydrolyzed at pH 6 at 20 to 25° C. for 15 hours with a proteinase from *Aspergillus sp.*

ORGANOLEPTIC AND OTHER PROPERTIES OF THE PREPARATIONS. The results in Table VIII show that the four peptides contained small amounts of NH_4Cl and ash; however, these compounds did not effect the sensory tests.

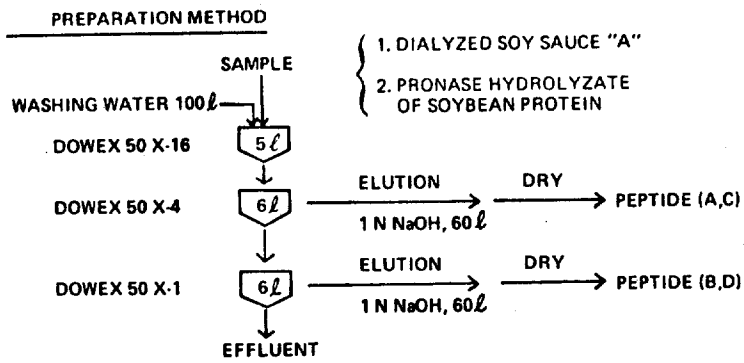

Figure 7. Preparation of peptide fractions from soy sauce and a soybean protein hydrolyzate

Table IX. Relationship Between Peptides, MSG, and Taste

pH 5.0; Panel size 9, trained
MSG at pH 5.0; RL 15[a]; TL 60[b]; DL 20[c]

Triangle Test

(X,Y,Y) X MSG	X Peptide	Y MSG	Y Peptide	(Mg./100 ml. of distilled water) Significance Peptide B	Peptide C
30	62	0	62	[d]	[d]
6	62	0	62	N.s.[e]	N.s.[e]
60	62	60	0		[f]
60	31	60	0	[g]	N.s.
60	6	60	0	N.s.	N.s.

Duo-trio Test

(X):(Y,Y′) X MSG	X Peptide	Y MSG	Y′ MSG	(Mg./100 ml. of 0.5% NaCl soln.) Sig. of Answer Y Peptide B	Peptide C
15	31	15	40	N.s.[e]	[d]
15	31	15	50	[d]	
40	31	40	15	N.s.[e]	N.s.[e]
65	31	65	15	N.s.[e]	N.s.[e]
85	31	85	15	[f]	[g]

[a] RL: Threshold value.
[b] TL: Limen at which specific taste can be clearly detected.
[c] DL: Differential limen.
[d] Significant at the 1% level.
[e] N.s.: Not significant.
[f] Significant at the 5%.
[g] Significant at the 0.1% level.

For each peptide the specific viscosity of a 9% aqueous solution (1.4% N) was approximately 1.4. This value was lower than that of a MSG solution adjusted to 1.4% nitrogen and much lower than that of whole soy sauce (specific viscosity: 2.2), and thus the peptides have no effect on the viscosity of soy sauce. The high viscosity of whole soy sauce is probably due to carbohydrates.

The contribution of peptides to the buffering capacity of whole soy sauce was slight (Figure 4).

Taste of the Preparations. Aqueous solutions of all four peptides tasted bitter and astringent. The thresholds were from 8 to 60 mg. per 100 ml., bitterness and astringency being easily detected at concentrations of 62 mg. per 100 ml. or higher (Figure 8).

To test what effect, if any, the peptide had on the taste of NaCl, a 0.1% solution of peptide was added to a NaCl solution (1.00 ± 0.015%). The solution exhibited the same saltiness as that in a solution containing 1.00% of NaCl alone, and hence the peptide had no effect on saltiness of NaCl.

Sensory tests were performed to determine the relationship between peptides B and C and MSG (Table IX). Taste differences were not significant between a solution containing 62 mg. per 100 ml. of peptide alone and a peptide solution containing subthreshold amounts of MSG or between a solution containing 60 mg. per 100 ml. of MSG alone and a solution containing subthreshold amounts of peptide. Differential limen (DL) of MSG in the mixture of MSG and either peptide was larger than that in MSG alone, indicating that the peptides depressed the intensity of the taste of MSG. The same results were obtained using the peptides and IMP or GMP.

The bitter and astringent taste of the peptides was similar to that of other soy sauce components such as monosodium succinate, sodium acetate, sodium lactate, and ammonium chloride; but there were significant differences in taste between solutions containing 62 mg. per 100 ml. of peptide alone and solutions containing subthreshold amounts of soy sauce components (Table X).

Sensory tests were performed to evaluate the effect of adding peptides to various soy sauce preparations. Imitation soy sauce (HVP liquid, Ajieki Type A, an acid hydrolyzate of soybean meal) had an unpleasant taste in the presence of peptide B compared to HVP alone; but a half-imitation soy sauce (a mixture of HVP and brewed soy sauce) had a more pleasant taste with added peptide

Table X. Relationship Between Peptides and Other Components of Soy Sauce Having Bitter or Astringent Taste

pH 5.0, aqueous solution;
Panel size 10, trained

Triangle Test

(X,Y,Y) $\begin{cases} X = \text{Peptide (TL)} + \text{Component (1/10 TL)} \\ Y = \text{Peptide (TL)} \end{cases}$

Peptide Conc., Mg./dl.	Component Name (RL)	Conc.	Significance Peptide B	Peptide C
62	Na-succinate (16)	13	[a]	N.s.[b]
62	Na-acetate (63)	13	[c]	[c]
62	Na-lactate (63)	25	[c]	[d]
62	Na-levurate (31)	6	[d]	[a]
62	NH_4Cl (16)	6	[a]	[a]

[a] Significant at the 5% level.
[b] N.s.: Not significant.
[c] Significant at the 0.1% level.
[d] Significant at the 1% level.

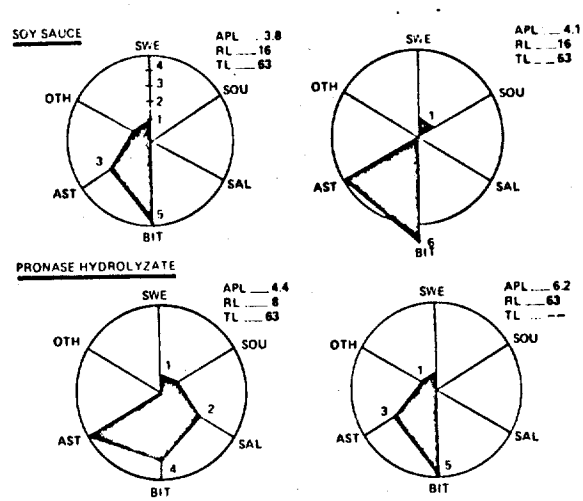

Figure 8. Taste profiles of peptide preparations

Radii represent taste intensities
100 mg./dl., pH 5.0–5.2, panel size 10, trained
AST: Astringency, OTH: Others
APL: Average peptide length
RL: Threshold value (mg./dl.)
TL: Limen at which specific taste can be clearly detected

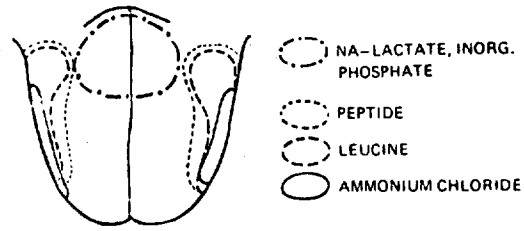

Figure 9. Area of the tongue stimulated by various components of soy sauce

Table XI. Effect of Peptides on Mixtures of HVP and Soy Sauce

pH 4.8 to 5.0; T-N 0.15 g./dl.; NaCl 1.8 g./dl.; panel size 10

Triangle Test (X,Y,Y) X: Testing base + Peptide B 160 mg./dl. Y: Testing base only

Testing Base			
Nitrogen Ratio HVP:Soy sauce A	Additives (Leu, NH_4Cl, NaH_2PO_4, and Lactate)	Significance	Effect of Peptide
10:0	0	***[a]	Unpleasant
6:4	0	***	Pleasant
6:4	Equivalent to 1/10 dilution of soy sauce A	***	Unpleasant

[a] *** = Highly significant.

Table XII. Partial Hydrolysis of Soybean Protein[a]

	HCl Concentration, *N*		
	0.03	0.01	0.006
A. P. L.[b]	3.8	7.7	11
Liberated Asp,[c] %	64	70	90

[a] 105° C., 72 hours, 1 part protein to 250 parts HCl (w./v.).
[b] Average peptide length.
[c] Liberated Asp/total liberated amino acids × 100.

than that without peptide. The half-imitation soy sauce, however, tasted less pleasant upon addition of peptide B and additives, such as leucine, NH_4Cl, NaH_2PO_4, and lactate (in the same amounts as contained in brewed soy sauce) than that without peptide added (Table XI).

Peptide added to the sauce stimulated both sides of the tongue (Figure 9). Although leucine imparted bitterness and astringency and NH_4Cl imparted saltiness and astringency, both stimulated the same area of the tongue. On the other hand, phosphate and lactate stimulated the central area of the tongue. When compounds stimulated comparable areas of the tongue, the taste of the sauce to which peptide had been added was more acceptable than sauce containing compounds which stimulated different areas of the tongue.

Finally, although the effect of peptides on the taste of soy sauce was different for each case mentioned above, these peptides were generally concluded to be bitter and astringent, as opposed to the universal taste enhancers, such as MSG, IMP, and GMP.

BITTER PEPTIDES IN PARTIAL HYDROLYZATES OF SOYBEAN PROTEIN

Peptides were prepared by extraction of natural products, chemical synthesis, and partial hydrolysis of protein using enzymes or other hydrolytic reagents. In particular, the hydrolytic action of very dilute hydrochloric acid on soybean protein was studied. Dilute hydrochloric acid was used to achieve a selective cleavage of protein at both sides of aspartyl residues. As shown in Table XII, the lower the concentration of hydrochloric acid, the more selective was the cleavage of aspartyl residues. The partial hydrolyzate had an APL of 3.8 and tasted slightly bitter.

Bitter peptides are a common product in the partial enzymic hydrolysis of soybean proteins. Fujimaki *et al.* (1968) identified the structures of seven peptides isolated from a peptic hydrolyzate of soybean protein and found that with the exception of one peptide, the amino- or carboxyl-terminal amino acid of these peptides was leucine. These bitter peptides can detract from the taste of foods, but a treatment using aminopeptidase, which hydrolyzes leucine-containing peptides, was effective in improving the taste of foods (Minamiura *et al.*, 1968).

SUMMARY

Based on the analytical and sensory results reported in this paper, the contributions of amino acids to the taste of foodstuffs can be summarized as follows: some amino acids contribute to the inherent tastes of foodstuffs themselves, some specific patterns of amino acid mixtures intensify the taste of foodstuffs and increase the mouthfulness without losing their inherent tastes, and the buffer action of amino acids can also contribute to the taste of foodstuffs.

The relationship between the chemical structure of dipeptides and their tastes may be utilized for improving foodstuffs as it points the way to the selective use of dipeptides in foods. Although further studies are required, it is presently concluded that peptides contribute to both the complexity and favorable balance of the taste of foodstuffs.

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KRAMPFAKTIVITÄT NACH INTRACEREBRALER INJEKTION VON L-GLUTAMAT

SEIZURES AFTER INTRACEREBRAL INJECTION OF L-GLUTAMATE

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Zusammenfassung—In akuten Versuchen an nicht narkotisierten, relaxierten, aufgespannten Katzen löst stereotaktisch in verschiedene Hirnregionen injiziertes Natriumglutamat eine generalisierte bioelektrische Krampfaktivität aus. Von allen untersuchten Regionen wurde für den Hippocampus die niedrigste Krampfschwelle nachgewiesen. Andere Hirngebiete sowie der Ventrikel reagierten nicht oder nur auf höhere Glutamat-Konzentrationen mit einem Krampf. Als krampfauslösende Ursachen werden Konzentrationsverschiebungen im Glutaminsäurestoffwechsel diskutiert.

Abstract—Sodium glutamate stereotactically injected into different cerebral regions of cats caused generalized electroencephalographic seizures. The hippocampus was shown to have the lowest seizure threshold of all regions investigated. Other cerebral regions and the ventricle did not react at all or only to higher concentrations of glutamate. Differences in the activity of the glutamic acid metabolism are discussed as releasing factors for seizures.

AUF EINE prinzipielle Bedeutung der Glutaminsäure für den Gehirnstoffwechsel beim cerebralen Krampfanfall kann die durch verschiedene Enzymreaktionen bedingte pathophysiologische Anhäufung der Glutaminsäure in der präkonvulsiven Phase hinweisen (WIECHERT und GÖLLNITZ 1969). In diesem Zusammenhang stellten wir uns die Aufgabe, zu untersuchen, von welchen Hirnregionen nach intracerebraler Injektion von L-Glutamat ein cerebraler Krampfanfall auszulösen ist und ob Beziehungen der Reizantwort zu der Injektionsmenge und dem Applikationsort bestehen.

METHODIK

Fünfundvierzig Katzen zwischen 2,5 und 5,0 kg wurden in Aethernarkose mit Succicuran (Succinylcholinchlorid, VEB Dtsch. Hydrierwerk Rodleben, DDR) relaxiert, durch ein Tracheostoma künstlich beatmet und in das stereotaktische Gerät gespannt und operiert. Nach Einführung und Fixierung der Elektroden wurde die Narkose abgebrochen. Mit bipolaren Elektroden erfolgte nach den Koordinaten von JASPER und AJMONE-MARSAN (1954) die intracerebrale Ableitung und die cranielle mit Nadelelektroden vom Schädelknochen. Eine 0,5 M-Lösung von L-Natriumglutamat (50–250 μl) wurde im Intervall bis zu 60 Minuten mit einer Mikroinjektionsspritze in 10–15 s appliziert. Nach fortlaufender Registrierung der bioelektrischen Aktivität mit dem 8-Kanal Schwarzer EEG wurden die Tiere ungefähr 60 Minuten nach der letzten Injektion durch Hexobarbital (*N*-Methyl-5-(zyklohexen-(1')-yl)-5-methylbarbitursäure-Natriumsalz, VEB Arzneimittelwerk Dresden, DDR) getötet und die intracerebralen Reiz- und Ableitungspunkte nach der Hess'schen Methode markiert. Das Gehirn wurde in 10%igem Formalin fixiert, später in 5 mm dicke Frontalschnitte zerlegt und der Sitz der Elektroden mit der Berliner Blaureaktion kontrolliert. Die Injektion erfolgte in 15 verschiedene Hirnregionen; bei jeweils drei Katzen immer die gleiche Lokalisation.

ERGEBNISSE

1. *Injektion von* L-*Glutamat in den Hippocampus dexter* (*H.*)

In fünf verschiedene Regionen des Hippocampus wurde injiziert. Die intracerebrale Ableitung erfolgte aus dem N. amygdalae bds. (F + 13,0, L 0,5, H — 7,5) und dem H. ventralis oder dorsalis bds. und vom Schädel temporal und occipital nach nasal (Abb. 1).

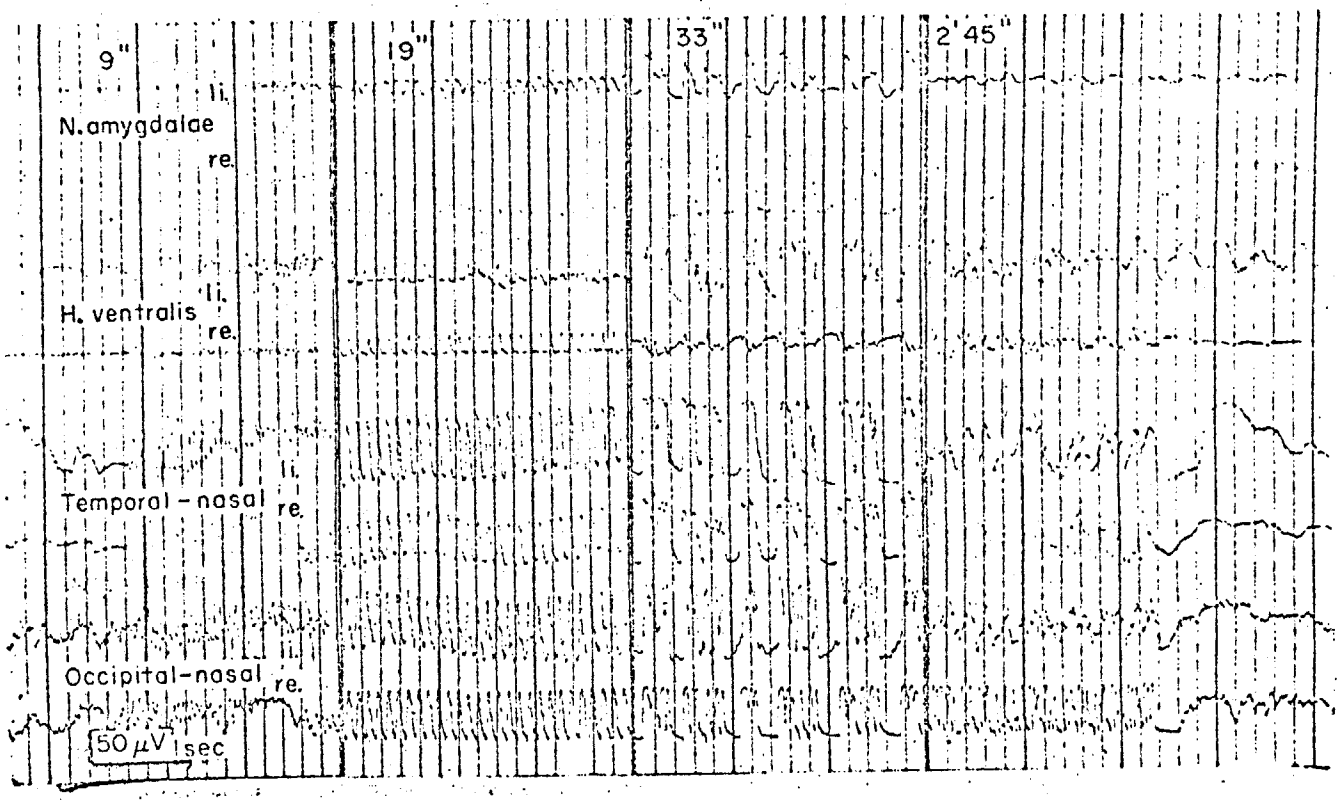


ABB. 2.—EEG einer Katze 9s, 19s, 33s und 2 min 45 s nach Injektion von 50 μl L-Glutamatlösung in den H. anterior ventralis dexter.

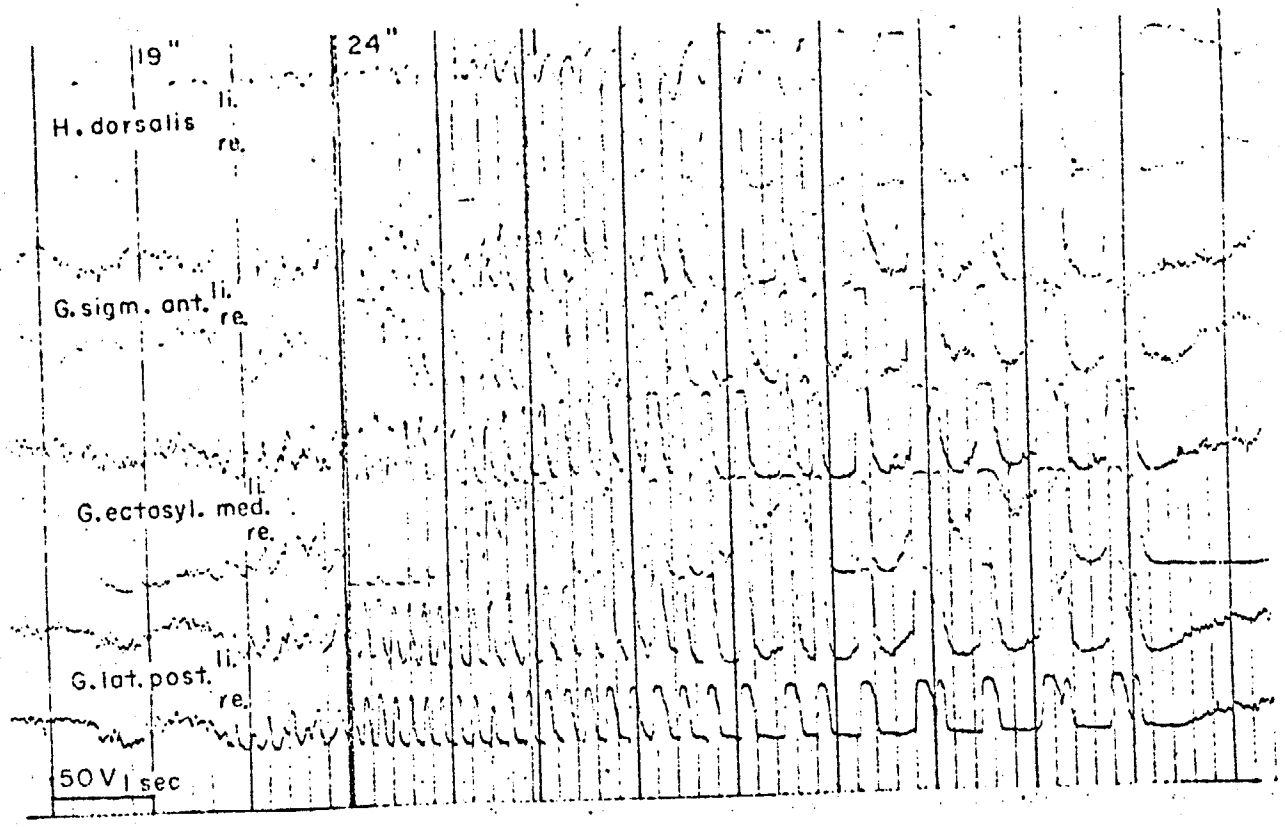
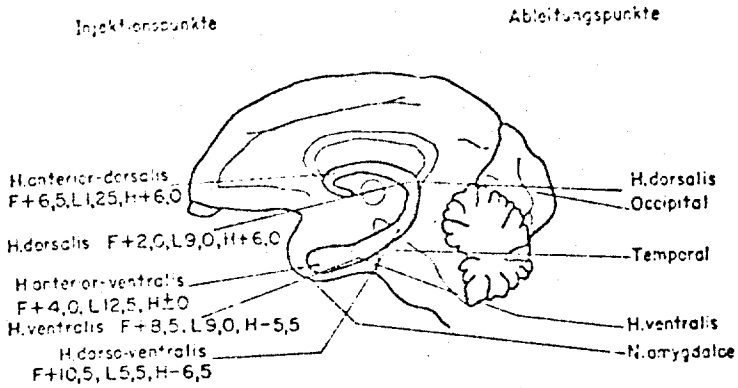


Abb. 5.—EEG einer Katze 19 s und 24 s nach Injektion von 50 μl L-Glutamatlösung in den Lobus piriformis dexter.



Injektion:	1. Katze			2. Katze			3. Katze		
	50 µl	50 µl	250 µl	50 µl	50 µl	250 µl	50 µl	50 µl	250 µl
H. anterior ventralis	+	+	+	+	+	+	+	+	+
H. ventralis	+	+	+	+	+	+	+	+	+
H. dorso-ventralis	+	−	+	+	+	+	+	−	+
H. dorsalis	−	−	+	+	−	−	+	+	+
H. anterior-dorsalis	+	+	+	−	+	+	+	−	+

Abb. 1.

(a) *Injektion in den H. anterior ventralis.* Bei 5–10 s nach Injektion von 50 µl L-Glutamatlösung in den H. anterior ventralis (F + 10,5, L 5,5, H − 6,5) trat bei allen Katzen eine sofort generalisierende Aktivität von 10–22/s Wellen auf, die sich nach wenigen Sekunden auf 8–10/s Wellen verlangsamte und dann erneut erhöhte. Mit zunehmender Krampfzeit unterbrachen langsame Wellen rhythmisch die Aktivität, und schließlich setzte der Krampf nach durchschnittlich 20–90 s abrupt aus (Abb. 2). Nach Injektionen von 250 µl dauerten die Anfälle bis zu 10 Minuten.

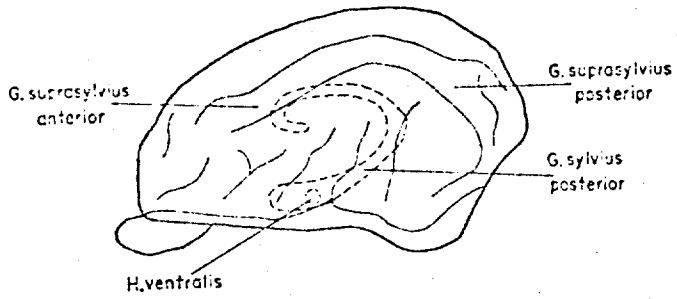
(b) *Injektion in den H. ventralis.* Bei 5–20 s nach Injektion von 50 µl L-Glutamat in den H. ventralis (F + 8,5, L 9,0, H − 5,5) fand sich ebenfalls eine innerhalb von 5 s generalisierende und im Hippocampus homolateral mit 20–30/s Wellen beginnende Krampfaktivität über 30 s, die bis zu 10 Minuten nach Injektion der größten Dosis anhielt.

(c) *Injektion in den H. dorso-ventralis.* Bei 10–30 s nach der ersten Injektion in den H. dorso-ventralis (F + 4,0, L 12,5, H ± 0) begann im Hippocampus die schnelle Aktivität, die entweder in 2–10 s generalisierte oder fokal blieb. Der Krampf dauerte 10–90 s. Nach der zweiten und dritten Injektion traten rechtsseitig langsame Wellen auf, auf denen sich die schnelle, generalisierende Krampfaktivität entwickelte.

(d) *Injektion in den H. dorsalis.* Nach Injektion in den H. dorsalis (F + 2,0, L 9,0, H + 6,0) begann nach 10–15 s im Hippocampus innerhalb von 10 s eine generalisierende paroxysmale Aktivität bis über 200 s Dauer.

(e) *Injektion in den H. anterior dorsalis.* Nach der ersten Injektion in den H. anterior dorsalis (F + 6,5, L 1,25, H + 6,0) trat nach 10–15 s eine im Hippocampus beginnende Krampfaktivität auf, die nach 1–5 s in einen generalisierten Krampf von 30–90 s überging. Nach weiteren Injektionen stellte sich nach einer Latenz von 30–40 s die gleiche Reaktion ein.

Ableitungspunkte



Injektion:	1. Katze 50 µl	1. Katze 50 µl	1. Katze 250 µl	2. Katze 50 µl	2. Katze 50 µl	2. Katze 250 µl	3. Katze 50 µl	3. Katze 50 µl	3. Katze 250 µl
N. amygdalae dext. F+13,0, L 9,5, H-7,5	–	–	–	–	–	–	–	–	–
N. caudatus dext. F+13,0, L 5,0, H+6,0	–	–	+	–	–	+	–	–	+
Septumregion dext. F+14,0, L 0,0, H+4,5	–	+	+	–	–	–	–	–	+
Thalamus dext. F+10,0, L 3,0, H+1,5	–	–	+	–	–	+	–	–	–
Hypothalamus post. dext. F+10,0, L 1,0, H-5,0	–	–.	+	+	+	–	–	–	–

Abb. 3.

2. *Injektion von L-Glutamat in andere Kerngebiete*

Die Ableitung erfolgte vom H. ventralis und vom G. suprasylvius posterior und anterior sowie vom G. sylvius posterior (Abb. 3).

(a) *Injektion in den N. amygdalae dexter.* Weder nach Injektion von 50 µl noch nach 250 µl waren in allen Versuchen fokale oder generalisierten Krämpfe zu beobachten.

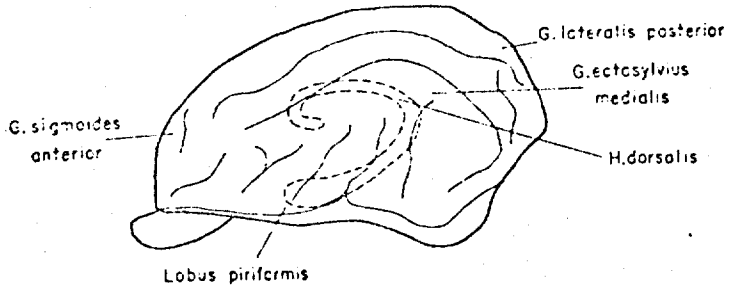
(b) *Injektion in den N. caudatus dexter.* Erst nach Injektion von 250 µl in den N. caudatus (F + 13,0. L 5.0. H + 6,0) trat aus einer schnellen Grundaktivität von 25–40/s Wellen nach 10–60 s eine generalisierte Krampfaktivität von 12–25/s Wellen über 60 s auf. Der schnelle Grundrhythmus konnte nach 30 Minuten noch nachgewiesen werden.

(c) *Injektion in die Septumregion.* In der Septumregion (F + 14, L 0,0, H + 4,5) trat nur vereinzelt bis zu 20 s nach der ersten oder dritten Injektion eine fokale Krampfaktivität im Hippocampus auf, die nach 1–5 s in einen generalisierten Krampf überging, der diffus von langsamen Wellen unterbrochen wurde.

(d) *Injektion in den Thalamus.* Im Thalamus (F + 10,0, L 3,0, H + 1,5) trat erst nach Injektion von 250 µl nach mehr als 60 s ein generalisierter Krampf von 30 s auf.

(e) *Injektion in den Hypothalamus posterior.* Injektion in den Hypothalamus posterior (F + 10,0, L 1,0, H – 5,0) führte nur vereinzelt nach 8–15 s zu einer diffusen Krampfaktivität ohne typische Generalisation bis zu 30 Minuten Dauer.

Ableitungspunkte



Injektion:	1. Katze 50 µl	50 µl	250 µl	2. Katze 50 µl	50 µl	250 µl	3. Katze 50 µl	50 µl	250 µl
G. sigmoides ant. dext.	–	–	–	+	+	–	–	–	–
G. lateralis post. dext.	–	–	–	–	–	–	–	–	–
G. ectosylvius med. dext.	–	–	+	–	–	+	–	–	+
Lobus piriformis dext.	+	+	+	+	+	+	+	+	+
Ventrikel dext.	–	–	+	+	–	+	+	+	+

Abb. 4.

3. *Injektion von L-Glutamat in die Hirnrinde*

Abgeleitet wurde aus dem H. dorsalis und vom G. sigmoides anterior, G. ectosylvius medialis und G. lateralis posterior (Abb. 4).

(a) *Injektion in den G. sigmoides anterior* löste einmal nach 5 s einen generalisierten Krampf von 40 s aus. 150 s nach der zweiten Injektion krampfte die Katze 10 Minuten lang, während die beiden anderen Tiere keine Veränderungen zeigten.

(b) *Injektion in den G. lateralis posterior dexter.* Vom G. lateralis posterior ließ sich kein Krampf auslösen.

(c) *Injektion in den G. ectosylvius medialis dexter.* Bis zu 10 Minuten nach Injektion von 250 µl in den G. ectosylvius medialis traten diffus 12–25/s Wellen auf, die nach weiteren 10–15 Minuten in einen generalisierten Krampf bis zu 15 Minuten Dauer übergehen konnten.

(d) *Injektion in den Lobus piriformis dexter.* Vom Lobus piriformis lösten alle Injektionen nach 8–20 s einen generalisierten Krampf aus, der im Hippocampus begann und 10–90 s lang anhielt (Abb. 5). Neben 20–30/s Wellen generalisierten auch 6–8/s Wellen bei weiterer Verlangsamung.

4. *Injektion von L-Glutamat in den Ventrikel*

Bei 10–30 s nach intraventriculärer Injektion von 250 µl begann diffus im Hippocampus eine schnelle Aktivität, die nach weiteren 10–90 s über 10 s bis zu 15 min generalisierte, dann in langsame Wellen überging, aus denen heraus erneut schnelle Wellen auftraten, die zu einem weiteren Krampf führten.

DISKUSSION

Aus den Versuchen ist zu entnehmen, daß von allen mit Glutaminsäure gereizten Hirnregionen die verschiedenen Bereiche des Hippocampus für diese Aminosäure die niedrigste Krampfschwelle besitzen. Bereits 50 μl Natriumglutamat führten in den fünf untersuchten Regionen des Hippocampus zu einer generalisierten Krampfaktivität. Nach dem Hippocampus sprach nur der Lobus piriformis auch auf niedrige Dosen mit einem generalisierten Krampf an. Andere Kerngebiete und Bereiche der Hirnrinde sowie der Ventrikel reagierten nicht oder nur auf höhere Dosierung mit einem Krampf. Dabei fällt auf, daß der von elektrischer Stimulierung her bekannte bioelektrische Phasenablauf des Hippocampuskrampfes (CREUTZFELDT, 1956; ELUL, 1964) nach seiner Auslösung mit Glutaminsäure uncharakteristischer verläuft. Dies wurde auch schon nach Reizung des Hippocampus mit Penicillin gefunden (VOICULESCU und VOINESCU, 1965). Abzuleiten ist, daß die Bereitschaft des Gehirns, nach Glutaminsäure mit einem generalisierten und nicht nur auf den Reizort beschränkten Krampf zu reagieren, sehr groß ist. Für den Hippocampus ist eine direkte Abhängigkeit von der Dosisgröße nicht zu erkennen.

Biochemisch läßt sich bei experimentellen Krämpfen in der präkonvulsiven Phase nur im Hippocampus eine Anhäufung der Glutaminsäure nachweisen (WIECHERT und GÖLLNITZ, 1970). In anderen Hirnregionen war die Konzentration der Glutaminsäure unverändert oder erniedrigt, was auch STONE, TEWS and MITCHELL (1960) fanden. Diese Beziehungen von neurochemischen Befunden einerseits, die bei experimentell ausgelösten Krämpfen, wie z.B. durch Glutamat, Pyridoxal-5-Phosphat, Elektroschock, Cardiazol-Schock und audiogener Reizung auf der Grundlage veränderter Enzymreaktionen zu einer Anhäufung von Glutaminsäure im Hippocampus führen und elektrophysiologischen Befunden andererseits, die diese Hirnregion empfindlich für Glutaminsäure markiert, unterstreicht die Bedeutung des Hippocampus für das cerebrale Anfallsgeschehen. MC LENNAN, HOFFMAN und MARSHALL (1968) schließen aus ihren Experimenten, daß es neben unspezifischen Receptoren, die auf alle excitatorischen Aminosäuren reagieren und in den meisten Neuronen vorkommen, auch spezifische durch Glutamat aktivierbare Receptoren gibt, die unter Umständen mit den excitatorischen synaptischen Receptoren identisch sind.

Denkbar wäre, daß die durch den pathologischen Stoffwechsel in der präkonvulsiven Phase verstärkt gebildete Glutaminsäure im Hippocampus über diese erwähnten excitatorischen synaptischen Receptoren sowohl bei experimentellen als auch bestimmten klinischen Anfallsformen den Krampf auslöst.

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CCXXX. METABOLISM OF AMINO-ACIDS.

IV. THE SYNTHESIS OF GLUTAMINE FROM GLUTAMIC ACID AND AMMONIA, AND THE ENZYMIC HYDROLYSIS OF GLUTAMINE IN ANIMAL TISSUES.

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1. *Oxidation of* l(+)*-glutamic acid without formation of ammonia.*

THE starting-point of this investigation was the observation that glutamic acid behaves differently from all the other α-amino-acids in guinea-pig and rabbit kidneys, in that, although it increases the oxygen uptake even more than any other amino-acid of the *l*-series, in most cases it actually diminishes ammonia formation. In guinea-pig kidney, the oxygen uptake is increased about 100–150% (Table I) whilst in most experiments no ammonia at all is detectable in the presence of *l*(+)-glutamic acid [Krebs, 1933, 2][1].

Table I. *Oxygen uptake and ammonia formation in the presence of* l(+)*-glutamic acid.*

Tissue	Without substrate		With *l*(+)-glutamic acid (*M*/50)	
	Q_{O_2}	Q_{NH_3}	Q_{O_2}	Q_{NH_3}
Kidney, guinea-pig	16·8	1·20	25·7	~0
	14·6	0·82	35·8	~0
	14·1	0·90	30·9	0·18
Kidney, rabbit	14·8	0·92	35·5	0·57
	13·8	0·37	23·4	0·40

However, if guinea-pig kidney is poisoned with arsenious oxide, ammonia is formed from glutamic acid (Table II). This could be explained by assuming that ammonia is formed in a primary reaction but disappears in a secondary reaction, the latter being inhibited by arsenious oxide. Experiments were therefore set up to see whether ammonia reacts in the expected way if it is added to kidney.

Table II. *Influence of* As_2O_3 *on ammonia formation in guinea-pig kidney.*

Concentration of As_2O_3	Without substrate		With *l*(+)-glutamic acid (*M*/50)	
	Q_{O_2}	Q_{NH_3}	Q_{O_2}	Q_{NH_3}
0	16·8	1·20	25·7	~0
M/5000	8·5	1·18	10·2	2·30
0	14·6	0·82	35·8	~0
M/1000	5·1	1·41	6·4	2·00

In muscle, too, glutamic acid causes an increase in oxygen uptake without influencing ammonia formation (D. M. Needham). This however is an effect different from that in kidney.

2. *Disappearance of ammonia from kidney in the presence of glutamic acid.*

If ammonium salts are added to guinea-pig or rabbit kidney in the presence of $l(+)$-glutamic acid, the ammonia disappears from the solution (Table III, Fig. 1). 11·79 mg. kidney, for instance, removed 95 μl. of ammonia in 30 mins., $Q_{NH_3}\left[\frac{\mu\text{l. NH}_3\text{ used}}{\text{mg.} \times \text{hours}}\right]$ being $-16{\cdot}1$. Similar figures were obtained with rabbit kidney (Table V). The rate of disappearance of ammonia is even higher than the rate of synthesis of urea in liver under average conditions.

Table III. *Disappearance of ammonia from guinea-pig kidney.*

11·79 mg. kidney in 2 ml. phosphate saline containing $M/50$ $l(+)$-glutamic acid and 166 μl. NH_3; 37·5°; O_2.

Time mins.	Ammonia present in 2 ml. μl.	Ammonia used μl.
0	166	—
30	71	95
60	14	152
90	0	166

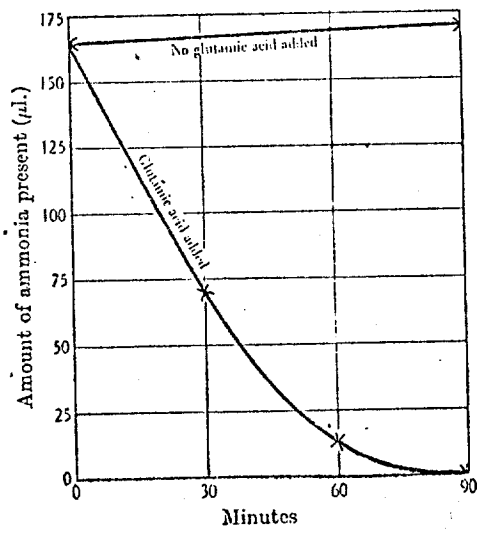

Fig. 1. Disappearance of ammonia in the presence of $l(+)$-glutamic acid (guinea-pig kidney).

No ammonia disappears from kidney in the absence of $l(+)$-glutamic acid; in the presence of amino-acids such as $d(-)$-glutamic acid, aspartic acid, β-hydroxyglutamic acid and numerous others the concentration of added ammonia is either unchanged or increased on account of deamination. The only two nitrogenous substances which behave similarly to glutamic acid are proline and hydroxyproline. The metabolism of these two substances will be dealt with in the next paper of this series.

In the presence of lactate or pyruvate, small quantities of ammonia can be utilised by kidney as shown in Table IV; the amount however is only 5–8% of that which disappears in the presence of glutamic acid.

Table IV. *Disappearance of ammonia from guinea-pig kidney in the presence of lactate and pyruvate.*

Substrate added	mg. tissue	NH_3 per flask (μl.) At start	After 60 min.	NH_3 used μl.	Q_{NH_3}
dl-Lactate (*M*/50)	10·64	86	74·0	12·0	−1·13
Pyruvate (*M*/50)	10·32	86	73·9	12·1	−1·17

3. *Formation of amide-nitrogen in kidney.*

If the solution from which ammonia has disappeared in the presence of glutamic acid is heated for 5 min. with 5 % sulphuric acid, the ammonia appears again in the solution (Table V). Amino-acids or amino-purines do not split

Table V. *Disappearance of ammonia and formation of amide-nitrogen in kidney.*

Phosphate saline; *M*/50 *l*(+)-glutamic acid.

Tissue	Dry wt. of tissue mg.	NH_3 added μl.	Duration of exp. min.	NH_3 at end μl.	Change NH_3 μl.	Q_{NH_3}	Amide-N found μl.	$Q_{Amide\text{-}N}$
Guinea-pig kidney	11·79	166	30	71	− 95	−16·1	124	+21·0
	18·82	202	20	21	−181	−28·8	191	+30·4
	7·66	232	60	38	−194	−25·3	222	+29·0
Rabbit kidney	15·00	232	60	53	−179	−12·0	187	+13·9
	9·33	224	80	70	−154	−12·4	186	+14·9
	9·87	218	60	25	−193	−19·5	236	+24·0

off ammonia when heated for 5 min. in dilute acid, whereas acid amides are hydrolysed by hot dilute acid [Sachsse, 1873]. Asparagine requires heating for one hour, whereas glutamine is completely hydrolysed within 5 min. by 5 % sulphuric acid at 100° (Table VI). We may therefore conclude that the kidney tissue has converted ammonium glutamate into glutamine.

The amount of glutamine formed is usually somewhat larger than the amount of ammonia which disappears (Table V). The source of the excess of amide-nitrogen is ammonia formed by deamination of the glutamic acid. Whilst one part of the added glutamic acid is deaminated, another part unites with the ammonia in the synthesis of glutamine.

Table VI. *Acid hydrolysis of acid amides.*

0·005 *M* amide heated with 5 % (final concentration) sulphuric acid at 100°.
Calculated for complete splitting 112 μl. NH_3 per ml.

Amide	Time of heating min.	NH_3 formed (μl.) per 1 ml.	% splitting
Glutamine	2	91·5	82
	4	111·5	99·6
	8	113·0	100·9
Asparagine	5	26·0	23·2
Urea	5	0	0

4. *Formation of glutamine in various tissues.*

In the kidneys of guinea-pig and rabbit synthesis of glutamine proceeds more rapidly than deamination of glutamic acid; thus no free ammonia but only glutamine accumulates in these kidneys after addition of glutamic acid

(Table VII). Kidneys of rat and sheep deaminate more quickly than they form glutamine, and ammonia and glutamine appear in the solution simultaneously. Added ammonia does not disappear in these cases, the tissue being already saturated with ammonia from deamination. On the other hand no amide-nitrogen is formed in the kidneys of pig, dog or cat if glutamic acid and ammonia are present.

Table VII. *Formation of glutamine in kidney cortex of various mammals.*

No ammonia added.

Tissue (kidney)	No glutamic acid added			$M/50$ $l(+)$-glutamic acid		
	Q_{O_2}	Q_{NH_3}	$Q_{Amide\text{-}N}$	Q_{O_2}	Q_{NH_3}	$Q_{Amide\text{-}N}$
Guinea-pig	14·5	0·98	0·20	38·0	0	6·36
Rabbit	13·8	0·37	1·38	24·6	0·40	2·92
Sheep	13·9	1·77	0·68	26·6	3·11	7·50
Rat	21·0	2·90	1·40	43·2	6·00	2·83
Pig	16·9	1·80	~0	21·7	2·23	~0
Cat	16·6	1·57	~0	24·4	2·74	~0
Dog	21·3	1·36	~0	27·2	2·69	~0
Pigeon	18·2	2·21	~0	26·4	4·40	~0

Thus the system which synthesises glutamine is not found in all kidneys, but it is found in the retina and central nervous system of all the vertebrates which have been investigated (various mammals, birds, tortoise, frog, trout (see

Table VIII. *Consumption of ammonia and formation of glutamine in brain cortex and retina.*

Solution: 3 ml. phosphate saline; $M/50$ $l(-)$-glutamic acid; $M/30$ glucose; 37·5°, unless otherwise stated.

Tissue	Dry weight mg.	Time min.	Total amount of NH_3 in experimental solution (μl.) At beginning	At the end	Q_{NH_3}	NH_3 found after acid hydrol. μl.	$Q_{Amide\text{-}N}$
Brain, guinea-pig	8·59	60	67·2	26·4	− 4·77	39·4	4·60
	13·08	60	80·0	49·5	− 2·34	13·4	1·03
Retina, guinea-pig	3·57	60	67·2	31·0	−10·1	32·8	9·20
	4·00	60	33·6	1·6	− 8·0	29·0	7·25
Brain, pig	18·82	80	33·6	0	—	38·1	—
Retina, pig	20·00	40	33·6	0	—	41·0	—
Brain, rat	9·54	120	85·0	28·6	− 2·96	44·4	2·34
	7·50	60	85·0	62·6	− 2·99	20·6	2·74
Retina, rat	2·58	90	66·0	36·0	− 7·75	24·4	6·30
Retina, sheep	30·98	120	431·0	48·0	− 6·20	227·0	3·67
	21·45	120	448·0	204·0	− 5·70	138·0	3·22
Brain, pigeon	11·12	80	67·2	20·6	− 3·10	61·5	4·11
	6·43	80	67·2	37·2	− 3·49	33·6	3·93
Retina, pigeon	19·10	80	67·2	5·0	− 2·42	61·0	2·40
	7·82	60	165·0	81·0	−10·7	88·0	11·2
	9·00	40	89·6	52·2	− 6·23	33·0	5·50
	10·87	40	134·0	68·2	− 9·12	46·2	6·35
Retina, domestic fowl	14·70	40	131·0	79·0	− 5·33	49·0	5·00
Brain, frog (27°)	9·16	180	39·0	9·2	− 1·08	41·5	1·51
Retina, frog (27°)	6·09	180	39·0	19·5	− 1·07	32·5	1·78
Brain, tortoise (30°) (*Testudo graeca*)	17·34	240	68·5	30·0	− 0·55	34·0	0·49
Retina, tortoise (30°) (*Testudo graeca*)	1·21	240	68·5	24·5	− 9·00	42·5	8·70
Brain, trout (30°)	22·4	60	127·0	95·0	− 1·44	33·0	1·47
Retina, trout (30°)	8·71	60	127·0	25·0	−11·7	88·0	10·1

Table VIII)). The retina of warm-blooded animals forms about 6–10 μl. of amide-nitrogen per mg. or 5–7 % of its own dry weight of glutamine per hour. In brain cortex the rate is about a third of that in retina.

Whilst in kidney, as mentioned earlier, the amount of amide-nitrogen formed is usually slightly higher than the amount of ammonia consumed, in brain and in retina the amount of ammonia which disappears is often greater than the amount of amide-nitrogen found. This will be further studied in section 10.

No formation of amide-nitrogen from ammonium glutamate was detectable in the following tissues of the guinea-pig: kidney medulla, liver, spleen, testis, placenta, chorion, muscle, heart, salivary glands, pancreas, white matter of brain, red blood cells, small intestine; or in Jensen rat sarcoma, mice Crocker tumour, fowl tumour and pigeon blood.

5. *Some properties of the glutamine-synthesising system.*

A. *Influence of* p_H. The optimum p_H for the synthesis of glutamine in guinea-pig kidney is 7·2–7·4, as shown in Table IX and Fig. 2.

Table IX. *Influence of* p_H *on the synthesis of glutamine.*

Guinea-pig kidney; 37·5°. p_H varied by varying the concentration of bicarbonate in the saline and the concentration of CO_2 in the gas mixture. Various concentrations of bicarbonate were obtained by mixing 1·3% bicarbonate with bicarbonate-free saline. $M/50$ $l(+)$-glutamic acid. Ammonium chloride: $2{\cdot}72 \times 10^{-3}$ M; 3 ml. solution for each flask.

Conc. of bicarbonate (M)	0·155	0·125	0·0950	0·0339	0·0136	0·00358	0·00358
Percentage of CO_2 in the gas mixture	2·5	5·0	5·0	5·0	5·0	5·0	20·0
p_H	8·58	8·19	8·07	7·62	7·22	6·64	6·04
mg. of kidney cortex	7·23	7·71	9·20	8·24	5·57	12·83	10·21
NH_3 at the beginning (μl. per flask)	201·0	201·0	201·0	201·0	201·0	201·0	201·0
NH_3 found after 40 min. (μl.)	204·0	148·5	114·0	105·5	132·0	100·0	172·0
NH_3 used (μl.)	3·0	−53·5	−87·0	−95·5	−69·0	−101·0	−29·0
Q_{NH_3}	~0·0	−10·4	−14·2	−17·4	−18·6	−11·8	−4·25
Amide-N found after 40 min. (μl.)	38·0	56·5	83·6	123·0	84·2	114·5	42·4
$Q_{Amide\text{-}N}$	7·9	11·0	13·6	22·4	22·6	13·4	6·2

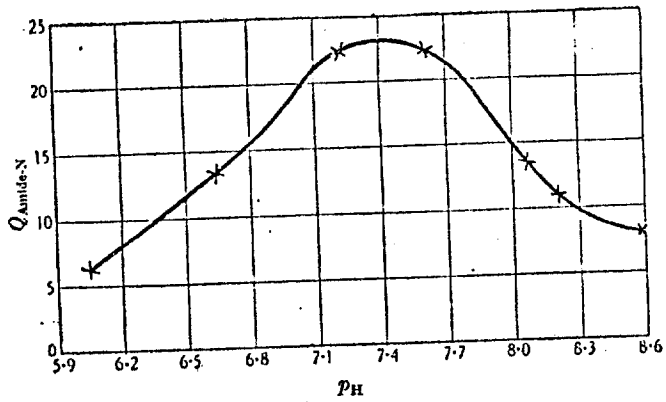

Fig. 2. Influence of p_H on the synthesis of glutamine (guinea-pig kidney).

B. *Concentration of glutamic acid.* It is only when the solution contains excess of glutamic acid that ammonia reacts completely to form glutamine in kidney (Tables III and X). In brain or retina however no excess of glutamic acid is

Table X. *Influence of the concentration of* l(+)*-glutamic acid on the synthesis of glutamine.*

Guinea-pig kidney; 3 ml. phosphate saline: initial concentration of ammonium chloride 0·00148 *M*; amount of tissue 4–6 mg.; time 80 min.; 37·5°.

Concentration of *l*(+)-glutamic acid *M*	Q_{O_2}	Q_{NH_3}	$Q_{Amide\text{-}N}$
0	11·5	+ 0·36	1·67
0·0033	29·1	− 10·9	8·80
0·0066	28·7	− 16·0	15·4
0·022	44·0	− 24·2	26·0

necessary; if glutamic acid is added in small quantities and ammonia in excess, practically the whole of the glutamic acid is converted into glutamine. The amount of ammonia which disappears is approximately equivalent to the amount of glutamic acid present (Table XI).

Table XI. *Disappearance of ammonia in the presence of small amounts of* l(+)*-glutamic acid and excess of ammonia.*

Sheep retina (about 30 mg. per experiment); 37·5°; 2 hours; bicarbonate saline with 0·3% glucose; 5% CO_2 in O_2.

Glutamic acid added µl.	NH_3 in the liquid (µl.) Initial	Final	Change in NH_3 µl.	Total NH_3 consumed by the tissue, corrected for blank µl.	Amide-N found µl.
112	350	264	− 86	101	72
224	350	140	− 210	228	158
0	0	18	+ 18	—	2

C. *Influence of glucose.* The synthesis of glutamine in kidney is unaffected by glucose; in retina and brain however the rate of synthesis is very small in the absence of glucose. All the figures recorded in Table VIII were obtained in glucose-containing saline. Table XII shows some examples of the effect of glucose on the rate of glutamine synthesis in brain and retina.

Table XII. *Influence of glucose on the rate of disappearance of ammonia and rate of synthesis of glutamine.*

Experimental conditions as in Table VIII.

Tissue	No glucose Q_{NH_3}	No glucose $Q_{Amide\text{-}N}$	*M*/50 glucose Q_{NH_3}	*M*/50 glucose $Q_{Amide\text{-}N}$
Retina, sheep	− 0·46	+ 0·58	− 6·03	+ 3·67
Brain, rat	− 0·70	+ 1·40	− 3·75	+ 3·72

D. *Grinding and extracting.* Ground kidney, suspended in 10 vols. of saline, does not synthesise glutamine. The synthesis is bound up with the structure of the cell, as might be expected since it requires energy.

E. *Relation to energy-giving reactions.* In kidney, respiration is practically the only reaction which can provide energy for endothermic reactions. It would

therefore be expected that inhibition of respiration would inhibit the synthesis of glutamine. This is actually the case. Under strictly anaerobic conditions, glutamine is not synthesised in kidney. Hydrocyanic acid inhibits the glutamine synthesis approximately in the same degree as it inhibits respiration (Table XIII).

Table XIII. *Inhibition by hydrocyanic acid.*

Guinea-pig kidney; phosphate saline; $M/50$ $l(+)$-glutamic acid; $M/400$ ammonium chloride; 37·5°.

Concentration of HCN (M)	Q_{O_2}	Q_{NH_3}	$Q_{Amide\text{-}N}$
0	−44·0	−24·2	26·0
10^{-4}	−17·6	− 5·66	6·65
10^{-3}	− 4·30	− 3·1	2·1

Unlike kidney cortex, brain and retina possess a second very active energy-providing system, namely lactic acid fermentation. It seemed of interest to test whether lactic acid fermentation can provide the energy for the synthesis of glutamine. 30·7 mg. of brain cortex were shaken for 130 min. in bicarbonate saline solution (0·027 M bicarbonate, 0·02 M $l(+)$-glutamic acid, 0·002 M ammonium chloride, 0·002 M pyruvic acid, 5% CO_2 in N_2) under strictly anaerobic conditions (wet yellow phosphorus in the inner cup of the vessel). After 130 min. 16 μl. amide-nitrogen were found in the solution. This corresponded approximately to the preformed amide-nitrogen so that no synthesis of glutamine had occurred.

Retinae of pig and pigeon however synthesise glutamine under strictly anaerobic conditions (Table XIV). In pig retina the rate of synthesis is smaller

Table XIV. *Synthesis of glutamine in retina under anaerobic conditions.*

$M/50$ $l(+)$-glutamic acid; 0·4% glucose; $M/500$ pyruvate; $M/40$ bicarbonate; 5% CO_2 in the gas mixture; 3 ml. solution.

Animal	Tissue mg.	Conditions	Time mins.	Initial conc. of NH_3 M	Amide-N found μl.	$Q_{Amide\text{-}N}$
Pig	20·65	N_2, phosphorus	80	0·002	93·5	2·78
Pigeon	8·03	N_2	60	0·0022	68·0	8·5
,,	7·82	O_2	60	0·0022	88·0	11·2
Pigeon	10·29	N_2, phosphorus	40	0·0012	30·2	4·42
,,	9·00	O_2	40	0·0012	33·0	5·50
Pigeon	9·56	N_2, phosphorus	40	0·0018	33·5	5·25
,,	10·87	O_2	40	0·0018	46·2	6·35

anaerobically than aerobically whilst in pigeon retina no appreciable difference is found in oxygen and nitrogen. The respiration of pigeon retina is extremely small; in bicarbonate saline or serum there is no measurable respiration [Krebs, 1927] (for technical reasons) whilst in phosphate saline a respiration of 7·5 is found (Table XV). The glycolysis (Q_N) of pigeon retina amounts to about 150. Pigeon

Table XV. *Respiration of pigeon retina.*

Phosphate saline; 0·4% glucose; O_2; inner cup: 0·3 ml. NaOH, 38°.

Number	Q_{O_2}
1	7·50
2	6·88
3	7·82

retina obtains its energy preferentially from the anaerobic lactic acid fermentation and therefore it is not surprising that the synthesis of glutamine, as an energy-requiring reaction, does not depend on the presence of oxygen. Accordingly $10^{-3}M$ HCN does not inhibit the formation of glutamine in pigeon retina.

F. *Inhibition by* d(−)-*glutamic acid.* If *d*(−)-glutamic acid and ammonia are added to kidney, no glutamine is formed. Racemic glutamic acid gives about half the yield of glutamine and half the increase in respiration given by *l*(+)-glutamic acid under the same conditions (Table XVI). This halving effect

Table XVI. *Inhibition of glutamine synthesis by* d(−)-*glutamic acid.*

Guinea-pig kidney; phosphate saline; 37·5°.

Substrates added (final concentrations)	Q_{O_2}	Q_{NH_3}	$Q_{Amide\text{-}N}$
l(+)-Glutamic acid (*M*/80); NH_4Cl (*M*/300)	−31·2	−25·3	29·0
dl-Glutamic acid (*M*/40); NH_4Cl (*M*/300)	−21·0	− 7·3	12·1
NH_4Cl (*M*/300)	−13·0	—	0·5
l(+)-Glutamic acid (*M*/80); NH_4Cl (*M*/150)	−29·0	−16·1	25·8
dl-Glutamic acid (*M*/40); NH_4Cl (*M*/150)	−19·6	− 6·65	11·1
NH_4Cl (*M*/150)	−12·0	—	—
l(+)-Glutamic acid (*M*/50); NH_4Cl (*M*/370)	−44·1	−24·2	26·0
l(+)-Glutamic acid (*M*/50); *d*(−)-glutamic acid (*M*/50); NH_4Cl (*M*/370)	−31·3	−14·6	13·1
l(+)-Glutamic acid (*M*/150); *d*(−)-glutamic acid (*M*/150); NH_4Cl (*M*/370)	−33·4	−17·5	17·1
l(+)-Glutamic acid (*M*/150); *d*(−)-glutamic acid (*M*/50); NH_4Cl (*M*/370)	−29·5	− 8·9	7·6
l(+)-Glutamic acid (*M*/150); NH_4Cl (*M*/370)	−28·7	−16·0	15·4
NH_4Cl (*M*/370)	−11·5	+ 0·4	1·7
d(−)-Glutamic acid (*M*/150), NH_4Cl (*M*/370)	−12·5	+ 1·0	1·3

is due to specific inhibition of the glutamine-synthesising system by *d*(−)-glutamic acid. Addition of *d*(−)-glutamic acid to *l*(+)-glutamic acid has the same result as addition of *dl*-glutamic acid. The figures in Table XVI show that it is not the absolute concentration of *d*(−)-glutamic acid which determines the degree of inhibition but the ratio $\frac{\text{concentration of } l(+)\text{-glutamic acid}}{\text{concentration of } d(-)\text{-glutamic acid}}$. For instance *M*/150 *d*(−)-glutamic acid inhibits the amide-nitrogen formation by 50 % if the concentration of the antipode is *M*/150, but only by 34 % if the concentration of the antipode is *M*/50.

The inhibition by *d*(−)-glutamic acid can be explained by the assumption that both isomerides combine with the enzyme and that the affinities of the enzyme for both are equal; only *l*(+)-glutamic acid however reacts to form glutamine.

Neither α-ketoglutaric acid, *dl*- and *l*(−)-aspartic acid nor *dl*-alanine influences the rate of glutamine formation. A slight inhibition is found when synthetic *dl*-β-hydroxyglutamic acid is added (Table XVII).

Table XVII. *Inhibition of the glutamine synthesis by* dl-β-*hydroxyglutamic acid.*

Guinea-pig kidney, phosphate saline, 37·5°.

Substrates added (final concentrations)	Q_{O_2}	Q_{NH_3}	$Q_{Amide\text{-}N}$
l(+)-Glutamic acid (*M*/60), NH_4Cl (*M*/184)	28·9	−25·3	+26·5
l(+)-Glutamic acid (*M*/60), NH_4Cl (*M*/184) + *dl*-β-hydroxyglutamic acid (*M*/60)	26·7	−19·3	+16·9

G. *Bicarbonate and phosphate buffers.* Varying the concentration of bicarbonate between *M*/40 and *M*/270, or replacing bicarbonate with phosphate does

not essentially influence the rate of glutamine synthesis in guinea-pig kidney if the p_H is kept constant (Table XVIII).

Table XVIII. *Synthesis of glutamine in bicarbonate and phosphate buffers.*

Guinea-pig kidney; $M/50$ $l(+)$-glutamic acid; $M/150$ NH_4Cl; p_H 7·4.

Buffer	Q_{NH_3}	$Q_{Amide\text{-}N}$
Phosphate ($M/100$)	−24·8	+26·0
Bicarbonate ($M/40$); CO_2 (5 vol. %)	−18·5	+22·8
Bicarbonate ($M/270$); CO_2 (0·8 vol. %)	−19·0	+25·0

6. *Enzymic hydrolysis of glutamine into glutamic acid and ammonia.*

If glutamine is added to extracts of brain, retina or kidney, the reaction described in the previous sections is reversed and glutamine is split into glutamic acid and ammonia (Table XIX). This reaction is not dependent on the cell structure and can be conveniently investigated in aqueous tissue extracts.

Table XIX. *Activities of asparaginase and glutaminase in kidney and liver extracts of rabbit and guinea-pig.*

Each test-tube contains 4·5 ml. veronal buffer (p_H 7·7; $M/10$), 1·5 ml. water, 0·5 ml. $M/5$ substrate (asparagine or glutamine) and 1 ml. tissue extract (1 part fresh tissue ground extracted with 10 parts water, centrifuged and supernatant fluid used), 1 hour; 37·5°.

Tissue	Substrate	NH_3 found after 1 hour's incubation µl.	NH_3 formed (corrected for blank) µl.
Rabbit liver	Asparagine	331	312
"	Glutamine	92	73
"	—	19	—
Rabbit kidney	Asparagine	18	3
"	Glutamine	291	276
"	—	15	—
Guinea-pig liver	Asparagine	1520	1484
"	Glutamine	368	332
"	—	36	—
Guinea-pig kidney	Asparagine	95	51
"	Glutamine	225	181
"	—	44	—

Enzymic hydrolysis of glutamine was observed by Hunter and Geddes [1928] and by Grassmann and Mayr [1933] in yeast extracts (see also Luck [1924]) when they were studying the specificity of asparaginase (which hydrolyses asparagine to aspartic acid and ammonia). These authors reached no conclusion as to whether glutamine and asparagine are split by one and the same enzyme. It can be shown however that there is a specific "glutaminase" in some tissues: rabbit kidney splits glutamine rapidly but hydrolyses asparagine very slowly (Table XIX). Rabbit liver on the other hand splits glutamine about five times more slowly than asparagine (under the same conditions). The ratio $\frac{\text{activity of asparaginase}}{\text{activity of glutaminase}}$ is about 1/100 for rabbit kidney and about 4 for rabbit liver extracts. If one enzyme were responsible for the breakdown of both amides, the above ratio should be constant. It follows from these experiments that glutaminase is a specific enzyme.

When glutamine is converted into ammonia and glutamic acid apparent amino-nitrogen must disappear from the solution, since 90% of the total

nitrogen of glutamine reacts in Van Slyke's method (the reaction time being 5 min.). Hence the disappearance of amino-nitrogen as determined by Van Slyke's method should amount to 80% of the ammonia formed. The experimental result agrees with the predicted figure within the limits of error: 0·6 ml. *M*/10 glutamine was mixed with 0·9 ml. brain extract (rat brain extracted with 10 vols. water) and 0·15 ml. *M* sodium bicarbonate. Immediately after mixing 785 μl. amino-nitrogen were found in 0·5 ml. solution. After one hour's incubation (37·5°) 341 μl. ammonia were found in 0·5 ml. Another 0·5 ml. was mixed with 1 ml. borate buffer, p_H 9·5, and evaporated *in vacuo* to 0·5 ml. in order to remove ammonia. The amount of amino-nitrogen found in this solution was 480 μl. Thus 305 μl. amino-nitrogen had disappeared, whilst 341 μl. ammonia appeared. The loss of amino-nitrogen is somewhat higher than calculated (89% found, 80% calculated). This might be expected since slight decomposition of glutamine during the evaporation, necessary for the removal of ammonia, is unavoidable.

Glutamine, heated (100°) in neutral solution, yields pyrrolidonecarboxylic acid and ammonia [Chibnall and Westall, 1932]. In this case two amino-groups disappear for one equivalent of ammonia formed; thus enzymic splitting and neutral heat hydrolysis yield different products.

7. *Some properties of glutaminase.*

A. *Specificity.* As mentioned in the previous paragraph glutaminase does not hydrolyse asparagine. Phenacetylglutamine and benzoylglutamine, kindly supplied by Dr N. W. Pirie, are not attacked by kidney or brain extracts. Glutamine peptides (glutaminylglycine and glutaminylglutamic acid) which Prof. Chibnall kindly gave me did not yield ammonia in the presence of rat brain extracts. *iso*Glutamine (also from Prof. Chibnall) is slowly hydrolysed, the velocity being only a few % of the rate of splitting of glutamine (Table XX).

Table XX. *Action of rat brain extract on glutamine derivatives.*

Brain extract: one part brain ground with 10 parts *M*/10 $NaHCO_3$. Each test-tube contained 0·5 ml. extract and 0·3 ml. *M*/10 neutralised substrate solution. p_H 8·5. Blank controls for brain extract and for the substrates. Incubation 2 hours; 37·5°.

Substrate added	NH_3 formed (corrected for blank) μl.
Glutamine	590
*iso*Glutamine	35
Benzoylglutamine	0
Phenacetylglutamine	0
Glutaminylglycine	12
Glutaminylglutamic acid	0

B. *Inhibition by glutamic acid.* A peculiar phenomenon is observed when the course of hydrolysis of glutamine is followed in extracts of kidney, brain or retina. The hydrolysis starts with a high velocity (Table XXI, Fig. 3), but the rate soon falls off. Initially 156 μl. ammonia were formed per mg. dry kidney per hour, but when only one-sixth of the total amount of glutamine was split the velocity fell to one-third of the initial rate, and when half of the substrate was decomposed the velocity was about 6 μl. per mg. per hour, that is 3·8% of the initial rate. This decrease of activity is not due to destruction of the enzyme, since addition of fresh extracts does not restore the initial rate, but increases

Table XXI. *Course of action of glutaminase.*

5 ml. phosphate buffer; p_H 7·8; 1 ml. pig kidney extract (1 part minced kidney cortex extracted with 4 vols. of water, centrifuged; 3 ml. glutamine ($M/20$); 1 ml. water. Control tube water instead of glutamine solution; 1 drop octyl alcohol. From time to time 1 ml. or 0·5 ml. analysed; calculated for complete splitting; 338 μl. per 1 ml.

Time min.	NH_3 in 1 ml. μl.	Time min.	NH_3 in 1 ml. μl.
2·5	26·4	100	154
5	52	380	216
10	68	2880	313
20	96	5760	340
40	127		

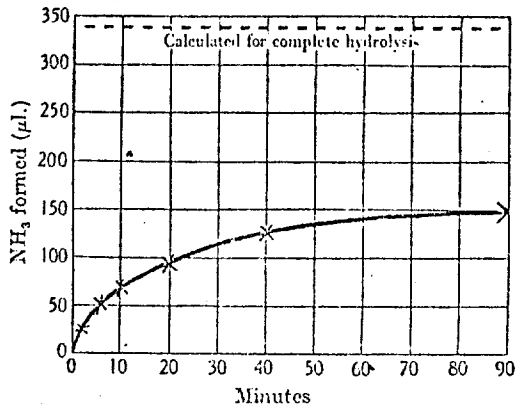

Fig. 3. The course of the hydrolysis of glutamine (pig kidney extract).

the rate only in proportion to the enzyme concentration. The diminishing activity is due to inhibition of glutaminase by the glutamic acid formed in the hydrolysis.

This inhibition of glutaminase by glutamic acid is shown directly in Table XXII. $2{\cdot}5 \times 10^{-2} M$ $l(+)$-glutamic acid produces 98 % inhibition of the

Table XXII. *Inhibition of glutaminase by glutamic acids.*

Each test-tube contains 2 ml. phosphate buffer (p_H 7·8), 0·5 ml. pig kidney extract (see Table XXI), 1 ml. glutamine solution (0·035 M) and 0·5 ml. of the solution given in the first column; control tube water instead of glutamine.

Solution added	NH_3 per test-tube after 40 min. incubation at 37·5° μl.	NH_3 formed (corrected for blank) μl.
Water	485	437
$l(+)$-Glutamic acid ($M/5$)	57	9
$d(-)$-Glutamic acid ($M/5$)	57	9
Control	48	—

splitting of $0{\cdot}87 \times 10^{-2} M$ glutamine. Both optical isomerides of glutamic acid are equally active as inhibitors. This inhibition is not due to thermodynamic equilibrium between glutamine and ammonium glutamate; the equilibrium of the reaction lies at practically complete hydrolysis of the amide (in physiological solutions). This is shown by the facts that the splitting of glutamine

goes to completion even in the presence of glutamic acid (though extremely slowly), and that no trace of glutamine is formed from glutamic acid and added ammonia in the presence of glutaminase.

The inhibition of glutaminase by glutamic acid could be explained however by a competition of glutamine and glutamic acid for the enzyme. Competitive inhibitions by compounds which are chemically similar to the substrate are known (*e.g.* in the cases of xanthine oxidase and of sucrase), but asparaginase is not inhibited by aspartic acid. It is remarkable that the inhibition of glutamic acid requires very small amounts of glutamic acid, indicating that the affinity of glutamic acid for the enzyme is much greater than that of glutamine. From the experiment given in Table XXI and Fig. 3, it may be calculated that the rate falls to 50 % of the initial rate when 18–20 % of the glutamine is hydrolysed. Hence the enzyme is equally distributed between glutamine and glutamic acid if the ratio of concentrations of glutamine to glutamic acid is 4 to 1.

The inhibitory action of glutamic acid is specific except that *dl*-β-hydroxyglutamic acid causes a slight inhibition (Table XXIII). Other amino-acids, or glutathione or α-ketoglutaric acid have no effect. The fact that *d*(−)-glutamic acid and *dl*-β-hydroxyglutamic acid inhibit glutamine synthesis and

Table XXIII. *Inhibition of glutaminase by* dl-*β-hydroxyglutamic acid.*

Each test-tube contained 0·5 ml. veronal buffer, 0·5 ml. *M*/10 glutamine, and 1 ml. brain extract (rat brain extracted with 10 parts water; 37·5°; 30 min.).

Solution added	NH_3 formed (μl.)
0·5 ml. water	144
0·5 ml. *M*/5 *dl*-β-hydroxyglutamic acid	112

glutamine hydrolysis to the same extent is evidence in favour of the identity of the enzymes concerned with the synthesis and the hydrolysis.

C. *Dry enzyme preparations.* To minced pig kidney 5 vols. of acetone were added. Extracts of the dried precipitate hydrolysed glutamine, though more slowly than extracts of fresh material. The activity was about the same whether the dry powder was extracted with water or with *M*/10 sodium bicarbonate. Acid extraction (1 % acetic acid) gave less active enzyme solutions (Table XXIV).

Table XXIV. *Glutaminase in acetone preparations of pig kidney cortex.*

0·5 g. powder extracted for 15 min. with 10 ml. solution;
1 ml. extract + 1 ml. *M*/10 glutamine + 0·2 ml. $NaHCO_3$ (*M*); p_H 8·5.

Solution used for extraction	NH_3 formed in 30 min.; 37·5° (corrected for blank) μl.
Water	33·7
M/10 $NaHCO_3$	36·8
M/60 acetic acid	23·7

Table XXV. *Influence of enzyme concentration.*

Guinea-pig brain extracted with 10 parts water; *M*/75 glutamine; bicarbonate buffer; p_H 8·5; total volume 3·2 ml.

Volume of brain extract ml.	NH_3 formed in 30 min.; 37·5° μl.
2·0	79
1·0	45
0·5	20
0·25	14

D. *Enzyme concentration.* The initial velocities are approximately proportional to the enzyme concentration as shown in Table XXV.

E. *Influence of glutamine concentration.* The inhibition by glutamic acid—a combination between catalyst and a product of the catalysis—complicates the kinetics of glutaminase. It is not proposed to analyse the kinetics exhaustively in this paper, but a few figures will be given showing the activity of glutaminase under different conditions. Table XXVI shows the influence of glutamine con-

Table XXVI. *Influence of glutamine concentration.*

Guinea-pig brain extracted with 10 parts water; 2 ml. extract + 0·3 ml. *M* $NaHCO_3$ + 2 ml. glutamine solution in each tube; p_H 8·5; 37·5°.

Final concentration of glutamine *M*	NH_3 formed in 30 min. µl.
0·02	188·0
0·01	69·5
0·005	20·5

Table XXVII. p_H *curve of brain, kidney and liver glutaminase.*

Buffer final *M* concentrations				NH_3 formed (corrected for blank) µl.		
Na_2CO_3	$NaHCO_3$	Vol. % CO_2 in gas	p_H	Brain	Kidney	Liver
0·09	0·01	—	10·8	38·5	53	10
0·05	0·05	—	9·8	134·0	215	21
0·01	0·09	—	8·8	525·0	288	72
—	0·1	5	8·1	230·0	312	75
—	0·025	5	7·5	32·0	190	218
—	0·005	5	6·8	10·0	145	191
—	0·005	20	6·2	4·0	116	26

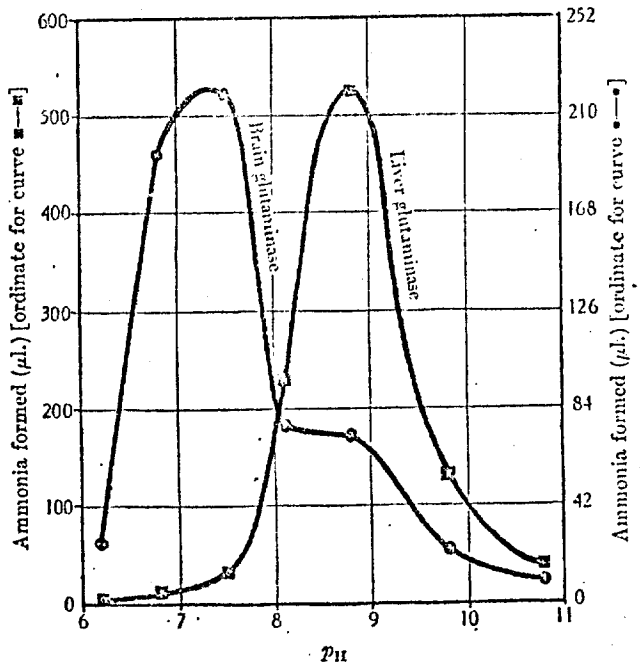

Fig. 4. p_H curve of brain and liver glutaminase.

centration on the initial velocity of hydrolysis. As expected from the preceding experiments an increase of the concentration of glutamine increases the velocity of the hydrolysis considerably.

F. *Influence of* p_H. 3 g. guinea-pig brain were ground with sand and extracted with 24 ml. water. 1 ml. of the centrifuged extract was mixed with 0·8 ml. $M/10$ glutamine solution and 0·2 ml. buffer. The flasks were incubated for 40 min. at 37·5°; ammonia was then determined. Blanks were carried out to measure the ammonia formation in the absence of added glutamine. The blank was found to be independent of p_H; it amounted to 11 μl. NH_3 per flask. The results are given in Table XXVII and Fig. 4. Similar experiments were carried out with kidney and liver extracts. Sheep kidney was extracted with 4 vols. of water. The liver extract was prepared by extracting guinea-pig liver with five parts of water. The results are also recorded in Table XXVII.

Owing to the complications in the kinetics before mentioned the curves in Fig. 4 give only an approximate picture of the influence of p_H on glutaminase. The optimum of brain glutaminase lies between p_H 8 and 9. The curves for kidney, liver and brain glutaminase are not identical. In the next paragraph further evidence will be adduced showing that the splitting of glutamine in various tissues is due to different "glutaminases".

G. *Glutaminase in various tissues. Existence of different glutaminases.* Glutaminase is found in those tissues in which the synthesis of glutamine occurs, but it is also found in some tissues which do not synthesise glutamine, for instance in spleen and liver of the guinea-pig and in pig kidney. Table XXVIII shows the relative activities of extracts from various tissues.

Table XXVIII. *Glutaminase in various tissues. Influence of glutamic acid.*

Veronal buffer; p_H 8·5; 37·5°.

Tissue	Water used for extract parts	Vol. of extract used ml.	Total vol. of fluid ml.	M conc. of glutamine	M conc. of $l(+)$-glutamic acid	Time min.	NH_3 formed (corrected for blank) μl.
Pig retina	30	1	2·2	0·0023	0	120	225
"	"	"	"	"	0·046	"	~0
Pig liver	4	1	2·5	0·02	0	30	238
"	"	"	"	"	0·04	"	238
Pig kidney	4	1	2·5	0·02	0	30	91
"	"	"	"	"	0·04	"	18
Guinea-pig spleen	12	1	2·5	0·02	0	60	27
"	"	"	"	"	0·04	"	3
Guinea-pig liver	5	1	2·5	0·02	0	60	740
"	"	"	"	"	0·04	"	740
Guinea-pig lung	5	1	2·5	0·02	0	60	68·5
"	"	"	"	"	0·04	"	3
Guinea-pig muscle	5	1	2·5	0·02	0	60	8
"	"	"	"	"	0·04	"	6
Guinea-pig testicle	5	1	2·5	0·02	0	60	0
Guinea-pig blood (laked)	3	3	7·0	0·02	0	120	27
" "	"	"	"	"	0·04	"	31
Rat brain	5	1	2·5	0·02	0	60	186
"	"	"	"	"	0·04	"	57
Rat liver	4	1	2·5	0·02	0	60	173
"	"	"	"	"	0·04	"	153
Rat muscle	8	2	4·0	0·02	0	140	7
"	"	"	"	"	0·04	140	7

The most characteristic property of glutaminase found so far is the inhibition of the enzyme by glutamic acid. But this inhibition is not found in all tissue extracts in which a glutaminase is found. Guinea-pig liver for instance hydrolyses glutamine rapidly, but glutamic acid does not inhibit the reaction (Table XXVIII). This proves again (see the preceding paragraph) that the glutamine-splitting enzyme in guinea-pig liver is different from the enzyme in kidney, brain or retina. There are at least two types of glutaminase distinguishable by their p_H optima and their inhibitions by glutamic acid ("brain type" and "liver type"). Some tissues, for instance rat kidney, seem to contain both types of glutaminase, since the splitting of glutamine is partly inhibited by glutamic acid and the p_H curves show two maxima.

The second glutaminase ("liver type"), too, is different from asparaginase. Guinea-pig liver splits both amides, asparagine and glutamine, and the rates of splitting are of the same order of magnitude. But blood serum from guinea-pig does not attack glutamine whereas it splits asparagine rapidly [Clementi, 1922]: 1 ml. serum hydrolyses up to 30 mg. asparagine per hour (p_H 7·4, 37·5°). Asparaginase is thus found in nature separately from the enzymes which split glutamine. This proves that asparaginase is not concerned in the splitting of glutamine.

8. *Glutamine as precursor of ammonia in tissues.*

Most animal tissues are capable of forming ammonia by anaerobic reactions when incubated at body temperature. The rate of ammonia formation is often much greater after destruction of the cells. Thus red blood corpuscles of birds form hardly any ammonia as long as the cells are intact and the medium contains oxygen and sugar; but laked cells, or cells which have been deprived of their substrates for energy-giving reactions, form ammonia. A difference between intact and ground tissues as regards ammonia formation has been demonstrated for muscle by Embden *et al.* [1928] and by Parnas and Mozołowsky [1927], for brain by Schwarz and Diebold [1932] and by Riebeling [1934]. I find it also in kidney, testicle and intestinal wall.

The precursors of the ammonia derived from anaerobic sources in tissues are not all known. In some tissues, adenylic acid seems to be the chief precursor. In blood (see Klisiecki and Heller [1935]), brain and other tissues the amount of adenylic acid is too small to account for the total ammonia formed.

Other possible sources of ammonia formed anaerobically are asparagine and glutamine. I have used the inhibitory effect of glutamic acid on glutaminase to decide whether some of the ammonia formed in mashed tissue is due to the action of glutaminase ("brain type"). Brain, retina and kidney (pig and guinea-pig) were divided into two equal fractions; the first fraction was ground with water, the second with $M/50$ $l(+)$-glutamic acid. The same amount of ammonia was found however in each fraction after incubating the tissues and the extracts at 38° at p_H 8·5 or 7·4. Hence we may conclude that glutaminase ("brain type") is not concerned with spontaneous ammonia formation in the tissues examined.

9. *Effect of* l(+)*-glutamic acid on the formation of ammonia in brain and retina.*

Slices of brain tissue or retina, when suspended in saline, produce considerable amounts of ammonia (for references see Dickens and Greville [1933]. $l(+)$-glutamic acid reduces the output of ammonia in brain and retina as shown in

Table XXIX. In some experiments no ammonia at all could be detected when $l(+)$-glutamic acid was present; but instead of ammonia amide-nitrogen was found. The sum of ammonia *plus* amide-nitrogen is approximately the same in

Table XXIX. *Formation of ammonia and amide-nitrogen in brain and retina in the presence of* l(+)-*glutamic acid.*

Tissue	Substrates added	Q_{NH_3}	$Q_{Amide\text{-}N}$	$Q_{NH_3}+Q_{Amide\text{-}N}$
Guinea-pig brain	—	1·68	0·71	2·39
"	$M/50$ $l(+)$-glutamic acid	~0	2·67	2·67
Guinea-pig brain	—	1·62	0·25	1·87
"	$M/50$ $l(+)$-glutamic acid	0·21	1·48	1·69
Guinea-pig brain	$M/70$ glucose	0·19	0·44	0·63
"	$M/70$ glucose + $M/50$ $l(+)$-glutamic acid	0·02	1·03	1·05
Rabbit brain	—	1·52	0·67	2·19
"	$M/100$ $l(+)$-glutamic acid	0·83	1·63	2·46
Cat brain	—	1·51	~0	1·51
"	$M/50$ $l(+)$-glutamic acid	0·70	0·76	1·46
Pig retina	—	0·63	0·18	0·81
"	$M/50$ $l(+)$-glutamic acid	~0	1·34	1·34
Pig retina	—	1·20	0·59	1·79
"	$M/50$ $l(+)$-glutamic acid	0·35	1·46	1·81
"	$M/70$ glucose	0·99	0·86	1·85
"	$M/70$ glucose – $M/50$ $l(+)$-glutamic acid	~0	1·12	1·12
Ox retina	—	1·25	0·33	1·58
"	$M/50$ $l(+)$-glutamic acid	0·14	0·77	0·91
"	$M/90$ glucose	0·43	0·33	0·76
"	$M/90$ glucose – $M/50$ $l(+)$-glutamic acid	0·28	0·67	0·95

the presence as in the absence of $l(+)$-glutamic acid. Thus whilst glutamic acid causes a change in the ratio $\frac{\text{ammonia}}{\text{amide-nitrogen}}$ it does not increase the sum ammonia *plus* amide-nitrogen. This is remarkable seeing that glutamic acid causes an increase in the oxygen uptake of brain and retina [Krebs, 1935].

The non-natural $d(-)$-glutamic acid does not inhibit the formation of ammonia in brain. This shows that the "inhibition" of ammonia formation by $l(+)$-glutamic acid is not due to inhibition of glutaminase, since glutaminase is equally affected by the stereoisomerides. The "inhibition" must be explained by the secondary disappearance of the ammonia through the synthesis of glutamine.

Warburg *et al.* [1924] and Dickens and Greville [1933] have found that slices of kidney, brain, and retina form less ammonia in the presence of glucose than in glucose-free saline. But glucose, unlike glutamic, acid does not increase the yield of amide-nitrogen (see the last experiment in Table XXIX).

10. *The fate of glutamine in brain and retina.*

When the amount of amide-nitrogen which is formed in brain or retina is compared with the amount of ammonia which has disappeared (see Table VIII) it will be seen that, in some cases, more ammonia is used than amide-nitrogen is found. The deficit is very considerable in some experiments on retina. Two explanations for this deficit suggest themselves: either ammonia may be used for another reaction, or a part of the glutamine formed may disappear by a secondary reaction. The following experiments favour the second explanation.

Retina was suspended in glutamine-containing saline at p_H 7·4. After incubating for one or two hours the tissue was well washed and the washings were

added to the experimental solution. Ammonia and amide-nitrogen were then determined in the solution. Only a small amount of ammonia was found, hardly more than in controls to which no glutamine was added. The amide-nitrogen on the other hand was considerably decreased (Table XXX). The sum of ammonia

Table XXX. *Disappearance of glutamine from retina.*

For each experiment 3 ml. bicarbonate saline, 0·4% glucose, 5% CO_2 in O_2; 35·0°. The deficit shown in the last column has not been corrected for blanks and therefore represents a minimum value.

Animal	Tissue mg.	Glutamine added µl.	Time min.	NH_3 formed µl.	Amide-N found µl.	Deficit µl.
Sheep	33·95	162	100	3·5	122	36·5
"	24·48	610	120	22·0	544	44·0
"	16·54	212	120	29·0	132	51·0
"	30·0	308	120	16·5	154	137·0
Pigeon	7·0	141	120	12·0	96	33·0

and amide-nitrogen as compared with the added glutamine shows a marked deficit. These results indicate that the tissue utilises glutamine but not by splitting it into glutamic acid and ammonia. The nature of the products of the conversion of glutamine remains to be investigated.

11. *Some experimental details.*

The methods used in this paper—tissue slices, manometric procedure, determination of ammonia—were essentially the same as in the preceding paper [Krebs, 1935]. Certain additional details are given in this section.

Glutamine was prepared according to Schultze and Winterstein from sugar beet or mangold wurzels [see Vickery *et al.*, 1935]. In the beginning of this investigation, I had at my disposal a large sample prepared by Dr N. W. Pirie.

Preparation of dl-*glutamic acid.* 50 g. of *l*(+)-glutamic acid were heated in an oil-bath for 50 min. at 170–180° and for 50 min. at 220°. The *dl*-pyrrolidonecarboxylic acid formed was hydrolysed with 3 times its weight of concentrated hydrochloric acid for 4 hours at boiling temperature. The *dl*-glutamic acid was then isolated in the usual way. Yield 17 g. recrystallised *dl*-glutamic acid. $[\alpha]_D$ +1·84° (in 2*N* HCl); amino-N 9·85%. This procedure is the method of Abderhalden and Kautsch [1910], except for the time of heating. The racemisation was very incomplete when the directions of Abderhalden and Kautsch were followed.

Preparation of d(−)-*glutamic acid.* 10 g. *dl*-glutamic acid were fermented with yeast according to Ehrlich [1914]. Yield 1·6 g. recrystallised *d*(−)-glutamic acid. $[\alpha]_D$ −34·6° (0·1936 g. in 20 ml. 2*N* HCl). Amino-N 9·43%.

Solutions. "Phosphate saline" and "bicarbonate saline" were made up as described previously [Krebs, 1933, 1]; 2–4 ml. were used for each flask. All substrate solutions which were added to the experimental fluid were neutralised with sodium hydroxide before they were added; ammonia was added as ammonium chloride.

Determination of amide-nitrogen (glutamine). The free ammonia was removed and determined in the apparatus of Parnas and Heller. The ammonia-free solution was transferred to a test-tube, neutralised with 14*N* sulphuric acid and mixed with one-tenth volume of 30% trichloroacetic acid. The volume of the fluid was now measured. The solution was filtered and an aliquot part of the filtrate was acidified with one-tenth of its volume of 50% sulphuric acid, heated for 5 min. in a boiling water-bath (see Table VI), cooled and made alkaline with 50% sodium hydroxide (thymolphthalein). Ammonia was then determined in Parnas's apparatus. Known amounts of glutamine were recovered by this method with an accuracy of 1–2%.

Blanks. Since glutamine solutions decompose spontaneously (though at a very slow rate), blanks were carried out in all experiments on the enzymic hydrolysis of glutamine to measure the spontaneous decomposition. Between p_H 7 and 9 about 0·2–0·4% of the glutamine dissolved

decomposed in 1 hour at 38° as determined by ammonia formation. This figure includes the hydrolysis which may occur during the determination of ammonia.

Tissue extracts were made by grinding the tissue with sand and water and centrifuging. The supernatant fluid was used as "extract".

Units. In order to make the metabolic changes comparable with respiration all substances are expressed in terms of gas volumes. 17 mg. NH_3, or 14 mg. amide-N, or 14 mg. amino-N are taken as equivalent to 22400 μl. at N.T.P.

12. *Discussion.*

A. *Reversibility of the glutamine synthesis.* The synthesis of glutamine in tissues can be reversed *in vitro*, for instance by changing the p_H. However, two facts indicate that the synthesis of glutamine is practically not reversed in the living cell. (1) The hydrolysis occurs in practice only outside the physiological range of p_H. (2) Glutamine disappears from brain or retina without forming ammonia. Thus there appears to be a cycle of ammonia in nervous tissue in which the conversion of ammonium glutamate into glutamine is one step. Nothing is known about the other stages.

B. *Physiological significance of glutaminase in brain and retina.* In a previous section (7 B) it has been shown that one and the same enzyme is probably concerned in the synthesis and the hydrolysis of glutamine in kidney, brain and retina. In the preceding paragraph it is suggested that the glutaminase found in extracts of these tissues is concerned *in vivo* with the synthesis only. It may well be that several hydrolysing enzymes found in tissue extracts are only components of synthesising systems. For instance hippuricase (histozym), or proteolytic and lipolytic enzymes are found in extracts of those tissues known to perform the respective syntheses *in vivo*.

C. *Glutamine synthesis and energy-giving reactions.* If the energy-giving reactions are inhibited (section 5 E) synthesis of glutamine ceases. This makes it evident that the system which synthesises glutamine consists of glutaminase and of an additional factor concerned with the transmission of energy. The transmission of energy results in a change in the thermodynamic equilibrium between ammonium glutamate and glutamine in favour of the latter. If this occurs the enzyme catalyses the attainment of the new equilibrium.

D. *Significance of the "glutamine system".* Nothing definite is known at present about the physiological function of the glutamine synthesis. Certain experiments suggest a connection between the system and the energy-giving reactions; *l*(+)-glutamic acid is the only amino-acid which increases respiration in brain and retina; unlike other amino-acids it inhibits anaerobic lactic acid fermentation. These experiments will be described in a later paper.

Summary.

1. Brain cortex and retina of vertebrates and kidney of rabbit and guinea-pig convert ammonium glutamate into glutamine. Under optimum conditions, kidney synthesises 10–20 % of its dry weight of glutamine per hour, retina 5–7 %, brain cortex 1–2 % (37·5°).

2. The synthesis of glutamine is an endothermic reaction and therefore depends on energy-giving reactions. Respiration supplies the energy in kidney and brain. In retina the energy can be derived from anaerobic lactic acid fermentation.

3. Extracts from those tissues which synthesise glutamine contain a specific enzyme which hydrolyses glutamine to ammonium glutamate ("glutaminase").

4. The synthesis of glutamine and the hydrolysis of glutamine are specifically inhibited by the non-natural $d(-)$-glutamic acid.

5. Some properties of glutaminase are described. Characteristic is the specific inhibition by glutamic acid.

6. Liver of mammals (pig, guinea-pig, rat) contains a glutamine-splitting enzyme which is not inhibited by glutamic acid and shows a p_H optimum different from the optimum of glutaminase from brain, kidney and retina. Both glutaminases ("brain type" and "liver type") are distinct from asparaginase.

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I wish to express my thanks to Sir F. G. Hopkins for his interest and help; to Prof. Chibnall for samples of *iso*glutamine and glutamine peptides; to Prof. Harington for a sample of *dl*-β-hydroxyglutamic acid; to Dr Pirie for samples of glutamine, benzoylglutamine and phenacetylglutamine, and to Mr V. H. Booth for his help in preparing the manuscript.

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Glutamate and γ-Aminobutyric Acid in Brain

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Evidence is accumulating that, in the brain, the amino-acids L-glutamate and γ-aminobutyric acid (GABA) are essential agents of communication and not just elements of an alternate metabolic pathway, as was generally believed for some years.

It has long been known that slices of brain can metabolize L-glutamate[1] transforming it, in a process which involves transamination and oxidation, to α-oxoglutarate (Fig. 1)[1,2]. The addition of glutamate thus increases the oxygen consumption of the brain. Slices of cerebral cortex can absorb large amounts of glutamate from incubating fluid, and a great deal of K^+ is taken up at the same time[3-6]. Although glutamate stimulates cerebral metabolism, glucose is the principal source of energy utilized by the brain: glutamate therefore seemed to serve some unknown function in the transport of K^+ (ref. 4).

Like other nerve cells, cerebral neurones are normally rich in K^+ and poor in Na^+. But the inward flux of Na^+ and outward flux of K^+ associated with depolarization and spike activity tend to reduce the difference between the internal and external concentrations of these ions. The normal steep gradients of concentration across the membrane are restored by an Na-K pump, which, through the expenditure of energy, can extrude Na^+ from the cell in exchange for external K^+ (refs. 7, 8). The high content of K^+ in the brain is thus a function of the state of polarization of the cells and can only be maintained by metabolism. It is therefore significant that glutamate has a powerful depolarizing action on cerebral neurones.

Excitation by Glutamate

The excitatory action of glutamate was first apparent in Hayashi's experiments[9] on the cerebral cortex. The effect is so strong and so rapid that it may not be recognized as excitation. Gross applications of glutamate to the brain surface give rise to "spreading depression"—a slowly moving wave of intense neuronal depolarization and inactivation—and so abolish all spontaneous and evoked activity[10].

Excitation is clearest when glutamate is released within the brain in minute amounts (10^{-13} equiv/s), by iontophoresis from a micropipette (Fig. 2*A* and *B*)[11,12]. The evoked discharge, which is typically rapid in onset and stops sharply when the release ends, is associated with depolarization of the neuronal membrane[11,13,14]. Most central neurones have this excitatory effect, but other cells (neuroglia) are not depolarized by glutamate[14]. The fact that neurones in spinal ganglia are also not depolarized by glutamate[15] suggests that the sensitivity to glutamate depends on the presence of synaptic contacts.

To excite neurones, glutamate must somehow trigger the entry of Na^+. Like acetylcholine at the muscle end-plate, it may do this by increasing the general permeability to cations. Alternatively, glutamate may activate a specific Na^+-carrier, similar to that which generates the nerve action potential[16]. There is some evidence against this, however. Tetrodotoxin, a specific inactivator of the Na^+-carrier[17], does not block very effectively the influx of Na^+ caused by glutamate[18].

Both glutamate and Na^+ are perhaps transported by the same carrier (Fig. 3), as in the mechanism suggested for amino-acid uptake in the gut[19]. In such a scheme, glutamate could be removed from its site of action without the need for a special glutamate pump, the energy being supplied by the electrochemical inward gradient of Na^+, maintained by the Na-K pump. It is assumed that the common carrier has separate sites for combination with

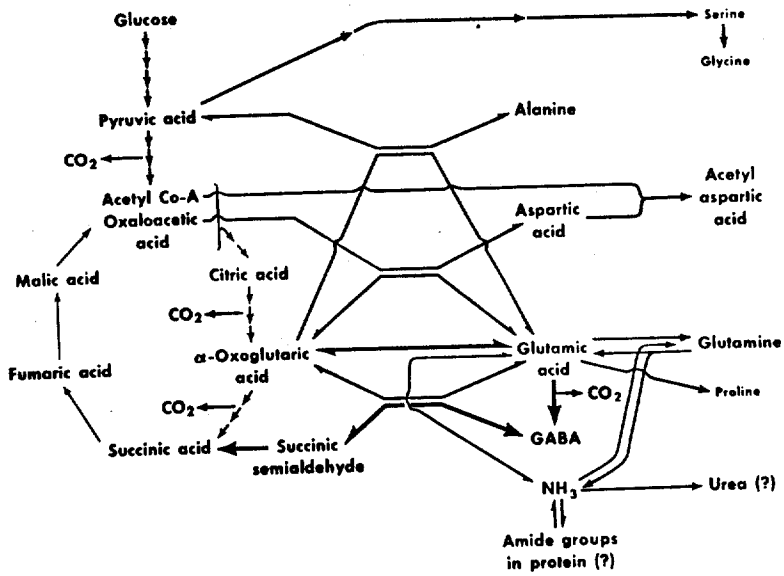

Fig. 1. Glutamate, GABA, and glucose metabolism in the brain (from ref. 90).

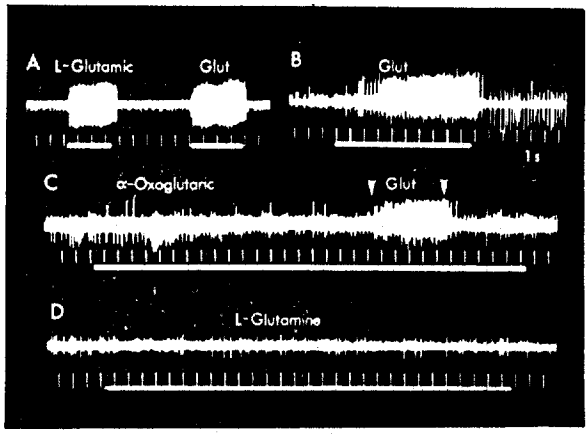

Fig. 2. Excitatory action of L-glutamate applied to cortical neurones by microiontophoresis (60–70 nA), at times indicated by white lines below oscilloscope traces. (*C*) α-Oxoglutarate (170 nA) had no effect and did not prevent action of glutamate applied between arrows. (*D*) Application of L-glutamine (100 nA) for a long time was also ineffective.

Na^+ and glutamate; that through a conformational change in the carrier, the affinity for Na^+ is much increased by the attachment of glutamate; and that the affinity for glutamate is itself a function of the Na^+ concentration. Thus external glutamate would promote the entry of Na^+, while being itself transported into the Na^+-poor cytoplasm, and the membrane would depolarize if this influx of Na cations exceeds that of glutamate anions. This hypothesis is given some support by the marked dependence of glutamate uptake on the presence of external Na^+ (ref. 5).

Glutamate excites so much more powerfully than much stronger chelators that its action cannot be ascribed principally to the binding of Ca^{2+} (refs. 12, 20).

Glutamate and Accumulation of Potassium Ions

Whatever its precise mechanism, the depolarizing effect of glutamate is probably caused by an influx of Na^+, and it should be followed by an efflux of K^+. What, then, is the reason for the observed increase in the K^+ content of brain slices?

An important additional observation is that glutamate causes a comparable increase in water content[4,21]. Because the cells swell, the internal concentration of K^+ remains approximately constant[21]. The complete sequence of events has been described by Harvey and McIlwain[8]. Within a minute of adding glutamate to brain slices, there is an increase in the Na^+ and a decrease in the K^+ contents of the "non-inulin" compartment (a useful index of the volume of intracellular water because bulky inulin remains outside the cells). At this stage there is no detectable movement of water. Thus the initial fluxes of Na^+ (in) and K^+ (out) can be explained as a direct consequence of the depolarizing action of glutamate.

In the next phase, the influx of Na^+ continues, but is accompanied by an influx of K^+, and there is an increase in intracellular water. These phenomena presumably follow the activation of the Na-K pump by the rise in internal Na^+. This active uptake of K^+ leads to a depletion of the high-energy phosphate compounds that provide the immediate energy requirements of the Na-K pump[22]. In spite of the continued rapid influx of Na^+ and K^+, a significant increase in internal osmolarity is prevented by the inflow of water. Glutamate causes similar changes in the Na^+, K^+ and water content of the isolated retina[4,23].

Glutamate as a Transmitter

The brain contains plenty of glutamate (about 10 μmole/g), more than its content of any other amino-acid[24,25]. This fact has perhaps militated against the idea that glutamate might be a transmitter—that is, a substance released by nerve endings at synaptic contacts to excite or inhibit other neurones. Neurotransmitters are often believed to be very scarce substances, difficult to extract or identify, and probably highly labile. Glutamate is the reverse of all these. Not only is it very abundant, but it is highly active in brain metabolism: if radioactive pyruvate, acetate or glucose is injected into an animal, labelled carbon is very rapidly incorporated into cerebral glutamate (Fig. 4)[26–31].

The general abundance of glutamate is not sufficient evidence of a transmitter function. The presence of adequate amounts of glutamate in excitatory nerve endings would be more significant. In an attempt to throw some light on this point, Whittaker and I[32] examined the excitatory properties of the contents of cortical nerve endings. Gray and Whittaker[33] had shown that nerve endings separate from nerve fibres when the cortex is homogenized and then centrifuged in a suitable density gradient. Suspensions of such isolated nerve endings were inserted into micropipettes and their contents released close to nerve cells in the cortex. Definite excitatory effects were observed, which could be ascribed to the glutamate content of the endings measured in separate experiments by Mangan and Whittaker[34]. Enough glutamate must therefore be present, at least in some nerve endings, for a significant synaptic action. Cerebral glutamate in general seems to be widely distributed, presumably in neuronal cytoplasm[35–37], but intracellular glutamate probably does not directly influence neuronal activity: unlike extracellular applications, intracellular injections of glutamate do not cause excitation[38].

The glutamate sequestered in isolated nerve endings forms *in situ* an active and readily accessible "pool". For example, it is rapidly labelled by systemic injections of

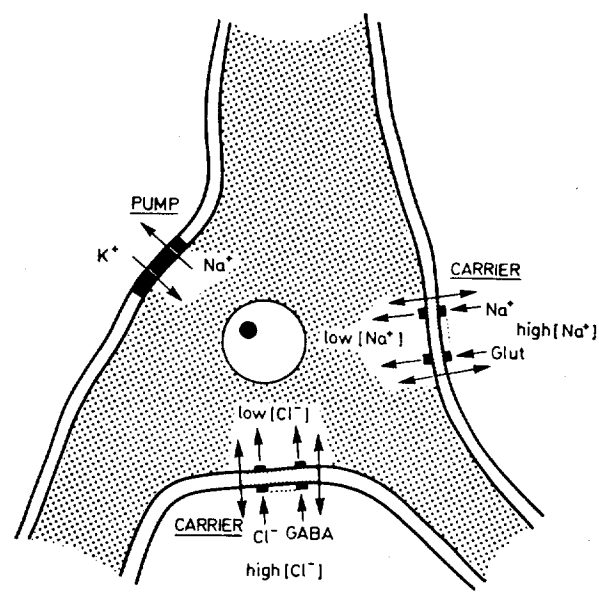

Fig. 3. Hypothetical model of glutamate and GABA carriers. Combination of glutamate with carrier on surface of cell promotes entry of Na^+, while combination of GABA with its carrier accelerates movement of Cl^- (see text).

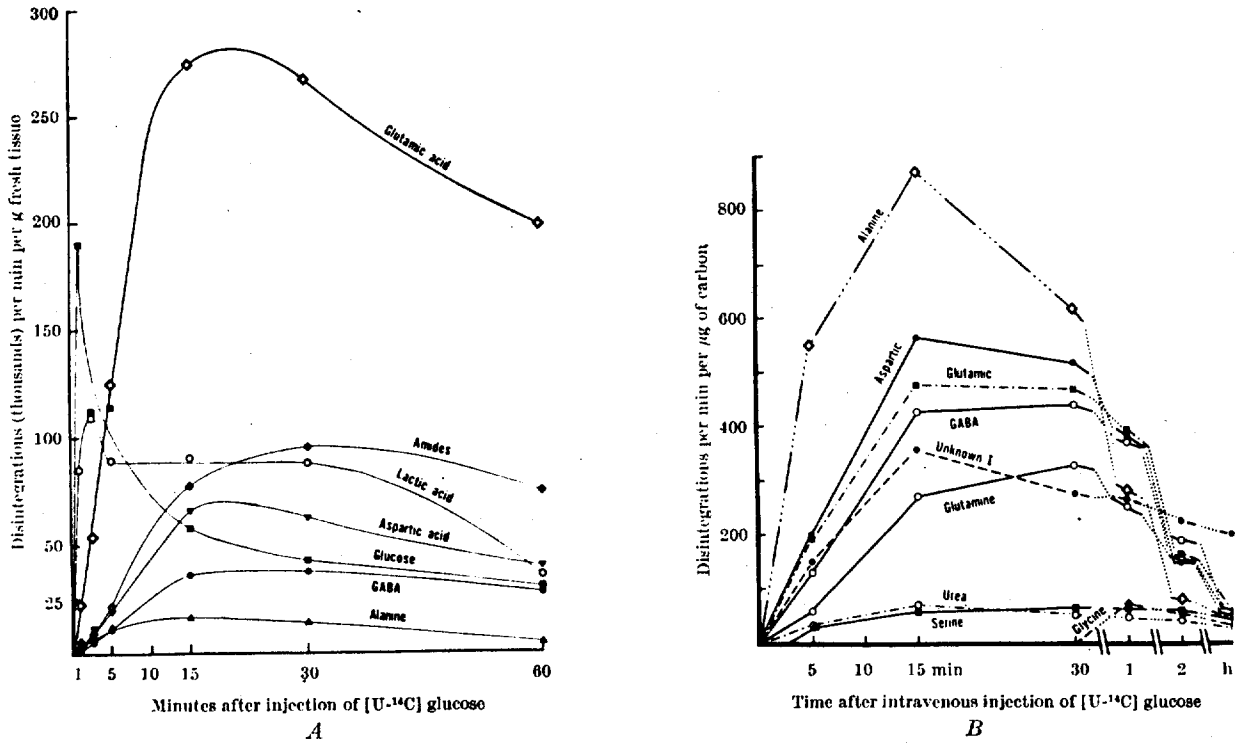

Fig. 4. Radioactive labelling of glutamate, GABA and other amino-acids in rat brain after intravenous injection of U-^{14}C-glucose. (*A*) Total radioactivity, (*B*) specific activity of ninhydrin-positive material (from ref. 31).

radioactive glucose[39]. And much of the uptake of glutamate into cortical slices finds its way into nerve endings[37]. By efficient synthesis and recapture, nerve endings evidently could supply the variable quantities of glutamate needed for synaptic release, without maintaining a large store.

Further evidence is required to confirm a possible transmitter function of glutamate: for example, that glutamate is released by excitatory nerve fibres. All that is known so far is that glutamate is continually being released at the surface of the brain[40]. The rate of release varies with cortical electrical activity, and it can be increased by stimulating the brain stem reticular formation. According to recent experiments[41], suspensions of isolated nerve endings selectively release glutamate and aspartate when they are stimulated electrically or with solutions rich in K^+. A serious problem is that re-uptake must prevent the escape of all but a small fraction of any glutamate released. If the process of uptake is driven by the Na^+ gradient, it will be difficult to block without abolishing all neuronal activity—except possibly by competitive inhibition.

Better evidence is needed to prove the identity between the action of glutamate and that of the natural excitatory transmitter. Some formidable technical problems are also likely to impede rapid progress in this direction. The reversal level of excitatory synaptic potentials is far removed from the resting potential, and therefore not easily attained with the small currents that can be carried by fine microelectrodes. Furthermore, many excitatory synapses may be situated remotely on the dendrites of pyramidal cells[42]; the corresponding synaptic events would give only small conductance changes and poorly defined reversal levels at the probable site of recording in the cell body. Finally, there are still no specific pharmacological antagonists of either excitation or of glutamate action.

Derivatives of Glutamate

L-Aspartic acid, the other dicarboxylic amino-acid found in the brain, has a strong excitatory action, comparable with that of L-glutamate[12,43]. But the longer-chain compounds, amino-pimelic and amino-adipic acids, are quite ineffective. The excitatory action depends rather critically on the presence of one amino and two acid groups: thus α-oxoglutaric acid (Fig. 2*C*), glutamine (Fig. 2*B*) and acetyl-aspartic acid are also practically ineffective; and the α-decarboxylation products of glutamate or aspartate (γ-aminobutyric acid or β-alanine) are powerful inhibitors of neuronal discharge discussed later. But a carboxyl group can be substituted with a sulphonic group; the resulting compounds are even more potent excitants than glutamate[12,43].

γ-Aminobutyric Acid and Inhibition

In contrast to glutamate, GABA was identified as an important brain constituent only relatively recently[44,45], probably because it is not found in any significant amount in other tissues, and its presence was therefore unexpected. Its functional role as a possible inhibitory transmitter became apparent when Elliott and his collaborators[46,47] identified GABA as the active agent in brain extracts which had a unique inhibitory action on crayfish stretch-receptors[48,49].

The powerful inhibitory effect of GABA on crustacean stretch-receptors and muscle was later shown to be indistinguishable from synaptic inhibition[50,51]. With the further demonstration that GABA is selectively released by stimulating inhibitory nerve fibres[52], it became probable that GABA is an inhibitory transmitter in crustaceans.

Hayashi[9] first noted that GABA and some other omega monocarboxylic amino-acids could inhibit mammalian cortical neurones. A depressant action was also observed by Purpura *et al.*[53] and Curtis and his collaborators[43,54], but neither group believed that GABA could be responsible

for the natural inhibition of central neurones. But further tests on the cerebral and cerebellar cortex[12] revealed such a powerful inhibitory effect (Fig. 5*A*) that GABA could not be ignored as a possible inhibitory transmitter. Later, intracellular recording and tests of membrane resistance[55,56] demonstrated a remarkable similarity between the effects produced by synaptic inhibition and by GABA. In both cases, there was a hyperpolarization—with a similar reversal level—and a great decrease in membrane resistance, chiefly attributable to an increase in Cl^- permeability (Fig. 6*A* and *B*)[38]. Comparable observations on neurones in Deiters's nucleus make it probable that GABA is the inhibitory transmitter released in the medulla by the endings of the cerebellar Purkinje cells[57].

Derivatives of GABA

Other short-chain omega-amino-acids and some related compounds such as guanidine derivatives are also potent inhibitors of central neurones (Fig. 5*B* and *D*)[9,12,43]. Physiologically, the most significant is glycine, which is probably an important inhibitory transmitter in the spinal cord[58]: many spinal neurones are more strongly inhibited by glycine than by GABA; and, like the best-known variety of spinal inhibition, the action of glycine is blocked by strychnine[59]. Glycine may be an inhibitory transmitter also in some parts of the brain stem[60,61]. But it is unlikely to have an important inhibitory function in the cerebral[62] or cerebellar[63] cortex, where it has only a very weak action (Figs. 5*C* and 6*C*).

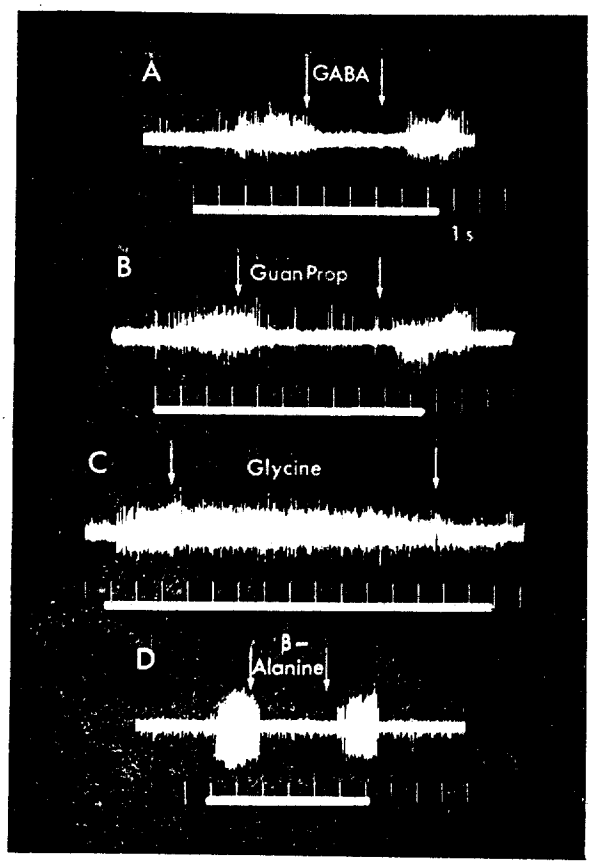

Fig. 5. Inhibitory action of GABA and some related amino-acids on cortical cells. In each case, neuronal discharge was initiated by release of glutamate (white line). (*A*) GABA (20 nA); (*B*) guanidino-propionic acid (20 nA); (*C*) glycine (100 nA), all tested on same group of cells; (*D*) effect of β-alanine (20 nA) in another experiment.

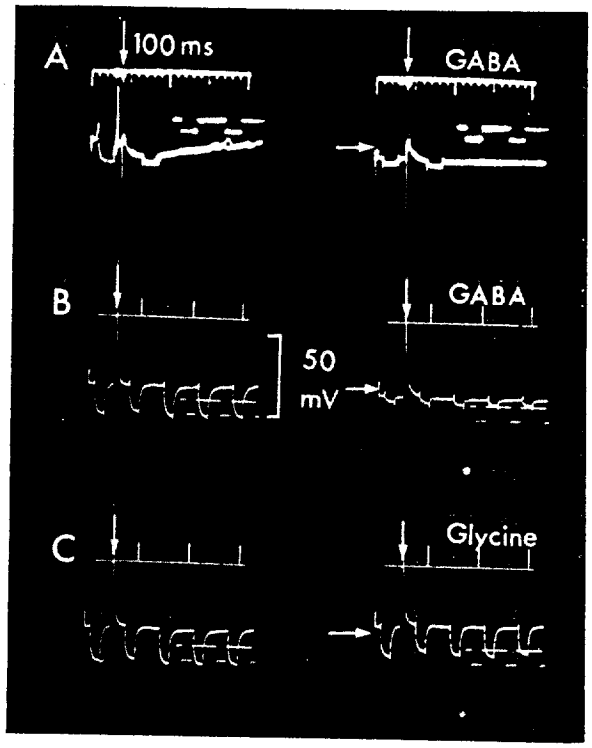

Fig. 6. Intracellular recording from cortical neurones showing inhibitory synaptic potentials (IPSPs) elicited by surface stimuli (at vertical arrows). Control traces are on the left. (*A*) IPSP shown after shock artefact as a downward (negative) deflexion, associated with a marked decrease in membrane resistance (note smaller voltage pulse developed by constant current pulses; the latter are monitored on another trace); when GABA was applied (140 nA), membrane potential became more negative (trace moved down) and resting resistance fell. (*B*) and (*C*) Traces obtained from another neurone: five resistance-testing pulses are visible in each. Note much smaller IPSP. GABA (28 nA) had a correspondingly smaller hyperpolarizing effect, but the membrane resistance was again much reduced. In contrast, a larger dose of glycine (56 nA) produced only a small change in resistance and some depolarization. Horizontal arrows indicate initial level of resting potential.

Metabolism and Localization in Brain

GABA is widely distributed in the brain[25,44,45]. Its production from glutamic acid (Fig. 1) is mediated by a highly specific enzyme, glutamic decarboxylase (GAD), which requires pyridoxal phosphate as coenzyme, and is inhibited by semicarbazide and hydroxylamine[64]. Other properties of this enzyme have been studied extensively[65]. A feature of some interest is that GAD is inhibited by Cl^-, so that an increase in the content of Cl^- in inhibitory cells, resulting from intense activity, could reduce substantially the rate of production of GABA[66]. GAD and GABA have an approximately similar distribution in the central nervous tissue[67,68]. The enzyme is not found in peripheral nerves or spinal roots and its concentration is greatest in areas of the brain stem that are exceptionally rich in GABA (such as the substantia nigra).

GABA-amino-transferase (GABA-T) is another relevant enzyme. By reversible transamination with α-oxoglutarate, it converts GABA to succinic semialdehyde, which, in the presence of an appropriate dehydrogenase[69], is oxidized to succinic acid (Fig. 1). Because succinic acid is a component of the tricarboxylic acid cycle, GABA is conveniently removed by this route. GABA-T is also present in the brain[70] but its distribution differs somewhat from that of GABA[67,71]. It seems that the content of GABA in a given region is determined by local GAD rather than GABA-T activity[67]. In the cerebellum and the hippo-

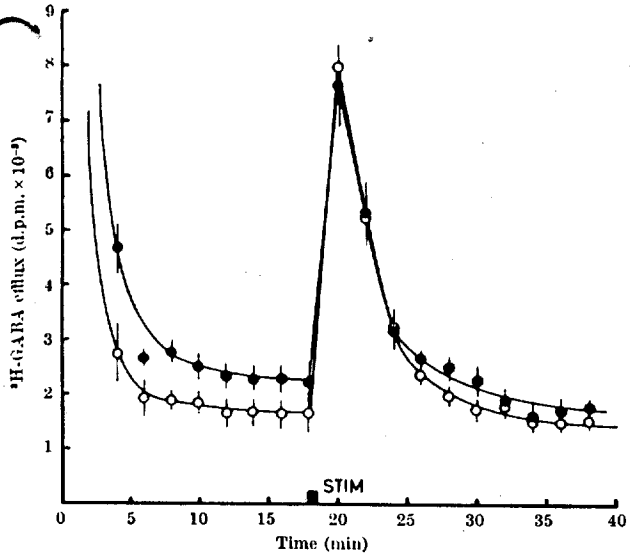

Fig. 7. Release of (^{3}H)-GABA from rat cortical slices by electrical stimulation (20 mA, 5 ms pulses at 100 Hz, for 30 s). ●—●, Normal; ○—○, Ca^{2+}-free. Mean values from seven experiments, with standard errors. (From Srinivasan *et al.*[84])

campus, the known situation of inhibitory neurones agrees with the distribution of GABA and the relevant enzymes[72,73].

GABA is too powerful an inhibitor to be freely distributed in the extracellular space of the brain. But GABA injected inside neurones does not alter their excitability[55]. GABA could therefore be stored inside cells. In fact, most of the GABA in the brain is probably actively kept in an intracellular compartment. Fluid left in contact with the surface of the brain contains at most only traces of GABA[40,74]. Slices of cerebral cortex absorb GABA, even against a very high concentration gradient[6,75]. This active process requires the expenditure of energy, and is therefore blocked by metabolic inhibitors.

Glutamate is apparently contained in more than one "pool" in the brain. One of these pools is in equilibrium with cerebral GABA but not glutamine; it is labelled very effectively through systemic glucose[30,31,39]. A second, smaller, pool seems to be accessible to systemic acetate, through which one obtains a high degree of labelling of glutamine but relatively little of GABA[27]; labelled glutamate injected directly into the brain[76] or systemically[77] also gives a high yield of labelled glutamine, probably by entering the same pool. These two pools may include, on the one hand, inhibitory nerve terminals which release or take up GABA and, on the other, excitatory terminals which release or take up glutamate. In the first pool, glutamate and GABA rapidly reach equilibrium through GAD and GABA-T activities. In the second, any excess glutamate may readily be stored in the inactive form of glutamine through glutamine synthetase.

Mechanism of Action and Uptake

A large increase in permeability to Cl^- fully accounts for the inhibitory action of GABA, because the gradient of Cl^- across the membrane is close to that for electrochemical equilibrium when the cell is in its resting condition; inward (repolarizing) movements of Cl^- therefore act as a powerful brake against any depolarizing (exciting) tendencies.

The precise mechanism by which GABA facilitates movements of Cl^- is not known. By analogy with glutamate, GABA might combine with a membrane carrier for Cl^-, increasing its affinity for the anion, and therefore accelerating Cl^- fluxes across the membrane (Fig. 3). If the binding of GABA to the carrier is a function of the ambient concentration of Cl^-, the usual low internal content of Cl^- would promote a net uptake of GABA. There is no direct evidence for this scheme, but some support is given by the observation that cortical slices absorb much less GABA in the absence of Cl^- or other halide anions (personal communication from Z. Gottesfeld and K. A. C. Elliott).

Although the active uptake of GABA by brain slices has been studied for some years, the precise localization of the absorbed GABA has been demonstrated only recently. According to Neal and Iversen[78], like endogenous GABA, nearly half of the labelled GABA taken up by cortical slices is found in nerve endings, which also contain most of the GAD activity in the tissue. These results thus confirm the presence of both GABA[32,34,39,79] and GAD activity[36,79,80] in cerebral nerve endings. Presumably at least part of the GABA absorbed by the slices is taken up directly by nerve endings, for even isolated nerve-ending particles can bind and take up external GABA[81,82]. In any case, when extracts of cortical nerve endings are tested directly on cortical cells[32], endings can be shown to contain enough GABA for a significant inhibitory action.

Release of GABA during Inhibition

If GABA is indeed an inhibitory transmitter, one should be able to demonstrate its release from inhibitory nerve endings, although only very small amounts are likely to escape from the tissue, even during the strongest inhibition, until a means is found of preventing the rapid uptake of extracellular GABA.

Some release of GABA from brain tissue has been demonstrated in experiments on brain slices, on isolated nerve endings or on the brain *in situ*. For example, if brain slices are incubated in a medium containing radioactive GABA, the subsequent efflux of labelled GABA into non-radioactive medium is very much accelerated by stimulating the slices electrically or with 40 mM K^+ [83,84]. Stimulating in a similar way a suspension of nerve endings—isolated by the technique of Gray and Whittaker[33]—causes a markedly greater proportional loss of GABA than of other amino-acids (except glutamate and aspartate)[41]. Jasper and his collaborators[40,74] have shown that appreciable amounts of GABA can sometimes be collected from the surface of the cortex; this efflux apparently depends on the functional state of the animal, being relatively large during sleep and practically undetectable after arousal. A more direct correlation with inhibitory function is evident when the efflux of cortical GABA is specifically accelerated by the activation of known inhibitory pathways; for example by stimulating the cortical surface, the thalamus[85], the caudate nucleus or the pyramids (personal communication from H. Jasper and I. Koyama).

Finally, Obata and Takeda[86] have shown that cerebellar stimulation increases the leakage of GABA into the fourth ventricle. This is particularly significant because the cerebellar cortex has a solely inhibitory action on nuclei adjacent to the ventricle[87]. Both in cerebellar and cerebral experiments, the yields of GABA are substantially greater when animals are pre-treated with inhibitors of GABA-transaminase.

In crustaceans the inhibitory action of GABA is blocked very effectively, and specifically, by the convulsant agent

picrotoxin[17,91,92]. Unfortunately, such a clear antagonism of GABA has not so far been observed regularly in the vertebrates, with picrotoxin or any other compound. As already mentioned, the inhibitory action of glycine is prevented by strychnine; but strychnine has little effect on inhibition by GABA[59,93,94]. Picrotoxin may antagonize the action of GABA on certain neurones in the brain stem and spinal cord[93,95], but really convincing antagonism has not yet been demonstrated in the cerebral or cerebellar cortex. Curtis *et al.*[96] have stated that the alkaloid bicucullin blocks selectively the inhibitory action of GABA in the brain and spinal cord, and that this drug should be useful for detecting GABA-mediated inhibition. But repeated tests by prolonged direct applications on cerebral cortical neurones have failed to confirm these observations[97]: it seems unlikely that bicucullin can be of great value as a specific antagonist of GABA.

In summary, there is now much evidence to suggest that the most important functions of L-glutamate and GABA are to act respectively as excitatory and inhibitory neurotransmitters. That the glutamate-GABA metabolic pathway (Fig. 1) can use up a substantial fraction of the total oxygen consumed by the brain[88] may be an indication not of its importance for general metabolism, but rather of the large amount of energy needed to maintain an adequate supply of these essential transmitters. Thus although cerebral glutamate first became well known because of its effects on the oxygen consumption and the sodium and potassium contents of the brain, these are probably secondary manifestations of transmitter functions. Of course, glutamate is also involved in the synthesis and breakdown of cerebral proteins[29,89]. But even these slower processes may be considered aspects of neurotransmission, for they probably determine the long-term synaptic facilitations or inactivations required for learning and memory.

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IONTOPHORETIC STUDIES OF NEURONES IN THE MAMMALIAN CEREBRAL CORTEX

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The interpretation of studies of the actions of various chemical agents on the cerebral cortex is peculiarly fraught with difficulties. Substances injected into the blood circulation may never reach cortical neurones on account of the blood-brain barrier, and they are quite likely to produce indirect effects by altering the blood supply to the brain, or by exciting peripheral structures with central connexions. Direct application to the surface of the cortex may not give a more definite answer, because excitability is abolished by inhibition or an excess of excitation and, in general, changes in electrical records from the surface can be quite ambiguous (compare the analyses of the action of γ-aminobutyric acid (GABA) by Purpura, Girado, Smith, Callan & Grundfest, 1959, on the one hand, and by Jasper, 1960, and Goldring & O'Leary, 1960, on the other hand). The cortex is not a homogeneous structure. Different neurones may not respond to drugs in the same way, but this need not be revealed by recording the activity of single units, since the numerous excitatory and inhibitory interconnexions between neurones may cause the reaction of the single unit to be altered radically by the massive discharge of other cells.

For all these reasons the iontophoretic method of applying substances to single units is the method of choice whenever the substance is ionized in solution. The amounts released are minute and therefore the concentration in the tissue negligible, except in the immediate vicinity of the neurones under observation. Many neurones can thus be studied in succession, in the same animal, little interference with the function of the brain being caused.

Iontophoresis has been used extensively in experiments on spinal neurones (e.g. Curtis & Eccles, 1958; Curtis, Phillis & Watkins, 1959, 1960) and also in two studies of neurones in the brain stem (Curtis & Koizumi, 1961, and Curtis & Davis, 1962).

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In the present paper we shall describe the excitation and inhibition of single cortical neurones by various agents applied iontophoretically, considering in particular the remarkable actions of dicarboxylic and ω-amino monocarboxylic acids, originally discovered by Hayashi (1954, 1956), as well as other interesting phenomena, such as the excitation of certain neurones by acetylcholine (ACh) and some effects produced by indole and catechol amines.

Brief reports of our results have already appeared (Krnjević & Phillis, 1961*a*, *b*).

METHODS

Experiments were performed on cats, rabbits and rhesus monkeys. In most cases the animals were anaesthetized with Dial compound, containing diallyl-barbituric acid (0·1 g/ml.) and urethane (0·4 g/ml.) (Ciba Ltd.), injected intraperitoneally (0·7 ml./kg). This was sometimes supplemented with intravenous pentothal sodium or sodium pentobarbitone (Nembutal; Abbott Laboratories). In a few experiments cats were anaesthetized with ethyl chloride and ether, and then either kept on ether, or given intravenous chloralose (80 mg/kg).

To eliminate a possible interference by anaesthetics, in four experiments we examined neurones in the unanaesthetized 'cerveau isolé' of the cat. This was prepared under ether, either as in the original description by Bremer (1937), i.e. by a section of the brain stem along the plane of the bony tentorium, or by a more inclined section (at 45°) beginning just behind the base of the tentorium. Neither approach was successful in preventing the frequent occurrence of cerebral oedema when the cortex was subsequently exposed. As a rule we made a wide exposure, to allow recording from different regions, including the symmetrical areas of both hemispheres. To prevent drying and to reduce cooling, the cortex was covered with one layer of transparent polythene and a second layer of black polythene (polythene mulch, Fablon). The area left free for the insertion of the micropipette was irrigated by a continuous drip of warm physiological fluid. The whole animal was kept at 37 ± 1° C by an automatically controlled heating pad under its abdomen (Krnjević & Mitchell, 1961).

The multibarrelled pipettes consisted of five hard glass tubes (diameter 6·5 mm, bore 4·5 mm) fused together for part of their lengths; the array of five tubes was heated and drawn out to a fine tip by a device similar to that described by Winsbury (1956). The micropipettes were filled by boiling in glass-distilled water. Before replacing the water with concentrated (nearly saturated) solutions of various substances, the shaft was broken back to give a tip diameter of 6–12 μ. This was done to ensure that barrels do not have an excessively high electrical resistance: as a rule, after filling with concentrated solutions of the various substances, the barrel resistances were in the order of 20–100 MΩ. In all the micropipettes the central barrel (which was always wider than the others, with a resistance only about one tenth as high for a given internal solution) was filled with 2·7 M-NaCl; it was connected by a short silver wire to the grid of the cathode follower at the input of the amplifying system. One barrel was usually filled with Na L-glutamate for the activation of quiescent neurones. Whenever we studied cholinoceptive cells, another barrel contained acetylcholine chloride (AChCl: Roche Products) at a concentration of about 3 M. Thus either two or three barrels were available for the various substances to be tested. They were made in approximately saturated solutions; for amino acids, strong acid or alkali was usually added to bring the pH well away from the iso-electric point, in most cases as in the experiments of Curtis & Watkins (1960).

Each of the barrels used for iontophoresis was connected by a flexible lead and a 60 MΩ resistor to a separate polarizing battery, the return circuit being completed through earth and an Ag-AgCl earthing electrode wrapped in moist gauze and fixed to muscles on the back.

of the neck. The polarizing currents could be varied and reversed, and their intensity was monitored continuously on a series galvanometer, with an accuracy of ±1 nA. Provision was also made for the constant application of a suitable braking current to neutralize the spontaneous outflux of substances from the tip of the pipettes (cf. del Castillo & Katz, 1955). The position of the micropipette was controlled by micromanipulator which allowed vertical movements in steps of 1 μ. To reduce pulsations a transparent Perspex pressor was applied to the surface of the brain with no impairment of blood flow through underlying cortical vessels (Phillips, 1956). Penetration by the micropipette was made easier by disruption of the pial layer, performed with fine forceps under a microscope.

The electrical activity of single neurones was amplified in the conventional manner. Spikes on a slow time base were easily photographed thanks to the excellent beam intensifier described by Pokrovsky (1960).

RESULTS

We have made observations on well over 4000 cortical units, in 56 cats, 4 rabbits and 4 monkeys. Most of them were recorded in the cerebral cortex, but in 5 experiments units were also examined in the cerebellar cortex of cats and rabbits.

Current effects

Iontophoresis depends on a flow of current into or out of the tip of the pipette. Since the tip must be very near a cell to record its activity, how much is the activity of the cell affected by the flow of current? This question is best answered by observing the effects of currents flowing from a multi-barrelled pipette containing solutions of NaCl in all its barrels. Under these conditions we found that most cells could be excited to some extent by a large inward current (i.e. one flowing from the tissue into the relatively negative tip of the micropipette) and conversely, they could be depressed by a large outward current (i.e. with tip positive). For a definite action, currents of at least 100 nA were usually needed.

The effect was seen best when the cell was discharging spontaneously, and therefore already in an excitable state. For instance, the unit in Fig. 1*B* was initially discharging spontaneously at a relatively high rate, but its activity could be blocked by an outward current of 100 nA. A few minutes later the same unit became much less active, and at this time an inward current of 120 nA had a sharp excitant action (Fig. 1*A*). Such powerful current effects were somewhat unusual, but these records demonstrate clearly several characteristic features: the instantaneous onset and termination (cf. the actions of various substances in Figs. 3, 5, 6, 9, 10, 12, 17, etc.), the tendency to adaptation, causing the effect to remain maximal for only 2–3 sec, and the rebound of excitability, lasting up to 15 sec after turning off the current.

On a few occasions we have seen current effects in the reversed direction: i.e. with excitation by an outward current and depression by an inward

current (Fig. 1*C* and *D*). These were associated with evidence that the tip of the pipette was extremely close to the cell membrane: spikes were positive and small currents unusually potent; there was a tendency to injury discharge, and electrical artifacts arising from movements. On account of the relatively large tips of our pipettes, we could not record very long under these conditions; the unit soon either reverted to the normal behaviour or it discharged paroxysmally and was lost.

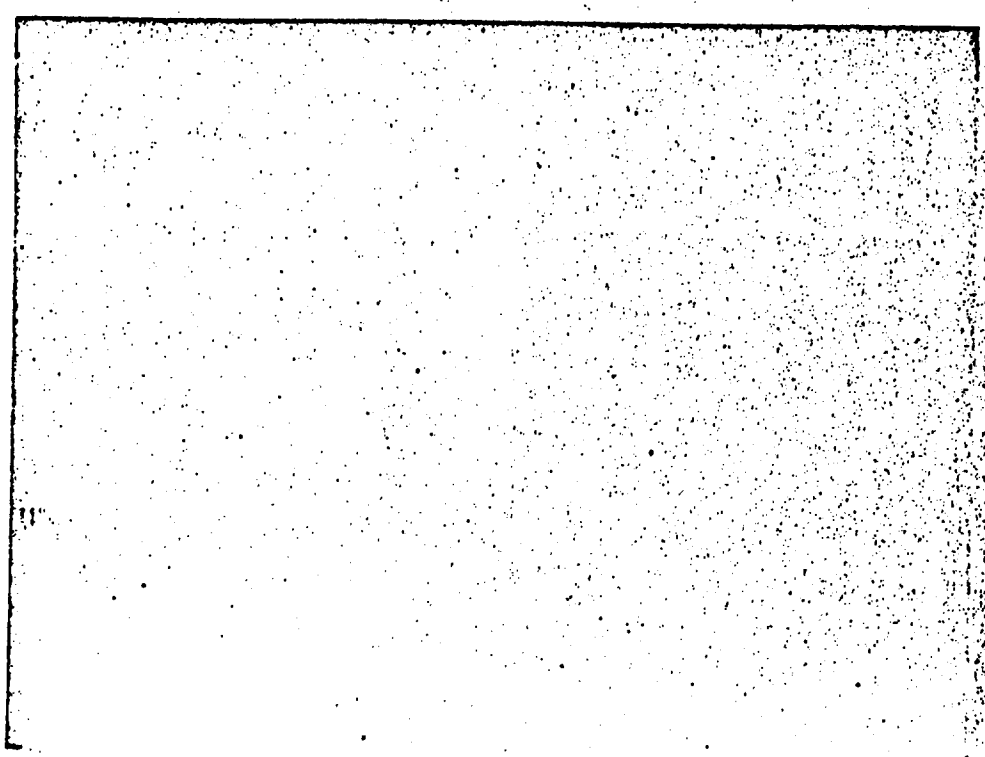

Fig. 1. Effects of current flow on cortical neuronal activity in cats. *A*, *B*: unit in cerveau isolé excited by an inward current (tip negative) and depressed by an outward current; pipette contained NaCl in all barrels. *C*, *D*: unit in animal under Dial giving positive spikes and showing exceptional effects of current flow, opposite to those in *A*, *B*. Note instantaneous changes in activity and tendency to accommodation and rebound.

Although current effects are probably never entirely negligible, they do not interfere to any serious extent with iontophoretic studies. The actions of powerful excitatory or inhibitory substances are hardly affected at all; at most there may be some tendency towards facilitation or depression, depending upon whether the substance is released as an anion or a cation, and the time course of the action may be slightly modified. On the other hand, the excitation or depression produced by less active substances might not be so readily distinguished from current effects. But with multi-barrelled pipettes, indifferent barrels are usually available, through which similar or greater currents can be passed as a control. This

procedure was done as a routine whenever there was the slightest doubt about the action of any substance (as shown in Figs. 2, 7, 10 and 17). Cerebellar cortical neurones seemed rather more sensitive to current effects than neurones in the cerebral cortex.

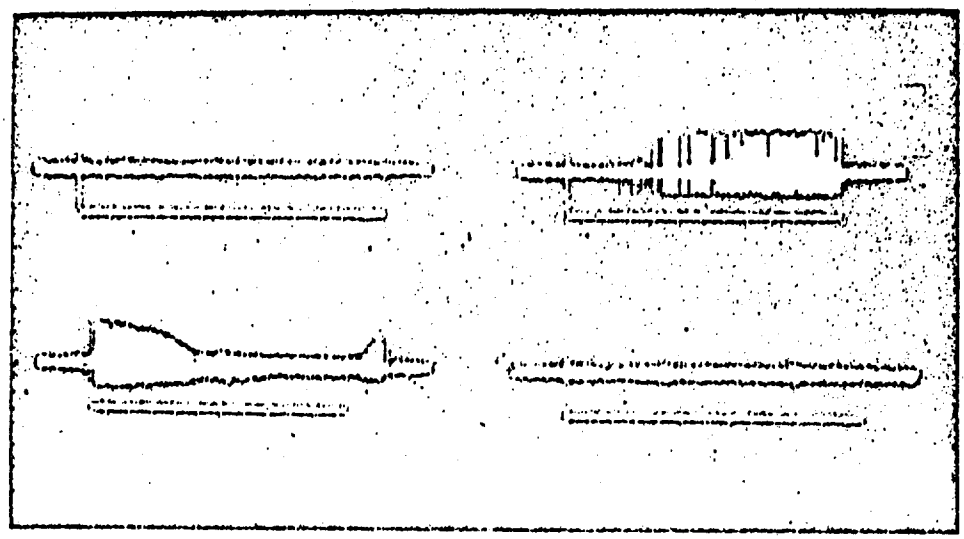

Fig. 2. Excitation by L-glutamate of unit in cerebellar cortex of cat under Dial. *A–C*: white lines indicate iontophoretic application by inward currents (tip negative). *D*: control for current effects; large inward current was passed through adjacent barrel containing AChCl.

Excitation by inward currents appeared to be somewhat greater if one of the other barrels contained L-glutamate. This was probably owing to some accumulation of glutamate by spontaneous outflux in the region just outside of the tip, where the four barrels have a common external resistance. Inward current would tend to carry some of this glutamate towards the cell. If the current is passed through one barrel, there is no appreciable current flow in the same direction through adjacent barrels; thus ACh+ is not released from one barrel by a very large current through an adjacent barrel (Krnjević, Mitchell & Szerb, 1963). A small current does pass in the opposite direction in the other barrels because they offer a parallel shunt to earth, via the polarizing circuits. However, this path has such a high electrical resistance (at least 80 MΩ for each barrel) when compared with the common external resistance (1–100 kΩ) as to be of negligible significance. Non-specific interference by currents is probably greatest when an outward current carrying one substance is superimposed on an inward current releasing L-glutamate. This is a convenient method of testing depressant substances: but it is clear that part of the outward current will tend to flow into the glutamate-containing barrel, reducing the outward transport of glutamate.

Excitation by L-*glutamate and certain related amino acids*

Identification of excitable units. As far as we can tell, all cortical neurones are excited by L-glutamate. Since we cannot observe the tip of the micropipette, our identification of the structures excited must rest on certain indirect considerations. Excitable units were found in large numbers at all depths of the cortex, but only occasionally in white matter, and most of them generated stable and substantial extracellular spikes recorded over relatively large distances (up to 100 μ). These facts suggest that L-glutamate excites the cell bodies (and perhaps large dendrites) of cortical neurones. We cannot tell at present whether some or perhaps all glial cells also respond to L-glutamate, but it is difficult to believe that axonal responses could be distinguished for any length of time by the relatively coarse tips of our pipettes. It should be noted that we do have good evidence of excitation of nerve cell bodies in the cortex by L-glutamate, since all the Betz cells that we have studied (see Krnjević & Phillis, 1962) could be excited in this way, and they were found at the expected depth (no responses to L-glutamate were given by axons of Betz cells in the white matter).

When a micropipette is gradually pushed through the cortex, neuronal activity can often be recorded (cf. Amassian, 1953; Li & Jasper, 1953; Mountcastle, Davies & Berman, 1957; Grüsser, Grützner & Baumgartner, 1958; Towe & Amassian, 1958), especially if the animal is not deeply anaesthetized. If L-glutamate is released continually from one barrel of the micropipette, the number of distinguishable neurones is greatly increased. Thus many cells lying quiescent within recording distance of the tip, most of which are not susceptible to excitation by peripheral stimuli or by the continuous synaptic bombardment suggested by the local slow waves, can nevertheless be brought into activity by L-glutamate. During routine searching for cells, up to about 20 useful units can be found in this way in the course of one track through the cortex. We have not tried to look systematically for the greatest possible number of distinguishable units. This would undoubtedly be much greater; small spikes can always be detected in the background noise, presumably from cells that are either very small or relatively far from the tip (or both).

If we assume that a distinct response can be recorded up to a distance of about 50 μ from the cell (Svaetichin, 1951; Li & Jasper, 1953; Mountcastle *et al.* 1957; Towe & Amassian 1958) the maximal number of neurones within reach of the tip during one track in the postcruciate gyrus of the cat would be approximately 320 (taking the mean density of neurones as $20/(100\ \mu)^3$: from Ramon-Moliner, 1961). However, L-glutamate probably excites many small cells, whose spikes are likely to be masked by the activity of larger or nearer cells. When the density of units recorded at different depths is compared with the known density of neurons it appears that our method may tend to discriminate against small cells (Krnjević & Phillis, 1963*a*).

Excitation by L-*glutamate.* The characteristic manner in which units are excited is illustrated in Fig. 2. A small amount of L-glutamate causes the cell to give occasional impulses. If one records activity with a long time constant of amplification, it is usually found that these impulses bear a close relation to negative peaks of slow waves of the electrocorticogram, e.g. Fig. 14*H*. With a stronger current of L-glutamate the discharge becomes more rapid, and also more regular (Fig. 2*B*). In the course of one application the frequency nearly always increases with time, probably because the concentration in the tissue does not reach its maximum immediately. Perhaps the most striking feature of glutamate excitation is its short latency of onset (seldom more than 1 sec) and even more rapid termination, typically within 1 sec of the end of the application (e.g. Figs. 2–6, 9, 10, 15, 18, 19). On the other hand, if too much is given the cell fires at an excessive rate which leads quickly to complete block, presumably by depolarization (Fig. 2*C*). Recovery then follows within a few seconds.

As already pointed out, one can easily prove that the excitation is not due to current flow by passing even larger inward currents through other barrels of the same micropipette. The trace in Fig. 2*D* shows that an inward current through another barrel containing AChCl (which would tend to release Cl^- from the pipette) only caused about as much excitation as a current 30 times smaller through the L-glutamate barrel (cf. Fig. 2*A*). Furthermore, L-glutamate is very effective if it is released from the micropipette by pressure injection. In Fig. 3 the upper record was obtained during the iontophoretic application of glutamate from one barrel (by a current of 40 nA), and the lower record when a hydrostatic pressure of 100 mm Hg was applied to another barrel, also containing Na-L-glutamate. Although the second method of release has a somewhat slower action, both in starting and stopping, the effects produced are comparable.

It has been shown that, given a 3 M-AChCl pipette and a tip resistance of 30–20 MΩ, with 100 mm Hg one can expect a flow rate at the tip of 10^{-10} to 10^{-9} ml./sec (Krnjević *et al.* 1963), equivalent to a release of 0·3–3 pmole/sec. With Na-L-glutamate in the barrel conditions are not likely to be grossly different, and the rate of release would therefore be comparable with that caused by an iontophoretic current of 40 nA.

Excitation by related compounds. If any doubts should remain about the potent action of L-glutamate, they can be dispelled by comparing it with that of D-glutamate. Every one of the 200 cortical cells tested proved much more sensitive to the laevo- than to the dextro-isomer. This can be seen in Fig. 4: identical inward currents (60 nA) were passed through barrels containing the L- and D-isomers of glutamate and aspartate, all made up in similar concentrations and at a similar pH (8·5). In spite of

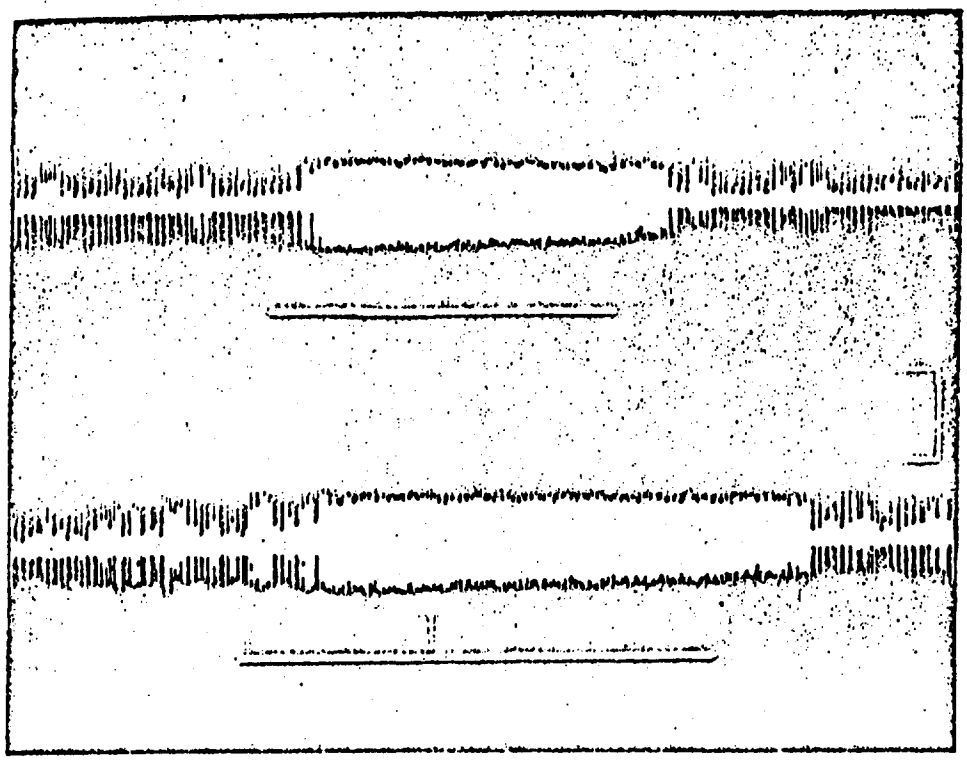

Fig. 3. Excitation by L-glutamate does not depend upon its release by a current. Comparable effects were obtained on unit in cat's cerebral cortex when L-glutamate was released from one barrel of micropipette by iontophorosis (*A*) and from another barrel by applying a head of pressure as indicated (*B*). Both barrels were filled with near-saturated solutions of Na L-glutamate.

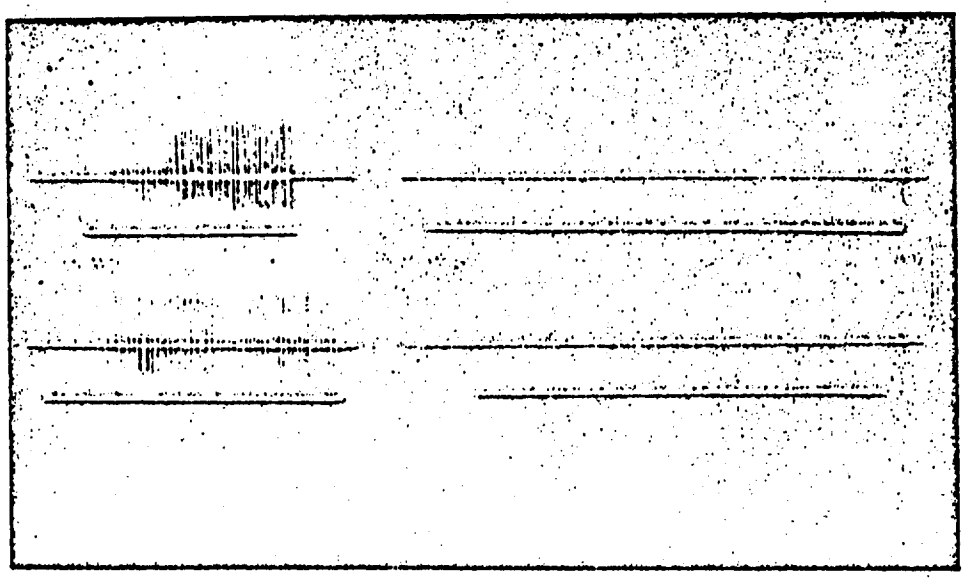

Fig. 4. Comparing actions of L- and D-isomers of glutamate and aspartate on unit in cat's cortex under Dial. Substances were in similar, near-saturated solutions, at pH 8·5; iontophoresis by identical inward currents of 60 nA.

a much longer application, D-glutamate caused only a slight excitation of the cell. Such consistent differences between L- and D-glutamate released by identical currents from solutions at the same pH demonstrate the specific action of the L-isomer, since any artifacts due to current flow, changes in pH etc. would be exactly similar in the two cases. As is shown here, in Fig. 4, D-glutamate was always much less effective than either L- or D-aspartate, but the two forms of aspartate in the aggregate seemed to have approximately the same potency; in some cases the dextro form was more effective, but more often, as in Fig. 4, the laevo form excited a little more strongly. The small variations probably arose from asymmetries in the shape of the micropipette tip.

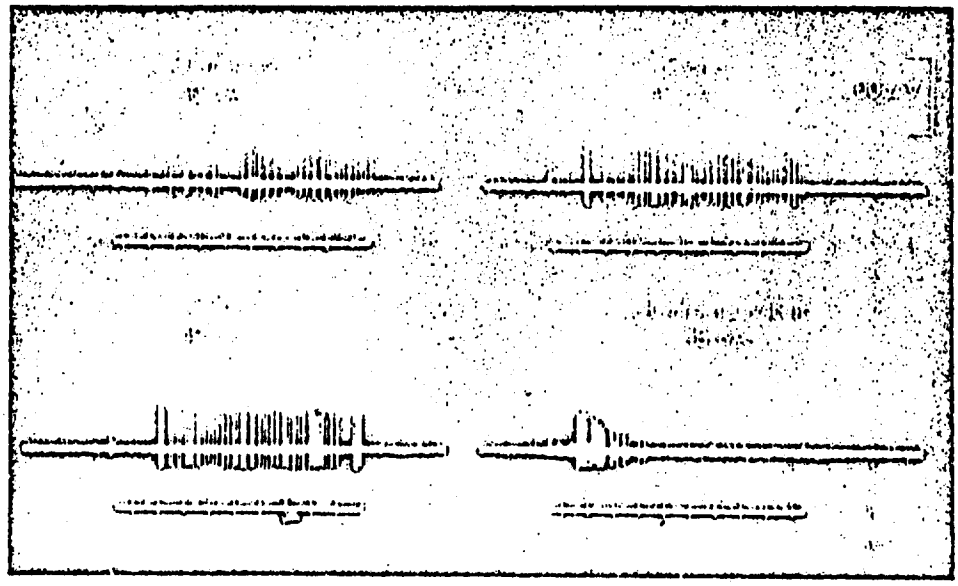

Fig. 5. Sulphonated derivatives of L-glutamate have similar or even stronger excitant actions. All substances were released by iontophoresis near a unit in a cat's cortex under Dial. Note excessive depolarization by homocysteic acid.

However, L-aspartate always had a weaker action than L-glutamate, and the same was true for most other related compounds which might occur in nervous tissues (for instance, glutamine and amino-adipic, amino-pimelic and α-keto-glutaric acids were almost totally inactive): the exception was cysteic acid (either in the DL or L form), whose activity was comparable with or even stronger than that of L-glutamate (Fig. 5). Homocysteic acid (Curtis & Watkins, 1961; Curtis, Phillis & Watkins, 1961*a*) is probably the most strongly exciting substance that we have tested, but it differed from L-glutamate in tending to cause a rapid depolarization block (see Fig. 5) which was not always reversible. Another powerful depolarizing agent is *n*-methyl-D-(or DL) aspartate, known to be particularly effective in causing spreading depression after a topical application (Curtis & Watkins, 1961); this substance was somewhat slower

than L-glutamate in its ability to initiate firing of cortical cells, but the effect usually continued for several seconds after the end of the application (Fig. 6). Moreover, as is also shown in Fig. 6, it tended to cause a prolonged reduction in the sensitivity of the cell to L-glutamate. We have not observed any interference of this kind by other related compounds, such as D-glutamate.

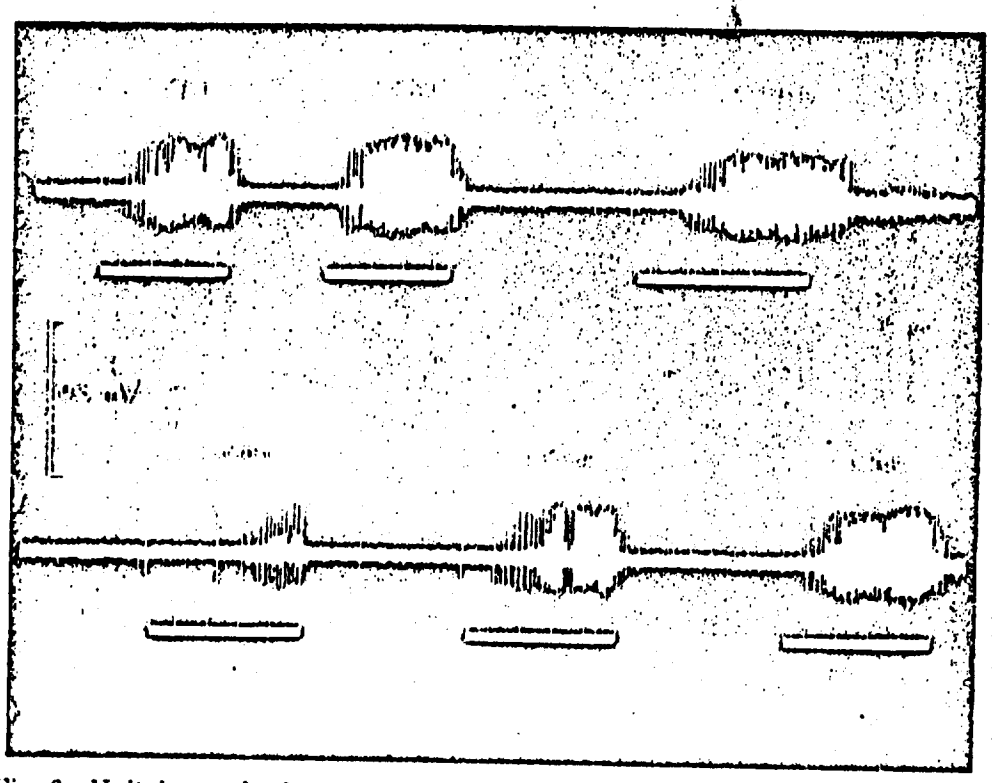

Fig. 6. Unit in cerebral cortex of monkey. Upper line: two applications of L-glutamate (70 nA) followed by one application of *n*-methyl-DL-aspartate (40 nA). Lower line, continuous with above: three more applications of L-glutamate (70 nA) show reduced sensitivity of unit for over 20 sec.

It has been said that dicarboxylic amino acids like L-glutamate have a non-selective *toxic* action on brain cells (Purpura, 1960). Yet one of the most impressive features of this type of excitation is that it can be repeated a great number of times without any evidence of a progressive deterioration in the condition of the cell. Even complete depolarization by an excess of L-glutamate leads only to a transient block (unless the application is continued). If L-glutamate is applied at a very slow rate, just sufficient to cause a cell to fire impulses steadily, the discharge can be maintained for many minutes with no sign of injury or obvious desensitization.

Minimal quantities needed for excitation. Cortical neurones varied greatly in sensitivity (compare Figs. 2 and 4), much of the variation being probably due to differing distances between the tip of the pipette and the

cells; but it appeared that some Betz cells with very large spikes (likely to be large cells) had a relatively high threshold for L-glutamate excitation. This would be in keeping with the observations of Curtis *et al.* (1960), who could not fire spinal motoneurones with L-glutamate.

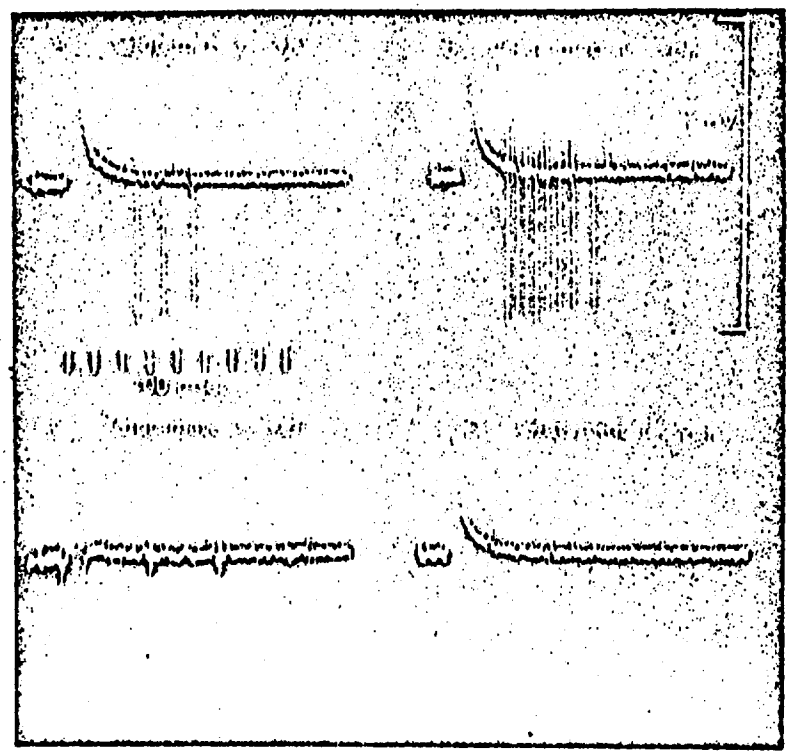

Fig. 7. Two units in cortex of cat under Dial, excited by L-glutamate released by 20 msec pulses of inward current. Voltage applied to pipette was 100 V in *A* and 150 V in *B*. In *C*, polarity of pulse was reversed, and in *D* a similar pulse of inward current was passed through another barrel containing glutamine.

When neurones were very sensitive to L-glutamate, its action could be demonstrated most clearly by inward pulses of current through the pipette. In Fig. 7*A*, a 100 V, 20 msec pulse of L-glutamate (seen as a large artifact near the beginning of the trace) excited two cells, one of them firing three spikes (large) and the other only one spike (small). Both cells fired many more impulses after a 150 V pulse. Outward pulses of 150 V (20 msec) through the same barrel were ineffective, as were inward pulses of the same magnitude through another barrel with a similar resistance but filled with glutamine. Similar results were obtained with several other cells (e.g. Fig. 8*A*, *B*). Under the best conditions, i.e. presumably when the tip was unusually close to the cell membrane, much shorter pulses were effective; the shortest had a duration of only 2 msec. With such pulses, the latency of excitation was reduced to 20–100 msec (cf. Fig. 8*B*). The most sensitive cells could be excited by passing a total charge of about

2×10^{-9} coulomb, corresponding to a release of some 10^{-14} mole of L-glutamate.

The same technique was used to deliver pulses of L- and D-aspartate, with comparable results. The latency of onset was not appreciably longer, and a similar discharge was obtained with somewhat stronger pulses, whereas D-glutamate was again much less effective.

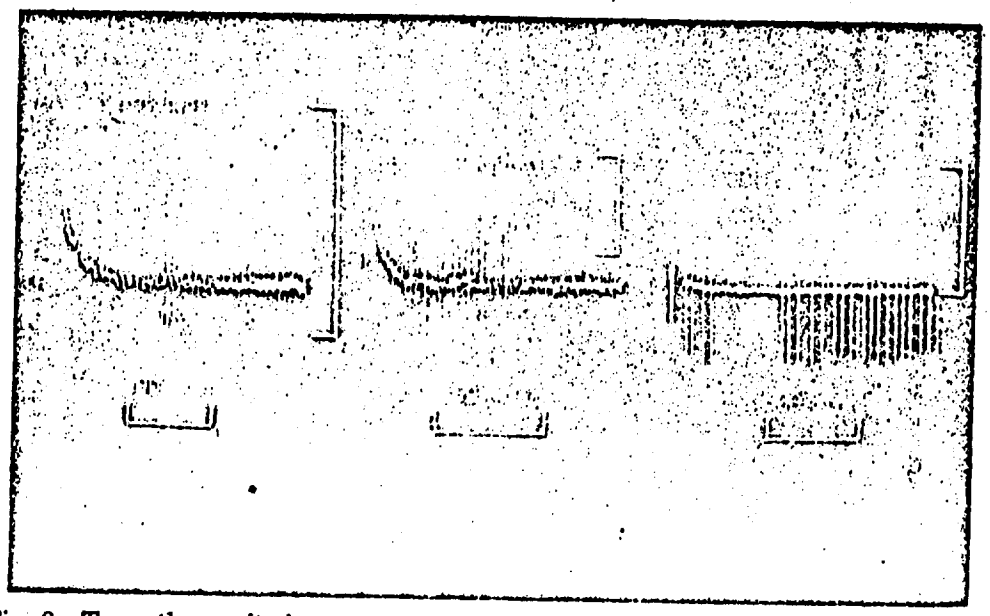

Fig. 8. Two other units in cat cortex under Dial, excited by L-glutamate released by 30 msec (*A*) and 5 msec (*B*) pulses of inward current (about 0·5 μA). Trace in *C* shows transient block of excitation of another unit by GABA released by a 20 msec pulse of outward current. Unit was excited by a background release of L-glutamate from another barrel (60 nA).

Other exciting substances

Amino acids not closely related to L-*glutamate.* The striking inhibitory action on cortical cells of γ-aminobutyric acid (GABA) and several other ω-amino-monocarboxylic acids is described below. Hayashi (1956) suggested that these ω-amino acids have both inhibitory and excitatory properties and that whereas the short-chain compounds (with fewer than 6 carbon atoms) mainly tend to inhibit cortical neurones, those forming a longer chain have a predominantly excitatory action. The later observations by Purpura *et al.* (1959) are consistent with this postulate, but these authors chose to interpret their results in a different way, which led them to believe that all the monocarboxylic ω-amino acids have depressant actions only, the different effects observed being ascribed to a specific blocking of excitatory synapses by the short-chain compounds, and an equally selective blocking of inhibitory synapses by the others.

In our experience the longer-chain ω-amino acids (ϵ-amino-caproic and ω-amino-caprylic, in particular) have proved somewhat ambiguous in

their actions, showing both inhibition and excitation. Of a total of 104 cells tested with these two substances, about one half showed either excitation or depression, the former being somewhat more common with ε-amino-caproic, and the latter when ω-amino-caprylic was applied. Examples of both types of actions are given in Fig. 9*A* and *B*. The delayed but maximal firing induced by these compounds (see Fig. 9*B*) is clearly very different from excitation by L-glutamate. It is much more akin to

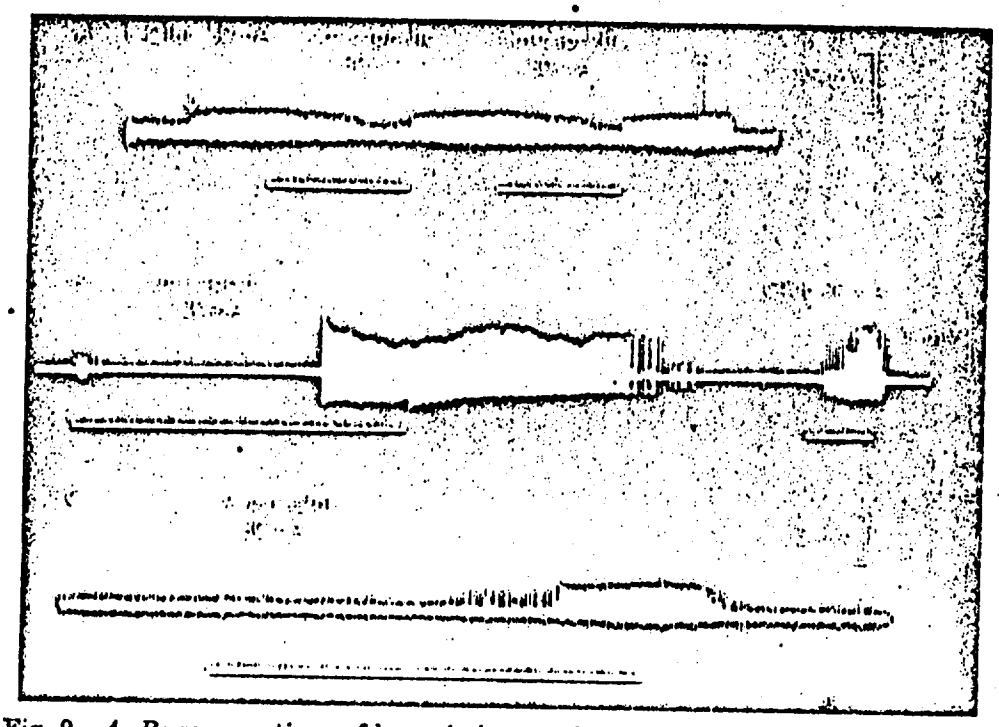

Fig. 9. *A*, *B*: some actions of long-chain ω-amino acids on cortical neurones. *A*: unit in cat cerebellum, at depth of 0·3 mm, during Dial anaesthesia. L-glutamate (50 nA) was applied throughout period between two arrows. During times indicated by white lines, ω-aminocaprylic acid was released as cation (80 nA) causing gradual block of discharge. *B*: 2 units in cerveau isolé of cat, at depth of 0·75 mm. ε-aminocaproic acid was released as cation (120 nA) during first signal. Both units were excited, with different latencies, but discharge of small unit was blocked almost immediately. At second signal L-glutamate was released to show that it was still effective. *C*: Excitation of unit in cat's cerebellar cortex under Dial anaesthesia by L-asparagine released as cation (80 nA).

the effects produced by various amines, like 5-hydroxytryptamine (Fig. 10) and nicotine (Fig. 11). The mixed effects observed with the long-chain ω-amino acids are thus fully in agreement with Hayashi's suggestion. On the other hand, we have confirmed the discovery by Purpura *et al.* (1959) and Van Harreveld (1959) of a substantial action of asparagine on cortical cells. Nearly all the 65 neurones tested with L-asparagine (released as a cation from solutions at pH 3·5–4) were excited after relatively long latencies of between 10 and 50 sec (Fig. 9*C*). This effect was observed less regularly with D-asparagine, and only occasionally with glutamine.

Certain amines. Many catechol and indole amines have a depressant action on cortical neurones when released iontophoretically (see below, and Fig. 10*A*). But with relatively large amounts of 5-hydroxytryptamine (5-HT), adrenaline and some other compounds, the cells were sometimes strongly excited instead, as can be seen in Fig. 10 *C* and *D*. This kind of paroxysmal discharge could be produced several times, with no evidence of any permanent damage to the cell. The records in Fig. 10 show that the firing cannot be ascribed to non-specific current actions, or to the creatinine sulphate with which 5-HT is usually associated.

Among other amines of biological interest which were also studied, nicotine requires special mention for its strong, though delayed, excitant

Fig. 10. Some depressant and excitant actions of 5-HT on unit in cerveau isolé of cat, at depth of 0·6 mm in post-cruciate area. *A*: unit excited throughout by a continuous application of L-glutamate (10 nA); discharge was blocked by 5-HT bimaleinate (first signal) and 5-HT creatinine sulphate (second signal), both released from solutions at pH 3·5, by outward currents of 70 nA. *B*: during similar excitation by L-glutamate (continued till arrow), control outward currents of 100 and 160 nA were passed through central, NaCl containing recording channel of multiple pipette (polarizing circuit shunted input thus reducing spikes; polarizing currents also caused temporary block of amplifier). *C* and *D*: Strong excitation of same unit by faster release of 5-HT (120 nA). In *C*, unit was excited 10 sec after starting application of 5-HT bimaleinate; onset of 5-HT creatinine sulphate release was before start of *D*, which shows excitation after 13 sec; two applications of L-glutamate (40 nA) indicate some temporary reduction in sensitivity.

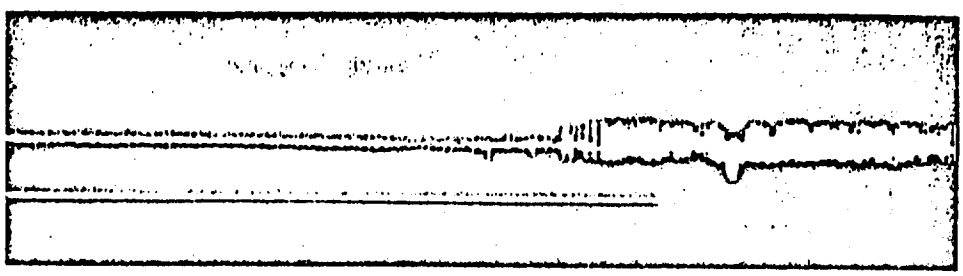

Fig. 11. Unit in cortex of monkey under Dial. Record begun 15 sec after starting iontophoretic application of nicotine as a cation (from a solution of nicotine hydrogen tartrate at pH 4); characteristic paroxysmal discharge went on for another 20 sec. Displacement of base line soon after end of nicotine release was an artifact.

actions observed on 54 cells out of 232 actually tested. Characteristically, after a continuous application of nicotine for 30–60 sec, a neurone would suddenly burst into activity, as in the record illustrated here. Figure 11 shows the last phase of a 45 sec iontophoretic application of nicotine, indicated by the white line below the trace. When firing began it was paroxysmal in a typical manner. The discharge continued for another 20 sec after the end of the trace shown in the figure and, as usual, it was not followed by a considerable period of reduced excitability. This action of nicotine resembles the excitatory action of curare (tubocurarine and dimethyl tubocurarine) also seen on a small number of occasions. Neither of these effects bears any relation to the excitation of certain neurones by ACh; they may help to explain previous observations of excitatory effects of nicotine and curare on the cortex (e.g. Chang, 1953; Rech & Domino, 1960; Morlock & Ward, 1961; Silvette, Hoff, Larson & Haag, 1962).

Acetylcholine. Although large numbers of neurones were tested thoroughly with ACh, as well as other related compounds, only a small proportion could be excited (about 10 % on the average). Most neurones were not affected in any way, showing neither an increase nor a decrease in excitability. The action of ACh was radically different from that of L-glutamate. One need only compare the two traces in Fig. 12 for this to be evident: L-glutamate excitation had the usually sharp onset and abrupt termination; in contrast, excitation by ACh only began after an appreciable interval, during which the spontaneous activity (a feature typical of cholinoceptive cells) tended to be depressed. This initial depression was probably a current effect and could be reproduced with similar outward currents through indifferent barrels. The effect produced by ACh long outlasted its application, as in Fig. 12, the after-discharge going on for 30–60 sec or even more. In this respect cholinoceptive cells in the cerebral cortex were similar to those found in the mid-brain by Curtis & Koizumi (1961).

Like other cortical neurones, cholinoceptive cells were readily excited with L-glutamate. But it was unusual for them to have a very low threshold for the action of L-glutamate, and in several cases the cell was excited much more effectively by ACh than by L-glutamate (cf. Fig. 12).

It is clear that ACh excitation cannot be easily confused with non-specific effects of current flow. ACh^+ was delivered by passing an outward current through the pipette (i.e. with the tip relatively positive). We have shown above that this type of current nearly always tends to depress adjacent neuronal activity (Fig. 1*B*); on the few occasions when an outward current excited a cell, the action was immediate, unlike that of ACh, and it did not persist after stopping the current (Fig. 1*D*).

The distribution and identity of cholinoceptive neurones in the cortex

and their pharmacological properties will be considered in more detail in two subsequent papers. It will be shown that these neurones are related especially closely to ascending pathways arising in the specific rather than the non-specific nuclei of the thalamus, and that the ACh receptors responsible for their activation have strongly muscarinic properties.

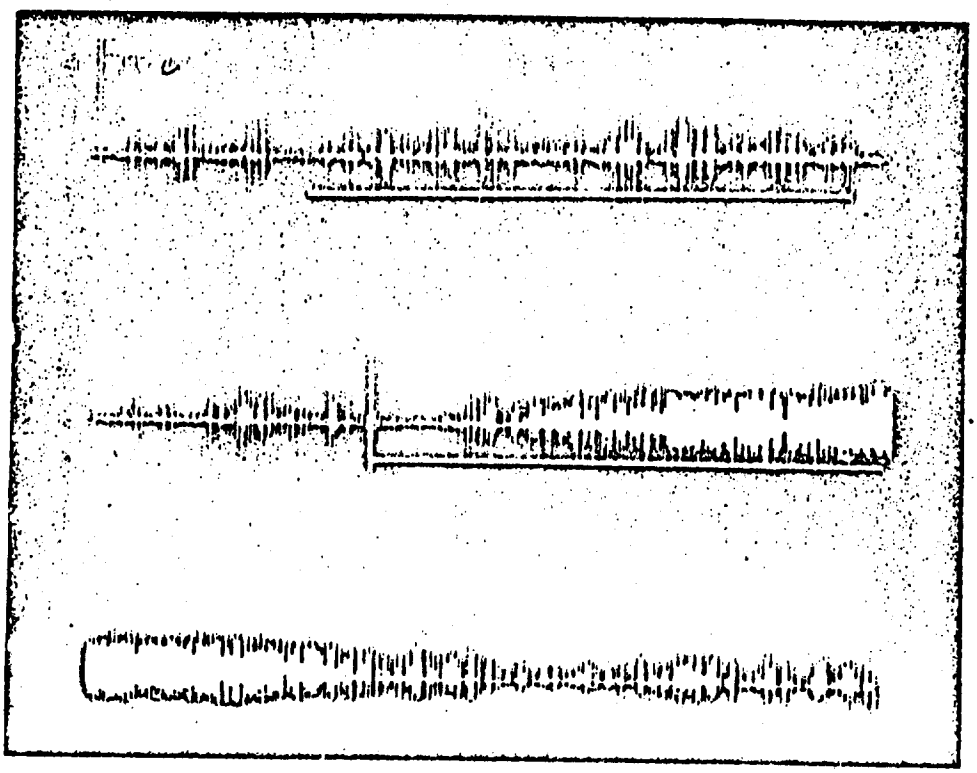

Fig. 12. Comparing excitatory actions of L-glutamate and ACh released iontophoretically by similar inward and outward currents (traces in *B* and *C* are continuous). Units were at depth of 0·95 mm in precruciate area of cat under Dial. Large unit was identified as a Betz cell by antidromic excitation of pyramidal tract.

Hydrogen ions. Most cortical cells can be excited by releasing comparatively large quantities of H^+ in their vicinity. This is probably a non-specific action, similar to the depolarization of peripheral nerves by acid solutions. The action of H^+ is less predictable than that of L-glutamate. The latency of onset is usually much longer and the effect more persistent, as is illustrated in Fig. 13. There is little gradation in the action of H^+ which tends to have an all-or-none character. Cells vary much in their sensitivity to H^+, possibly because of local differences in buffering capacity.

Apart from intrinsic interest, an important reason for the study of H^+ is that it must be released iontophoretically to some extent by outward currents which are passed through acid solutions. It may thus interfere with the action of other substances, or even cause excitation by its own presence. We have examined this latter possibility by studying the

behaviour of nearby cells while passing outward currents from pipettes filled with concentrated NaCl solutions at various levels of pH. Our observations suggest that excitation by H^+ is very unlikely if the internal pH exceeds 2·5, in agreement with previous observations on spinal neurones (Curtis, Phillis & Watkins, 1961*b*).

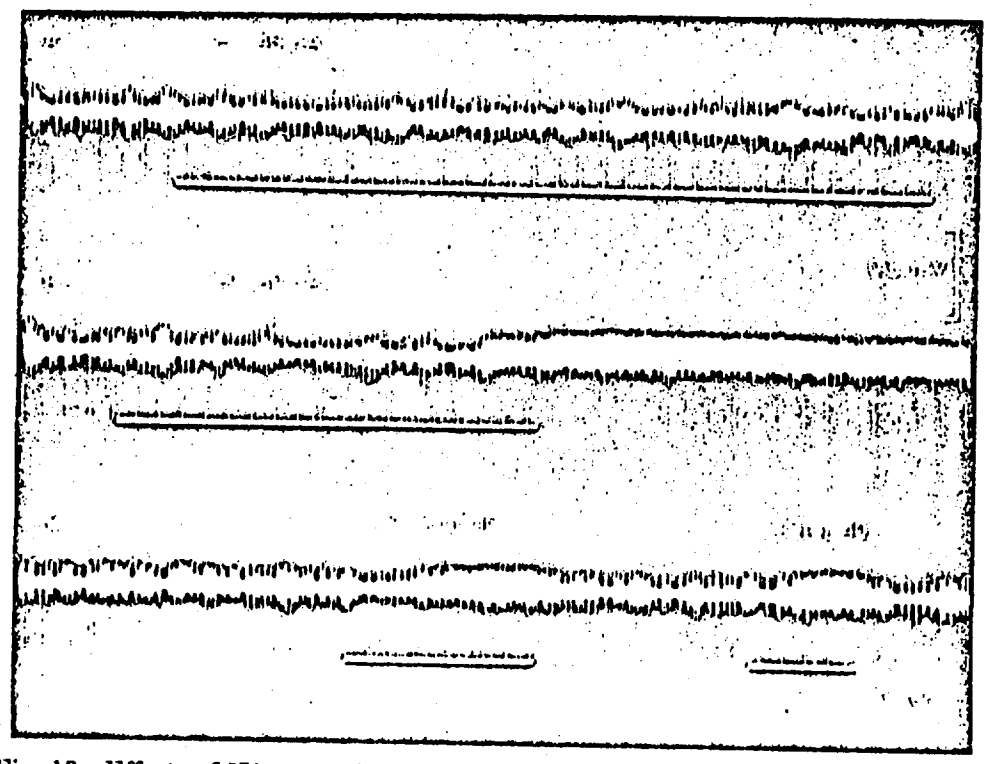

Fig. 13. Effects of H^+ on cerebral cortical unit in cat under Dial. *A*: during signal H^+ was released from 0·1 N-HCl by current of 40 nA. *B*: H^+ now released by current of 60 nA. *C*: record follows 110 sec after end of *B*; it shows remnants of excitation by H^+ and effects of two further applications of L-glutamate.

The excitation produced by nicotine, long-chain ω-amino acids, 5-HT and adrenaline bears some resemblance to that induced by H^+. Since these compounds were all released from acid solutions, we have considered the possibility that they all excite by causing a sufficient increase in tissue acidity. Without excluding this factor entirely, it seems to be relatively unimportant for several reasons. In many cases, as a control, we could release similar substances from equally acid solutions in adjacent barrels: for instance, 5-hydroxytryptophan applied to the same cells, under the same conditions, as 5-HT, did not tend to excite. Effects were obtained even when the solutions were only mildly acid (pH 5). Other amino acids such as leucine, citrulline and tryptophan did not have an excitant action like ϵ-amino caproic acid. Nicotine only excited about a quarter of the cells tested, and usually only after a very long delay; whereas H^+ excites most cells within 10–15 sec. It seems likely that any agent capable of pro-

ducing a sudden break-down in membrane resistance will cause firing of this type.

Cortical neurones are more resistant to OH^- ions, which only sometimes caused a relatively mild depression when applied in very large amounts (iontophoretic currents of > 100 nA for 1 min).

Potassium and some other agents. Potassium may be of significance in the causation of spreading depression (Grafstein, 1956; Marshall, 1959). The extracellular concentration of K in the brain is probably identical with that of the cerebrospinal fluid, i.e. about 2–3 mM (Davson, 1956). If the brain cells behave like peripheral nerves (Stämpfli, 1959), no appreciable change in membrane potential is to be expected unless the external K concentration is at least doubled (cf. Libet & Gerard, 1939) and there may not be much change before it is raised to some 10–20 mM.

A comparison between the excitatory effects of L-glutamate and K^+ released under the same conditions and in the same manner is therefore particularly instructive, since it gives an indication of the probable effective concentration achieved in the vicinity of the cell by iontophoresis, and also gives a clue to the relative contributions of K^+ or L-glutamate in the causation of spreading depression if both agents are released by depolarized cells.

This experiment was done on sixteen cells. A typical example is given in Fig. 14, which shows a unit found in the posterior sigmoid gyrus, firing spontaneously in close relation to local slow waves (*H*) and also in response to a peripheral stimulus (*G*). It was readily excited within a few seconds by L-glutamate (*A*, *B*) and ACh (*C*, *D*) but K^+ applied with a much larger current failed to cause more than a relatively slow and irregular discharge, even after a period of 90 sec. The majority of cells showed no appreciable excitation by K released in similar quantities, and in no case did K induce a cell to fire rapidly.

Unlike K^+, NH_4^+ consistently had no action at all on the neurones tested, in agreement with the observations of Hillman & McIlwain (1961).

On the other hand, many neurones were excited quite strongly by EDTA (diaminoethane tetra-acetic-acid) (Fig. 15), normally after a relatively short latency when compared with its action on spinal neurones (Curtis, Perrin & Watkins, 1960). However, EDTA, which was released as an anion, was always less effective, and often very much less so than L-glutamate (cf. Fig. 15*C*, *D*).

Inhibitory substances

γ-Aminobutyric acid (GABA). The inhibitory action of GABA on cortical neurones is just as remarkable as the excitatory action of L-glutamate. The two substances complement each other very satisfactorily; both are universally effective, having quick actions that are rapidly reversed, with no

apparent damage to the cells, so that they can be safely applied many times. The amounts of GABA required to cause marked inhibition have varied between 4 and 100 nA of outward current, values of 20–50 nA being most common. When cells were particularly sensitive, it was possible to demonstrate its quick action by passing pulses of outward current through the pipette. For instance, in Fig. 6*C* a 20 msec pulse of GABA at the start

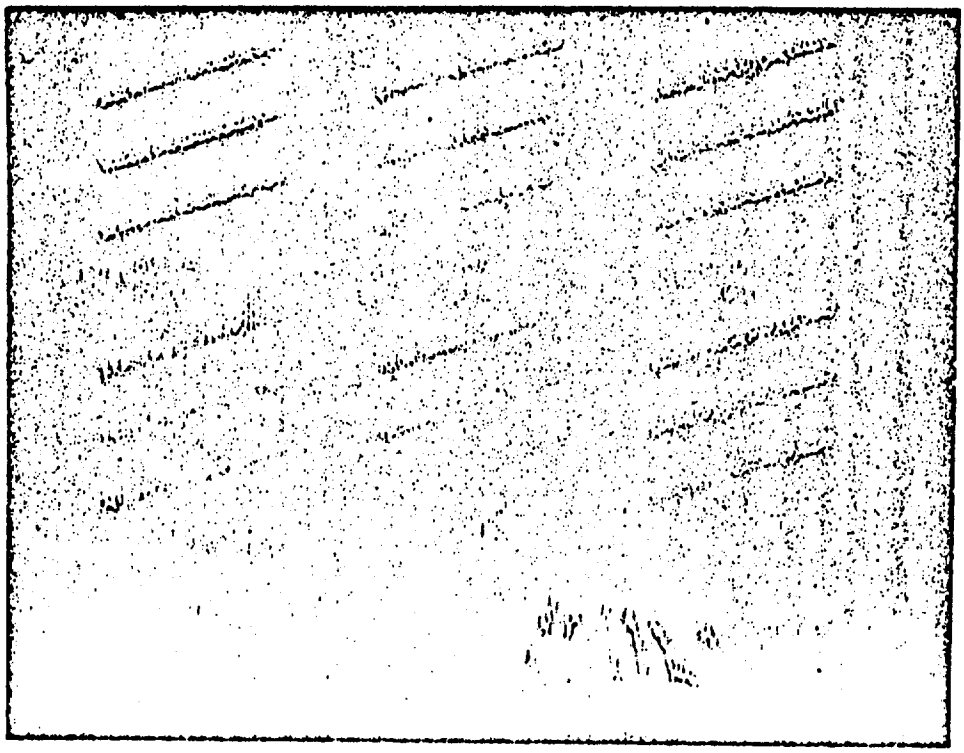

Fig. 14. Comparing effects produced by iontophoretic release of L-glutamate, ACh and K ions. Unit was found at depth of 1·1 mm in post-cruciate area of cat anaesthetized with Dial: it discharged impulses spontaneously in close relation to negative peaks of slow waves (*H*) and could be activated by electrical stimulation of contralateral forepaw (*G*; recorded with short time constant of amplification). *A*, *C* and *E* are control records of oscilloscope sweeps at 4/sec. *B*, *D* and *F* were taken at various times after starting release, as indicated. Some spikes have been retouched.

of the trace caused a transient block of the continuous discharge of a cell induced by the steady release of L-glutamate from another barrel of the same pipette. The time course of the inhibition is not unlike that of the excitation caused by comparable pulses of L-glutamate, as would be expected if both effects were simply dependent on the concentration in the tissue.

As in some other tissues (Curtis *et al.* 1959; Edwards & Kuffler, 1959), the effect of GABA is initially at a maximum, and then diminishes appreciably after several seconds during a prolonged application, owing to desensitization or an increased rate of inactivation.

The interaction between L-glutamate and GABA is shown even more clearly in Fig. 16, where 9 pulses of GABA (visible in the record as prominent vertical artifacts) were responsible for regular interruptions of the rapid firing produced by a continuous release of L-glutamate (indicated by the white line below the trace).

GABA was usually released as a cation by passing an outward current through a concentrated solution at pH 3·5–4 (pK_1 = 4·23). It has been

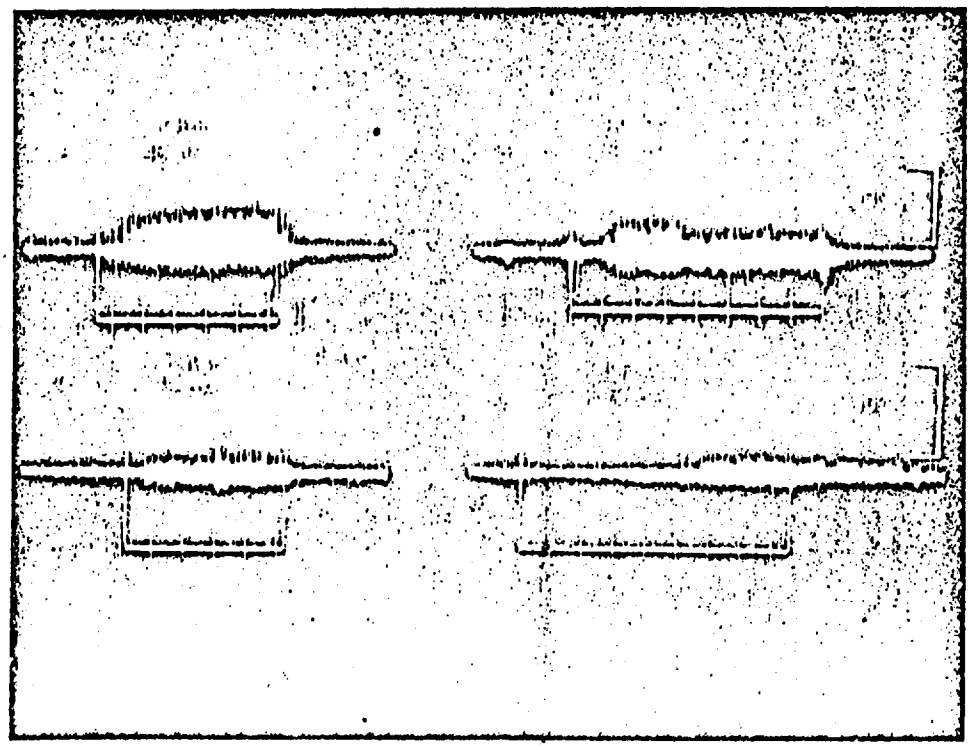

Fig. 15. Excitation by EDTA, released as anion from near saturated solution of di-sodium salt. Both units were in post-cruciate cortex of a cat, anaesthetized with Dial, and they were excited even more effectively by L-glutamate; compare *A* with *B*, and *C* with *D*.

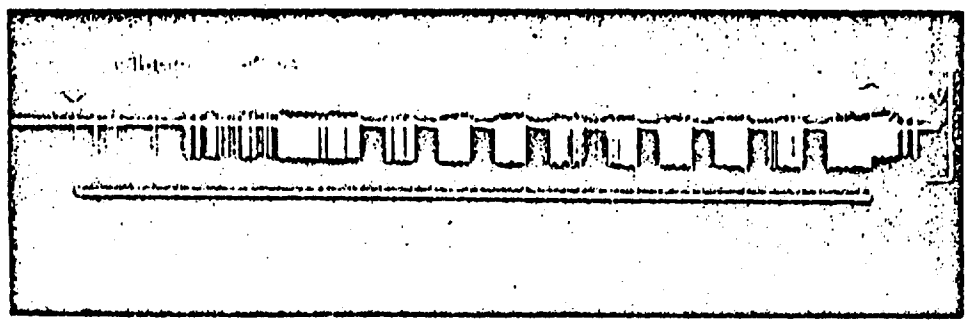

Fig. 16. Iontophoretic release of L-glutamate (between two arrows) initiated a continuous discharge of unit in cortex of cat under Dial; excitation was blocked nine times by GABA released from another barrel by 20 msec pulses of outward current (indicated by large electrical artifacts).

suggested that in its inhibitory properties, GABA released iontophoretically as a cation may differ substantially from the zwitterion, the predominant form at a body pH (Kuno, 1961; Muneoka, 1961). This criticism is probably unjustified, because inhibition is obtained easily if GABA is released either by pressure injection or by an outward current, from a pipette containing a neutral solution. The latter may seem surprising since GABA is at its iso-electric point at pH 7·5. The probable explanation is that in the iso-electric region GABA solutions in contact with hard glass have a substantial zeta potential, reaching a peak of 36–38 mV between pH 7 and 10 (unpublished observations). This gives an electro-osmotic mobility of 3 μcm/V sec, which would easily account for a significant release of neutral GABA (cf. Krnjević *et al.* 1963). It is not possible to give at present a strictly quantitative comparison of GABA cations and zwitterions, but our results do not indicate any marked difference between their

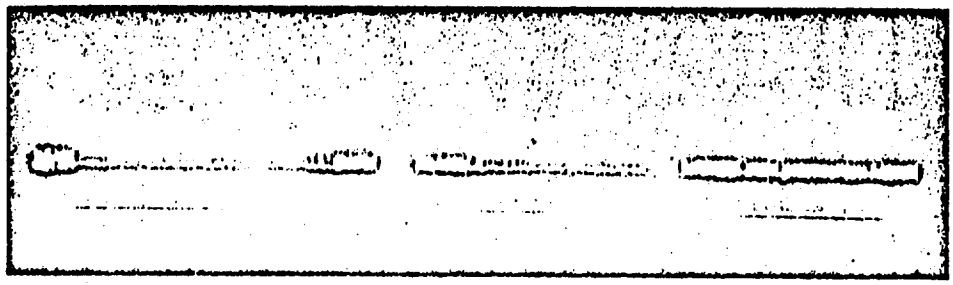

Fig. 17. Block of fast spontaneous discharge of cerebellar unit, in cat under Dial, by iontophoretic release of GABA and α-alanine from solutions at pH 3·5. An even larger outward current through another barrel containing Na salt of cysteic acid had only a slight depressant effect (*C*).

actions. The region of high zeta potentials is distributed very asymmetrically about the iso-electric point of GABA, being much more pronounced on the alkaline side of pH 7; this probably accounts for the difficulty experienced in trying to release GABA as an anion from alkaline solutions (Curtis & Watkins, 1960).

GABA is just as effective in blocking the natural activity of a neurone, i.e. activity arising 'spontaneously' or from the excitation of various pathways, as that initiated by L-glutamate. The fast spontaneous discharge of the unit illustrated in Fig. 17 was temporarily stopped by the iontophoretic release of GABA (*A*); as a control, a somewhat larger current was passed through another barrel of the same pipette, with no significant effect (*C*).

We have tested GABA on some 400 neurones including several Betz cells, in most areas and at various depths of the cerebral and cerebellar cortex, with and without anaesthesia; apart from one or two doubtful responses, we have never failed to observe a clear inhibition. Betz cells

were identified by short latency antidromic activation from the bulbar pyramids (see Krnjević & Phillis, 1963*a*). Such a widespread action is not consistent with the claim that GABA only blocks superficial, excitatory axodendritic synapses (Purpura, 1960). Although in our experiments GABA prevented the excitation of Betz cells by L-glutamate and by synaptic activity, antidromic invasion was not readily blocked.

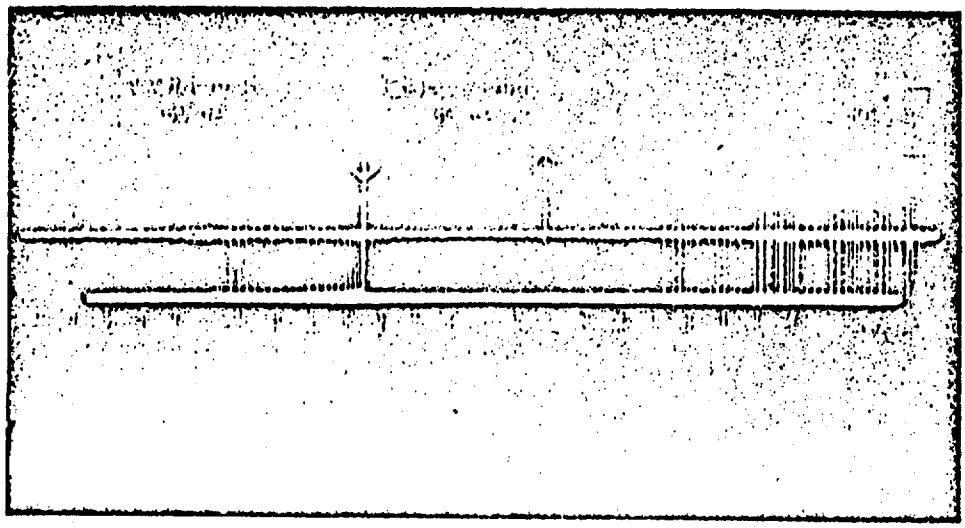

Fig. 18. Depressant action of glycocyamine (guanidino-acetic acid) on excitation by L-glutamate; both substances were released iontophoretically, by outward and inward currents, respectively. Unit in cortex of cat under Dial.

Other inhibitory substances

Amino acids related to GABA. Several close derivatives, including γ-aminocrotonic, β-hydroxy-γ-aminobutyric, δ-aminovaleric acids and β-alanine (but not taurine), as well as the ω-guanidine compounds, guanidino-acetic (Fig. 18) and β-guanidino-propionic (but not γ-guanidino-butyric) acids all have inhibitory activities comparable with that of GABA. The strong action of some guanidino-amino acids confirms previous observations on the cat's cortex (Purpura *et al.* 1959), the toad's spinal cord (Curtis *et al.* 1961*a*) and the crayfish stretch receptor (Edwards & Kuffler, 1959), but it is in marked contrast with their reported inactivity on cat spinal neurones (Curtis & Watkins, 1960). Even amino acids which are less closely related, such as α-alanine (Fig. 17*B*) and glycine are appreciably active. But the long chain ω-amino acids have only a relatively weak and inconstant inhibitory action, often masked by excitatory effects (Fig. 9). We have also looked for a specific block of inhibitory synapses by these substances (cf. Purpura *et al.* 1959). Surface stimulation with single shocks (10 V) causes a strong inhibition of many cells in the cortex, lasting over 100 msec. This could not be reduced by large amounts of ϵ-aminocaproic acid given by iontophoresis or by lavish surface appli-

cation of a 1 % solution. It is interesting that with regard to the actions of inhibitory amino acids, cortical neurones in general behave remarkably like crayfish stretch receptors (Edwards & Kuffler, 1959).

Some indole and catechol amines. Many compounds in this group have been applied to cortical neurones iontophoretically (mostly as cations, released from acid solutions). A systematic survey cannot be given here, but it should be noted that, in agreement with previous observations by Marrazzi (1961), there was a general tendency towards a depression of the activity of most cells (although large doses sometimes caused excitation,

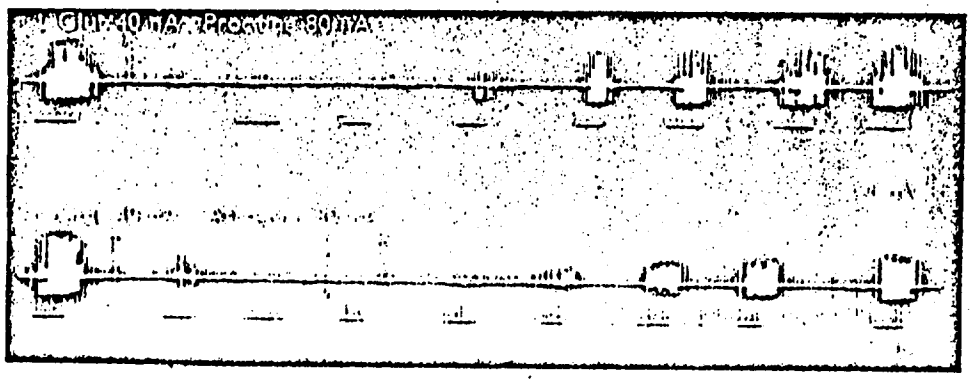

Fig. 19. Comparing depressant actions of similar iontophoretic currents of procaine and atropine on same cortical unit in cat under Dial; periods of release are indicated by two arrows. Excitability was tested repeatedly by applying L-glutamate during times shown by horizontal white lines below traces.

as described above). The depressant action was usually shown by first exciting units with L-glutamate. Among the most effective compounds were ephedrine, dopamine and 5-HT (see Fig. 10), while somewhat weaker depressions were seen with adrenaline, noradrenaline, amphetamine and histamine. Even the most potent were much less active than GABA.

In contrast to these comparatively short-lasting effects, LSD (lysergic acid diethylamide) and several derivatives (cf. Curtis & Davis, 1962) had a depressant action that was only slowly reversible (full recovery required a period of at least 30 sec); this effect was rather similar to the general blocking action of atropine described below.

Procaine, atropine and hyoscine. Like spinal neurones (Curtis & Phillis, 1960), most cortical units are readily depressed by atropine and hyoscine released iontophoretically. An example is given in Fig. 19, which shows clearly the associated characteristic reduction in spike size, an effect that persists for 30–60 sec after the end of the application. This blocking action of atropine is no doubt due to its local anaesthetic properties, and it is, indeed, almost identical in its time course with the effects produced by a similar iontophoretic current of procaine (Fig. 19). But procaine, unlike atropine, does not cause a prolonged interference with the size of the spike.

This type of depression by atropine and hyoscine is fully reversible within 1 min and it bears no relation to the ACh sensitivity of certain cells. It must therefore be sharply distinguished from the specific, prolonged block by atropine of the excitation of cholinoceptive neurones by ACh (Krnjević & Phillis, 1962, 1963*b*).

DISCUSSION

Current effects

Our observation that outward currents (with micropipette tip relatively positive) in general tend to depress neuronal activity, while inward currents have the opposite action, are in agreement with some previous reports based upon studies with micropipettes of comparable tip dimensions (Burns & Salmoiraghi, 1960; Curtis & Koizumi, 1961). The effect is not surprising, since the inward current flow will tend to depolarize the nearer portions of the neurone, and this may be enough to trigger spikes.

With very fine micropipette tips it is possible to approach much closer to the cell. If the tip is moved until the recorded spike becomes nearly positive, it is likely that the tip is now pushing against the cell membrane and causing local inactivation. Under these conditions the changes observed should resemble those seen when current flows into a cell from an intracellular electrode, i.e. an outward current depolarizes and therefore excites the neurone and vice versa (Strumwasser & Rosenthal, 1960). This effect will be only seen occasionally with large tips, which are very unlikely to approach a cell sufficiently without causing fatal damage, as in the present study. It is therefore very surprising that Spehlmann & Kapp (1961) have found consistently this kind of behaviour while studying neurones in the visual cortex of the cat with pipettes comparable to ours.

Tasaki, Polley & Orrego (1954) have calculated that the extracellular spikes recorded from cells in the visual cortex are consistent with an external current flow in the order of 10^{-7} A (for a 10 μ cell). This is similar to the currents which often caused appreciable changes in the cellular firing when applied from micropipettes and would seem to support the belief that the excitability of cells can be altered by adjacent neuronal activity (Bremer, 1941; Gerard, 1942).

Actions of amino acids

Possible transmitter functions. One cannot fail to be impressed by the powerful complementary actions of L-glutamate and GABA. These substances, together with glutamine, form a very closely related trio, potentially capable of a full control of neuronal activity. Glutamine itself is rather inactive and so suitable for storage; L-glutamate apparently excites all cortical neurones in a manner which lends itself to fine gradations of

intensity and duration, without desensitization or damage when applied repeatedly or for a prolonged period; while GABA inhibits neurones no less effectively and with a similar flexibility. It is therefore all the more significant that all three compounds are not only present in the brain in abundance but are very common currency in the cerebral metabolic exchanges (McKhann, Albers, Sokoloff, Mickelson & Tower, 1960; Albers, 1960).

Clearly, in addition to the substantial quantities of L-glutamate and GABA normally present in the cortex, a copious supply is thus assured from metabolic processes. Although there is no conclusive evidence at present that glutamate and GABA function in the brain as excitatory and inhibitory transmitters, it is obvious that their quick and reversible actions and ready availability make them eminently suitable for such roles.

Glutamate in the cortex. The normal L-glutamate content of the mammalian brain is about 10 μ-mole/g (Berl & Waelsch, 1958; Tower, 1960). If this was evenly distributed in the cerebral tissues, the concentration in the extracellular fluid would be approximately 10 mM. This is certainly much more than the threshold concentration for excitation and would probably keep the cerebral neurones permanently depolarized (cf. Hillman & McIlwain, 1961).

The iontophoretic method does not permit a very accurate estimate of concentration in the tissues, but it is possible to calculate approximately the probable concentration obtained by diffusion at various distances from the tip of the pipette, taking the latter as an instantaneous or a continuous point source, in an infinite homogeneous medium (del Castillo & Katz, 1955; Carslaw & Jaeger, 1959; Curtis *et al.* 1960).

The analysis based on the continuous source (with constant current) is of limited value, because we have little indication of the distance between the pipette tip and the neurone under observation. When short pulses of current are used, the tip of the pipette becomes an 'instantaneous' point source, and there is a simple relation between the time at which the maximum concentration is reached at any point, and its distance from the source (as pointed out by del Castillo & Katz, 1955). Thus, in view of the extremely rapid action of L-glutamate it is a reasonable assumption that the peak concentration coincides with the peak of the neuronal response: we can then calculate both the concentration and the distance between the tip and the neurone. The peak maximum concentration is given by $C = \frac{Q\exp(-1{\cdot}5)}{8(\pi DT)^{1{\cdot}5}}$, where Q is the amount of substance released 'instantaneously', D its diffusion coefficient and T the time to the peak.

If we take as an example the response to a pulse of L-glutamate shown in Fig. 7*A*, the total charge passed through the pipette was about 10^{-8} C. Taking the transport number for glutamate as 0·5, the value of Q is about 5×10^{-14} mole. The coefficient of diffusion of glutamine in aqueous solutions at 25° C is $7{\cdot}62 \times 10^{-6}$ cm²/sec (Longsworth, 1953). If we assume that glutamate diffuses at a similar rate, and make allowances for the higher temperature (a factor of about 1·5) and for the slowing of diffusion by various obstructions in the brain (a factor of about 2, cf. McLennan, 1957), the probable effective diffusion coefficient is approximately 5×10^{-6} cm²/sec. From Fig. 7*A* it is clear that the peak response occurs at

about 0·3 sec: calculating C in the above equation thus gives a value of $1{\cdot}4 \pm 10^{-7}$ mole/ml. or 0·14 mM. The corresponding diffusion distance is obtained from $l = \sqrt{6DT}$, i.e. 30 μ in this instance. Similar calculations for the responses shown in Fig. 8*A* and *B* gave values of 0·095 mM for both peak concentrations and 47 and 25 μ for the respective distances, while the comparable discharge of another cortical unit, in a monkey, corresponded to a peak concentration of 0·12 mM and a diffusion distance of 17 μ. It is clear that the calculated effective concentrations fall within a fairly narrow range. Moreover, the calculated diffusion distances are well inside the limits beyond which substantial spikes are not likely to be recorded.

Glutamate is not likely to diffuse freely in all directions in the brain, as in the theoretical model. Although there is almost no true extracellular space in the brain, a substantial fraction (0·2–0·5) of the total volume, possibly made up of astroglia, is relatively accessible to several 'extracellular' ions (De Robertis & Gerschenfeld, 1961). If we assume that glutamate also moves freely through this space, and make the appropriate correction, the true tissue concentrations of L-glutamate which caused a fast discharge in our experiments were probably in the region of 0·5–1·2 mM. The weak and inconstant excitation produced by prolonged applications of K currents tenfold greater give some support to this analysis, since an increment of 5–10 mM in external K is likely to be the minimum required for an appreciable depolarization.

L-glutamate can therefore only be stored in the brain inside cells. If we take the intracellular fluid fraction as 0·5–0·8 the amount of L-glutamate, inside an average nerve cell (with a volume of 6620 μ^3 according to Ramon-Moliner, 1961) would be $13{\cdot}2$–$8{\cdot}3 \times 10^{-14}$ mole. The smallest amount needed to excite a cell by iontophoresis was about 10^{-14} mole.

Glutamate in spreading depression. Our observations evidently support the suggestion that spreading depression may be caused by the liberation of intracellular glutamate (Van Harreveld, 1959), although K might also leak out of depolarized cells, and so contribute to the general spread of depolarization. According to Curtis & Watkins (1961) spreading depression is elicited most effectively by *n*-methylaspartate. This can be accounted for by its characteristic prolonged action (though relatively slow in onset) and the fact that it is probably not metabolized rapidly. The depression of the sensitivity of the neurones to L-glutamate, which persists for as much as 30 sec after a short iontophoretic application of *n*-methyl-DL-aspartate (cf. Fig. 6), may be an important additional factor if L-glutamate is concerned in normal transmission. These features strongly suggest that the binding between *n*-methyl-DL-aspartate and the glutamate receptor is only slowly reversible; its action would therefore in some respects be analogous to that of decamethonium at peripheral cholinergic synapses (del Castillo & Katz, 1957).

Mechanism of L-glutamate and GABA actions. Although it has been

suggested that L-glutamate and GABA act mainly inside cells (Elliott, 1960; Hillman & McIlwain, 1961) their actions are so rapid (with latencies corresponding to the expected diffusion times) as to be necessarily in the region of the surface membrane. In any case, since there is already so much glutamate and GABA inside, the entry of a fraction of 10^{-14} mole would not change the intracellular content significantly. It is known that cells in general can accumulate amino acids against a concentration gradient (Christensen, 1960), and the uptake of L-glutamate by cortical cells has been demonstrated under various conditions (Stern, Eggleston, Hems & Krebs, 1949; Tower, 1960). The simplest explanation for the action of L-glutamate is one which would account for both its transport into the cell, and the simultaneous apparent increase of Na^+ permeability. If we postulate the existence in the membrane of a carrier molecule with a resultant positive charge, capable of reacting with glutamate, it is not too difficult to suppose that this positive charge may be so placed as to prevent the inward movement of Na^+. Combination with L-glutamate might then allow an appreciable influx of Na^+ until the return of the carrier to its original position. This mechanism would ensure the rapid removal of glutamate from the external fluid and thus help to explain the lack of an after-discharge. The inward movement of the carrier is itself apparently related to the Na^+ influx in some way, since glutamate accumulation ceases in the absence of external Na^+ (Takagaki, Hirano & Nagata, 1959).

We know little about the properties of the L-glutamate receptor (? carrier). Our results are consistent with the hypothesis of a 3-point receptor for excitatory amino acids, with fairly rigid steric specificity (Curtis & Watkins, 1960). In fact, cortical neurones seem to be much more discriminating in this respect than spinal neurones, which are almost equally sensitive to L- and D-glutamate, according to the same authors. Although D-glutamate is much less active, this is apparently compensated by the slow rate of its utilization by the tissue (Weil-Malherbe, 1936; Stern *et al.* 1949; Takagaki *et al.* 1959) so that a little may go a great deal further, especially in the initiation of spreading depression (Van Harreveld, 1959). Unlike *n*-methyl-DL-aspartate, it does not interfere markedly with the action of L-glutamate, presumably because of its low affinity for the receptor. If the postulated carrier molecule is, or includes, a metal ion, the substantial excitant action of EDTA would be readily understood.

In the case of the neutral, short-chain ω-amino acids, we are dealing presumably with a different carrier, which may be involved in the accumulation of GABA by cortical slices, demonstrated by Elliott & van Gelder (1958). Amino-acid transport is said to be closely related to the accumulation of K (Christensen, 1960) so that it is conceivable that movements of the ω-amino-acid carrier are somehow associated with the opening of

smaller channels, permeable to K^+ and possibly Cl^- but not Na^+, which would tend to stabilize the membrane potential near its resting level and so reduce excitability.

Actions of other substances. The most common action of the various indole and catecholamines has been a depression of neuronal activity, in agreement with previous observation on the effects of intracarotid injections (Marrazzi, 1961); but all these compounds were less effective than GABA (even dopamine, cf. McGeer, McGeer & McLennan, 1961). It seems a remarkable fact that neurones in some other parts of the central nervous system are apparently quite insensitive to all these drugs (e.g. neurones in the mid-brain and the medulla, Curtis & Koizumi, 1961, and in the spinal cord, Curtis, Phillis & Watkins, 1961*b*; Curtis, 1962); even in the lateral geniculate body several of these compounds block synaptic transmission but not excitation by L-glutamate (Curtis & Davis, 1962). The ease with which the firing of cortical neurones by L-glutamate is depressed by 5-HT and other compounds suggests that these substances do not cause a specific block of all synaptic transmission in the brain.

SUMMARY

1. Many substances have been applied to single units in the cerebral and cerebellar cortex of cats, rabbits and monkeys, under various conditions of anaesthesia, by iontophoretic release and, in some cases, also by pressure injection from multibarrelled micropipettes.

2. L-glutamate and several related amino acids (but not D-glutamate) excited all neurones quickly and powerfully. The effect was almost instantly reversible and could be obtained with minute amounts of excitant (10^{-14} mole). Repeated or prolonged applications did not lead to desensitization.

3. Other amino acids with weaker excitatory actions included long-chain ω-amino monocarboxylic acids and asparagine; glutamine was very ineffective.

4. A small proportion of neurones were excited in a characteristic manner by ACh: the onset of firing was slow, and there was a prolonged after-discharge.

5. General excitation was also caused sometimes by curare and nicotine (this effect was not related to ACh-sensitivity), by H^+, EDTA, and relatively large doses of compounds like 5-HT and adrenaline.

6. GABA and several other short-chain ω-amino acids, as well as some ω-guanidino derivatives, had a powerful and quick depressing action on all neurones, comparable in effectiveness with the exciting action of L-glutamate.

7. The excitability of all cortical neurones could be blocked temporarily with procaine, atropine and hyoscine, recovery taking place within 30–60 sec.

8. Various indole and catechol amines, such as ephedrine, dopamine, LSD, 5-HT adrenaline and amphetamine also depressed neuronal activity in a reversible manner.

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HISTORY & DEVELOPMENT OF FLAVOR NUCLEOTIDES

Akira Kuninaka,
Masajiro Kibi,
and Kin'ichiro Sakaguchi

See Cover

"FLAVOR THROUGH NUCLEOTIDES"

■ MSG AND IMP BEGINNING AS FLAVOR ENHANCERS. Since ancient times both sea tangle, *Laminaria* sp., and dried bonito have been generally used for seasoning Japanese meals, because they can be well preserved for a long time and their extracts have characteristic flavors.

Ikeda (1908, 1909) reported that the main flavor component of sea tangle extract was MSG (the abbreviations used herein are explained in a note following the article). It was possible to start industrial production of MSG as a chemical seasoning almost simultaneously because it had been known that L-glutamate was present in protein hydrolysate. At present, MSG is produced not only by protein hydrolysis but also by direct fermentation or chemical synthesis, and it is used as an excellent flavor enhancer not only in Japan but also in all the world.

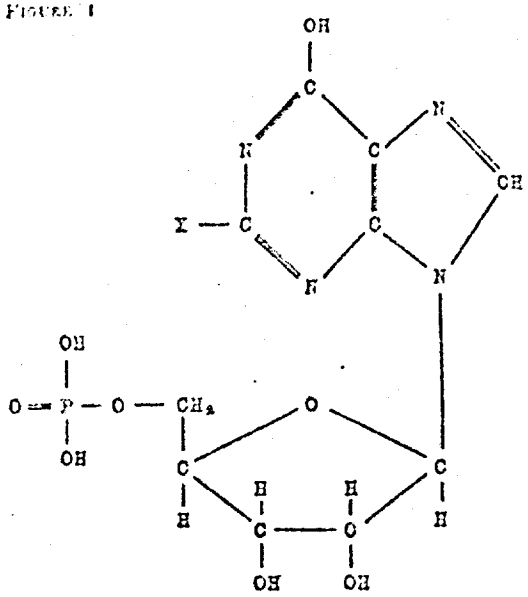
On the other hand, Kodama (1913) reported that the principal flavor component of dried bonito was the histidine salt of IMP. However, IMP has not been produced as a chemical seasoning on a large scale until quite recently, because both the biochemical background and the flavor property of IMP are more complicated than those of MSG.

For example, there are two types of glutamate—L- and D-isomers whereas there are three types of IMP: 2'-, 3'-, and 5'-isomers. L-Glutamate is a natural component of protein, but D-glutamate is not. Flavoring action is recognized only in L-glutamate, and not in D-glutamate. The relationship between isomerism and flavoring action of IMP had never been reported, nor had experiments been made that would allow one to define the interaction between IMP and histidine. Furthermore, the biochemistry of nucleic acid is a more modern field than that of protein or carbohydrate, so the biochemical position of IMP was clarified much later than that of L-glutamic acid.

Discovery of Flavor of 5'-IMP and 5'-GMP, and their Production from RNA.

In 1951, the authors started to study both the enzymic degradation of nucleic acid by microorganisms and the taste of the degradation products. Several *Aspergillus* enzymes involved in the RNA degradation were found, and the conditions for their activities were clarified [Kuninaka (1954, 1955, 1956, 1957, 1959)]. Using the enzyme system of *Aspergillus oryzae*, 3'-IMP could be obtained from RNA. However, the 3'-IMP obtained had little or no flavor. The mixture of 2'- and 3'-IMP derived from alkaline hydrolysis of RNA

FIGURE 1



X = H, NH_2, (or OH)

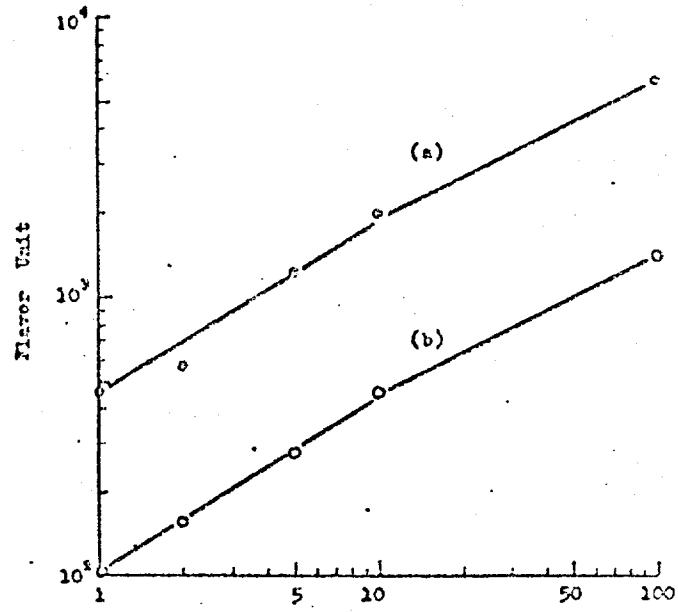


FIGURE 2

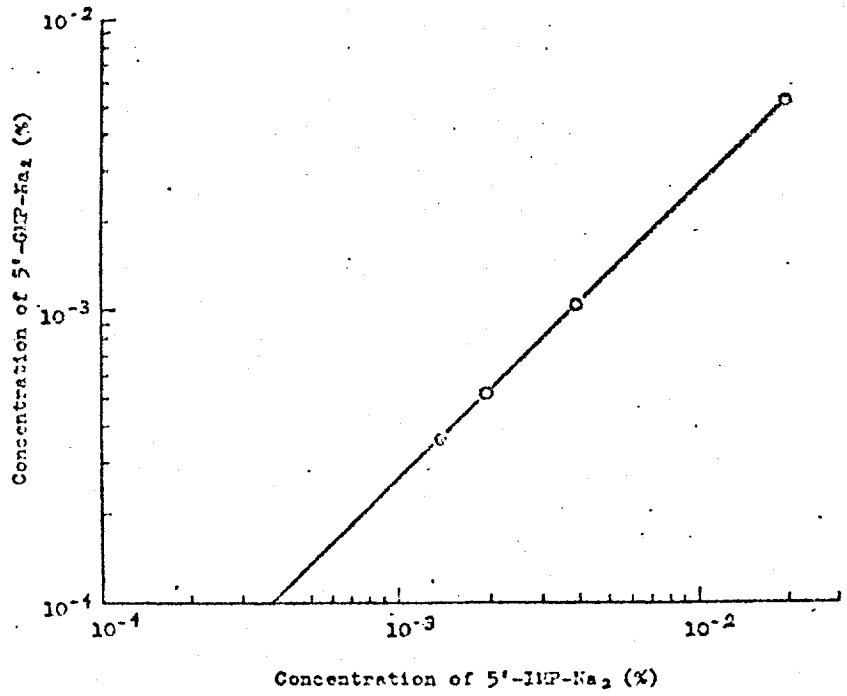
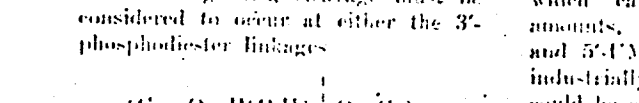


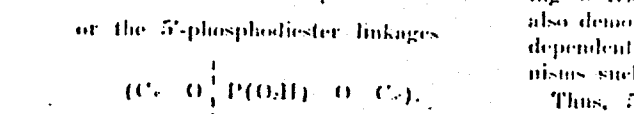
FIGURE 3

was also found not to be flavorous. On the other hand, 5'-IMP free from histidine, which was prepared from muscle of rabbit or bonito, was confirmed to have a specific flavor, especially together with MSG. Thus, it was demonstrated that, among three isomers of IMP, only 5'-IMP was flavorous.

The authors therefore undertook to prepare 5'-IMP from RNA. Recently, it has become evident that the RNA is a polynucleotide in which individual nucleoside residues are joined, one to the other, by phosphodiester linkages between the 3'- and 5'-positions. When RNA is degraded, cleavage must be considered to occur at either the 3'-phosphodiester linkages



or the 5'-phosphodiester linkages



Most of RNA-degrading enzymes split 3'-phosphodiester linkages in RNA, giving rise to nucleoside-2',3'-cyclic phosphates or 3'-nucleotides. Alkali splits the same linkages, giving rise to nucleoside-2',3'-cyclic phosphates, which are further cleaved to form 2'- and 3'-nucleotides.

Only so-called unspecific phosphodiesterases of snake venom and intestinal mucosa had been demonstrated to split 5'-phosphodiester linkages in RNA, giving rise to 5'-nucleotides. However, it is very difficult to obtain large amounts of these enzymes free of phosphomonoesterase activity. Thus, chemical or enzymic production of 5'-nucleotides from RNA was very difficult, and economical industrial production thereof was quite impossible.

Kuninaka *et al.* (1957a,c) demonstrated that 5'-phosphodiesterases, which split 5'-phosphodiester linkages in RNA, giving rise to 5'-nucleotides, were formed by several microorganisms such as *Penicillium citrinum*. Using the microbial 5'-phosphodiesterase, which can be obtained in large amounts, 5'-AMP, 5'-GMP, 5'-CMP, and 5'-UMP could be produced first industrially from yeast RNA. 5'-IMP could be easily obtained by deaminating 5'-AMP. Omura *et al.* (1958) also demonstrated similar enzymes independently in several microorganisms such as *Streptomyces*.

Thus, 5'-IMP has been produced microbiologically and used as a new seasoning. Furthermore, establishment of this RNA degradation process also led to recognition of the flavor of 5'-GMP, one of the RNA degradation products, for the first time. 5'-GMP was found to have stronger flavor than 5'-IMP (Sakaguchi *et al.*, 1958; Kuninaka, 1960).

Japanese Food Additive Petitions for 5'-IMP·Na_2 and 5'-GMP·Na_2 were respectively approved on September 10th, 1960, and on June 1st, 1961. The specifications of the Japanese Official Standardizations are summarized in Tables 1 and 2. Note that 5'-IMP·Na usually contains 7.5 molecules of crystallization water, and 5'-GMP·Na does not contain crystallization water. Used in the experiments reported here were standardized 5'-IMP·Na_2 and 5'-GMP·Na_2.

Relations between Flavor and Chemical Structure of RNA Derivatives

The tastes of various RNA derivatives were summarized by Kuninaka (1960) as follows: Purine and pyrimidine bases, nucleosides, and polynucleotides had little recognizable taste. On the other hand, mononucleotides had good taste. The taste of 5'-nucleotides was much stronger than that of 2'- or 3'-nucleotides. Especially, 6-hydroxypurine ribonucleoside 5'-monophosphates (5'-GMP, 5'-IMP, and 5'-XMP) had very agreeable good tastes (5'-GMP > 5'-IMP > 5'-XMP). Furthermore, the equimolar mixture of inorganic phosphate and inosine (or guanosine) or the equimolar mixture of ribose 5-phosphate and hypoxanthine (or guanine) had no flavor. Thus, both ribosidic and phosphate ester linkages were considered to be essential for flavoring action.

From these findings, the chemical structure necessary for the flavoring action may be summarized as follows:

Fig. 1. General constitutional formula for the flavor nucleotides.

Fig. 2. Difference of flavor unit of nucleotide MSG composition and that of MSG.

(a): [Flavor unit of composition of each amount of 5'-GMP·Na_2 and 100 g of MSG] − [Flavor unit of 100 g of MSG]

(b): [Flavor unit of composition of each amount of 5'-IMP·Na_2 and 100 g of MSG] − [Flavor unit of 100 g of MSG]

1 Flavor unit was defined as flavor strength of 1 g of MSG.

The following relationship is suggested to exist between (a) and (b):

$$\log(a) - \log(b) = \log\frac{(a)}{(b)} = \log(3.57 \text{ to } 4.68)$$

$$(a) = (3.57 \sim 4.68)(b)$$

Fig. 3. Comparison of flavor activity of 5'-GMP·Na_2 and that of 5'-IMP·Na_2 in their synergistic action with MSG.

[5'-GMP·Na_2] corresponding to [5'-IMP·Na_2] were determined in the MSG sodium chloride solution by means of paired comparison tests.

[5'-GMP·Na_2]: concentrations of 5'-GMP·Na_2

[5'-IMP·Na_2]: concentrations of 5'-IMP·Na_2

MSG concentration: 0.2% (○) or 0.67% (●)

Sodium chloride concentration: 1.2%

The following relationship was suggested to exist between [5'-GMP·Na_2] and [5'-IMP·Na_2]:

$$\log[5'\text{-GMP}\cdot Na_2] = \log\frac{0.00165}{0.00430} + \log[5'\text{-IMP}\cdot Na_2]$$

$$3.8\,[5'\text{-GMP}\cdot Na_2] = [5'\text{-IMP}\cdot Na_2]$$

Therefore, flavor activity of 5'-GMP·Na_2 is 3.8 times as much as that of 5'-IMP·Na_2 in the synergistic action with MSG.

Table 1. Japanese official standardization.

Disodium 5'-inosinate	
Colorless or white crystals that have a specific taste	
Nitrogen (dried)*	14.0~14.6%
Phosphorus (dried)*	7.6~8.2%
Max. U.V. absorption (in 0.01N HCl)	250 ± 2 mμ
Ribose (formed) (reaction)	positive
Organic phosphate	positive
Paper chromatography*	U.V. absorbing one spot
Sodium	positive
Solution (0.5 g→10 ml)	colorless, little or no turbidity
pH (1 g→20 ml)	7.0~8.5
Arsenic	negative
Nitrogen	Max. 2 ppm
Heavy metal	Max. 20 ppm
Guanylic acid	negative
Loss on drying*	Max. 26.5%

* 120°C for 4 hr.
* A mixture of saturated ammonium sulfate, tert-butanol, and 0.025N ammonia solution (160:3:40) is employed as the solvent.

Table 2. Japanese official standardization.

Disodium 5'-guanylate	
Colorless or white crystals or powder that has a specific taste.	
Nitrogen (dried)*	16.2~17.2%
Phosphorus (dried)*	[illegible]
Max. U.V. absorption (in 0.01N HCl)	256±2 mμ
Specific U.V. absorption (in 0.01N HCl)	270~280 mμ
Ribose (formed) (reaction)	positive
Organic phosphate	positive
Paper chromatography*	U.V. absorbing one spot
Sodium	positive
Solution (0.1 g→10 ml)	colorless and almost clear
pH (1 g→20 ml)	7.0~8.5
Arsenic	negative
Nitrogen	Max. 2 ppm
Heavy metal	Max. 20 ppm
Guanylic acid	negative
Loss on drying*	Max. 8%

* 120°C for 4 hr.
* A mixture of saturated ammonium sulfate, tert-butanol, and 0.025N ammonia solution (160:3:40) is employed as the solvent.

1) The base moiety is to be a purine nucleus containing a hydroxy group in the 6-position. (An amino group in the 6-position reduces the flavoring action. The influence of the kind of the group in the position 2 on the taste is not so much. The tastes of pyrimidine nucleotides are inferior to those of purine nucleotides.)

2) The 5'-position of the ribose moiety is to be esterified with phosphoric acid.

The general constitutional formula for the flavor nucleotides may be shown in Fig. 1. In brief, both the 6-hydroxypurine moiety and ribose-5-phosphate moiety are essential to flavoring action of RNA derivatives.*

It is very interesting that the conditions necessary for the structure of nucleotides that are substrates of *Aspergillus* ribosidase[5] are the same as those for the flavor nucleotides.

It may be suggested that there is a kind of analogy between the specific relation of a flavor nucleotide to the human gustatory bud and that of a substrate for the enzyme. Therefore, it seems possible to select unknown flavor substances by means of enzyme action.

By the way, mosquitoes were demonstrated by Hosoi (1961) to be specifically fond of 5'-AMP or its derivatives but not fond of the other nucleotides, nucleosides, bases, ribose, and amino acids such as glycine, glutamic acid, cystein, arginine, tyrosine, and histidine. Thus, the human and mosquito palates are similar to each other in the selectivity of 5'-nucleotides. However, there are differences between their detailed specificities: the human palate is an *Aspergillus* ribosidase type, whereas the mosquito palate is an *Azotobacter* ribosidase type.

The taste of 6-hydroxypurine ribonucleoside 5'-monophosphates (5'-IMP, 5'-GMP, and 5'-XMP) was milder or softer than that of MSG, while the aftertaste of the former was much more remarkable than that of the latter. 5'-GMP has the strongest taste in these three 5'-nucleotides, and 5'-XMP has the weakest taste. The threshhold level for recognition of these 5'-nucleotides was suggested to be about 0.01%. Toi *et al.* (1960) and Wagner *et al.* (1962) later respectively reported that the threshold level for recognition of 5'-IMP·Na₂ was 0.025% and 0.02%. Fujita *et al.* (1961) reported that the threshold levels for recognition of 5'-IMP·Na₂ and 5'-GMP·Na₂ were respectively 0.012% and 0.0035%.

Synergistic Action between MSG and 5'-Nucleotides

There are many kinds of interaction in taste among various components of foods or beverages. For example, an additive effect is recognized when two or more different acids or two or more different sugars are mixed; a contrastive effect is recognized when a small amount of salt is added to sugar; and a harmonization effect was recognized when MSG was added to saccharin (Maeda *et al.*, 1956) or salt.

The 5'-nucleotides were considered to have a same kind of taste, and an additive property was recognized between the taste of 5'-GMP and that of 5'-IMP. Succinate and basic amino acids, such as histidine, lysine, and arginine, were not recognized to enhance significantly the flavorous taste of 5'-GMP or 5'-IMP. On the contrary, MSG was recognized to enhance remarkably the flavorous taste of 5'-GMP or 5'-IMP, and vice versa.

For example, addition of 5'-IMP or 5'-GMP to MSG remarkably strengthened the taste of MSG, as follows: MSG and 5'-IMP·Na₂ or 5'-GMP·Na₂ were mixed in various ratios. Each mixture was dissolved in 1.2% sodium chloride solution at various concentrations. The tastes of the resulting solutions were compared with the taste of 1.2% sodium chloride solution con-

* Lately 5'-deoxy inosinate and 5'-deoxy guanylate were also reported to have flavorous taste (Nakao *et al.*, 1960). Furthermore, 2'- and 3'-hydroxy groups seem not to be essential for the flavoring action (Honjo *et al.*, 1963).

* According to the prevailing theory, the phosphoric acid group in the nucleotides has to be removed before the ribosidic linkage can be resolved by enzymes. Kuninaka (1956, 1957, 1959) furnished the first evidence for the conclusion that hydrolytic enzyme (ribosidase) for the cleavage of the bond between ribose 5-phosphate and hypoxanthine or guanine does exist in *Aspergillus oryzae*. This enzyme cleaves specifically the ribosidic linkages of 6-hydroxypurine ribonucleosides (inosine and guanosine) or their 5'-phosphates. 5'-AMP, 5'-CMP, 5'-UMP, adenosine, cytidine, uridine, and various 3'- or 2'-mononucleotides are not cleaved by the enzyme. No evidence has been found for the requirement of ATP or inorganic ortho or pyrophosphate. Hurwitz *et al.* (1957) also independently demonstrated the enzymic cleavage of ribosidic linkage in 5'-AMP, using *Azotobacter vinelandii*. Both *Aspergillus* and *Azotobacter* ribosidases are nonphosphorolytic hydrolases, and act on 5'-nucleotides but do not act on 2'- or 3'-isomers. However, these enzymes are distinguishable from each other in regard to detailed substrate specificity and relation to ATP. While 5'-AMP-ribosidase of *Azotobacter* requires ATP, *Aspergillus* ribosidase does not.

Table 3. Comparison of MSG synergistic action between 5'-IMP and MSG in flavor.

Ratio of components in combination	Concentration of [illegible] which have flavor strength equal to that of 0.3% MSG solution
MSG : {5'-IMP·Na₂ / (5'-GMP·Na₂)}	
1:0	0.3%
1:1	0.0[illegible]% ([illegible])
10:1	0.0[illegible]% ([illegible])
20:1	0.05% (0.024%)
50:1	0.12% ([illegible])
100:1	0.15% (0.05%)

Each solution contained 1.2% of sodium chloride.

taining 0.3% MSG by *paired comparison tests*, respectively. Then the concentration of each mixture solution, that had a taste strength corresponding to that of 1.2% sodium chloride solution containing 0.3% MSG, was determined as shown in Table 3. The results show that a small amount of 5'-IMP or 5'-GMP can synergistically increase the srtength of the taste of MSG.

As shown in Fig. 2, the synergistic action between MSG and 5'-GMP was about four times as much as that between MSG and 5'-IMP. Another experiment also indicated that 5'-GMP·Na₂ was as effective as about 3.8 times as much 5'-IMP·Na₂ when dissolved in 0.2% or 0.07% of MSG solution (Fig. 3).

Although there was a small harmonization effect between the taste of 5'-GMP or 5'-IMP and that of histidine or succinate, the synergistic action between MSG and 5'-nucleotides was much more remarkable than such harmonization effect. Furthermore, this synergistic action was recognized both in the presence and in the absence of the other components. Such remarkable synergistic action as mentioned above had been never conceived, at least in the field of taste chemistry.

By the way, the threshold level for recognition of 5'-IMP·Na₂ or 5'-GMP·Na₂ in 0.1% MSG solution was reported to be 1/129 or 1/117 that in water (Fujita *et al.*, 1961). Independently, the threshold levels for recognition of 5'-IMP·Na₂ in 0.1% sodium aspartate solution, MSG solution, sodium β-hydroxyglutamate solution, sodium α-aminoadipinate solution, and sodium homocysteinate solution were reported to be 1/10, about 1/100, 1/50, 1/10, and 1/100 of that in water, respectively, and the threshold levels for recognition of sodium aspartate, MSG, sodium β-oxyglutamate and sodium α-aminoadipinate in 0.01% 5'-IMP·Na₂ solution were confirmed to be 1/46, 1/15, 1/15, and 1/100 of those in water, respectively, too (Toi *et al.*, 1963).

The specific synergistic action indicated above is to be the basis for application of 5'-IMP, 5'-GMP, or their mixtures as new chemical seasonings, because many foods and beverages contain a rather large amount of MSG in themselves whereas 5'-nucleotides are rather unstable intermediates in living tissue so that the distribution of 5'-nucleotides, especially 5'-GMP, in foods and beverages is much more limited than the distribution of MSG. Therefore, addition of 5'-IMP, 5'-GMP, or mixtures of them to those foods or beverages remarkably strengthens and improves the taste of those foods or beverages according to the synergistic action between 5'-nucleotides added and MSG present there originally. Furthermore, the mixture of MSG and 5'-nucleotide(s) is a very effective chemical seasoning for all kinds of dishes.

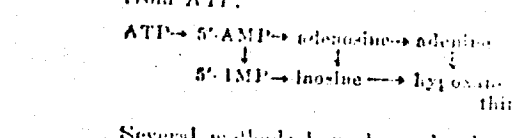
A composition of MSG with 5'-IMP·Na₂ was first sold in Japan as a new chemical seasoning in 1960. At present 5'-IMP·Na₂, 5'-GMP·Na₂, and mixtures of them are used mainly in food processing, whereas compositions of MSG with 5'-nucleotide(s) are mainly used in home life. The following ratios are generally being used: MSG:5'-IMP·Na₂ = 88:12, 92:8, or 96:4. MSG:5'-IMP·Na₂:5'-GMP·Na₂ = 95:2.5:2.5.

At any rate, it is interesting from the viewpoint of biochemistry that there is a synergistic action in taste between MSG derived from protein and 5'-nucleotides derived from nucleic acid, because the interaction between protein and nucleic acid is well known as one of the most important biochemical reactions.

Production of 5'-Nucleotides

There are several methods for production of 5-nucleotides.

Extraction from animal tissues. Mammalian muscle has been well known to contain 5'-IMP. The IMP in fish muscle was also confirmed to be 5'-isomer, which might be derived from ATP.



Several methods have been developed in Japan to get 5'-IMP from marine-animal muscle economically.

Separation of 5'-IMP from fish muscle. According to the author's experiments, fresh muscle of marine fish, such as mackerel-pike and sardine, contained a large amount of 5'-IMP and a very small amount of 5'-AMP. Probably the reactions forming 5'-IMP from ATP are carried out in a fishing-boat on the way to port. During the time muscle was stored at room temperature, 5'-IMP was observed to be degraded enzymically to inosine or hypoxanthine. To steam fish muscle was an effective way of stopping the enzymic degradation of 5'-IMP. Thus the intact waste juice or stick water liberated from fish muscle by steaming, the first general process of canning, was a good source. The juice was equal to about 20% by weight of the intact fish and contained about 0.1% of 5'-IMP.

The impurities that obstruct crystallization of the nucleotide could be removed from the waste juice by three steps (Kuninaka *et al.*, 1957b, 1960): 1) depolymerization of protein by bacterial protease; 2) exclusion of cationic impurities by treatment with cation exchange resin; and 3) precipitation of inorganic phosphate by addition of barium or calcium ion. After these treatments, 5'-IMP was directly crystallized as barium or calcium salt. To separate IMP from fish muscle extracts, electrolytic dialysis was also effective (Yoshida *et al.*, 1953).

Deamination of 5'-AMP of cuttlefish muscle. Saito (1960) reported that cuttlefish muscle lacked adenylic deaminase and that 5'-AMP derived from ATP was accumulated in the muscle effectively without further degradation. Thus, 5'-IMP was produced by deaminating the 5'-AMP accumulated.

The method of preparing 5'-IMP from marine animals is not entirely satisfactory, because the raw material is not stable from either an economical or biochemical viewpoint. Furthermore, the nucleotide that can be separated from marine animals is only 5'-IMP, or 5'-AMP, and it is impossible to separate 5'-GMP economically, though a very small amount of the

[illegible] observed to be present [illegible] nucleic

[illegible] of RNA by 5'-phosphodiesterase. Using the microbial [illegible] diesterase, four 5'-nucleotides, 5'-AMP, 5'-GMP, 5'-CMP, and [illegible]MP can be produced simultaneously from RNA (Kuninaka *et al.*, 1959, 1961; Ohnara *et al.*, [illegible], Ogata *et al.*, 1963). 5'-IMP [illegible] produced enzymically or chemically from 5'-AMP. It is not necessary to purify RNA or the 5'-phosphodiesterase before the enzyme reaction.

RNA can be separated from any [illegible] or plant tissues. It is convenient, however, to utilize microorganisms containing a large amount of nucleic acid. At present, yeast is the best source of RNA in Japan. It is expected that RNA, protein, and vitamin will be separated from yeast and utilized effectively in respective fields. Since yeast is rich in protein and vitamin as well as nucleic acid, nucleic acid might be regarded as a by-product of yeast protein or vitamin. In fact, removal of nucleic acid is considered to enhance the value of the yeast as a protein fodder. The 5'-phosphodiesterase is formed during culture of the screened microorganism. The cultured liquid or the cell extract, as is, can be employed as the enzyme [illegible]

The pharmaceutical value of each of 5'-nucleotides produced from RNA is being disclosed. (Now that 5'-nucleotides can be supplied economically in large amounts for their biological tests, their pharmaceutical uses are expected to develop rapidly. For example, Hirata *et al.* (1962) disclosed the role of 5'-nucleotides as *Bifidus* factor, and Kato *et al.* (1962, 1963) recognized [illegible] remarkable effects of 5'-IMP on [illegible]very from the insulin-shock coma.) [illegible]ppears that valuable products for food and or pharmaceutical uses can be obtained simultaneously from the economical raw materials with this simple procedure. This is the reason [illegible] this RNA process has been recognized as the most effective for producing 5'-nucleotides. This process is expected to be further developed as pharmaceutical uses of 5'-nucleotides are developed.

Direct fermentation (biosynthesis). The direct fermentation process is now being studied most actively in Japan.

Several nucleosides have been observed to be accumulated in rather [illegible] amounts. For example, 1.55 g/L, [illegible] 5 g/L, 2.7 g/L, or more [illegible] g/L of inosine was accumulated by an auxotroph of *Bacillus subtilis* (Aoki *et al.*, 1959; Ichiba *et al.*, 1959, 1961; Nara *et al.*, 1963; Aoki *et al.*, 1963), and 4 to 5 g/L of xanthosine was also accumulated by a guanine-requiring auxotroph of *Aerobacter aerogenes* (Nakayama *et al.*, 1963).

In these cases, the nucleosides might be synthesized *de novo*. On the other hand, adenosine was observed to be produced by a nonexacting purine-requiring auxotroph of *Bacillus subtilis* during its growth in a medium containing adenine (Hara *et al.*, 1962a), and inosine was also observed to be produced from hypoxanthine by an adenine-requiring auxotroph of *Bacillus subtilis* (Kuninaka *et al.*, 1963).

As for the nucleotide, 859 mg/L of 5'-AMP was accumulated by a wild strain (Furuya *et al.*, 1963). It seems more difficult to accumulate large amounts of 5'-nucleotides using microorganism than to accumulate the same amounts of nucleosides.

Chemical synthesis. In several Japanese laboratories chemical synthesis is being studied to produce 5'-nucleotides economically. For example, inosine was reported to have been economically phosphorylated to 5'-IMP (Kato *et al.*, 1963).

Combination. There are various possibilities to combine the above four processes. The following two methods are actually going to be developed in Japan.

Method A (Hara *et al.*, 1962b)

Malononitril, Thiourea → adenine → adenosine → 5'-AMP
(chemically; by bacteria; by yeast)
→ 5'-IMP
(chemically or enzymically)

Method B (Akoki *et al.*, 1963; Kato *et al.*, 1963).

Carbon source, Nitrogen source, Phosphate → inosine → 5'-IMP
(by bacteria; chemically)

Conclusions

5'-Nucleotides and their compositions with MSG are now reforming Japanese food practices. As the processes for their production continue to be improved, they are expected to attain world-wide recognition as new, excellent flavor enhancers. It also appears that improved availability of these compounds will stimulate the development of many other uses of interest to food technologists, nutritionists, and physiologists.

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THE ABBREVIATIONS used herein are as follows: adenosine monophosphate (AMP); inosine monophosphate (IMP); guanosine monophosphate (GMP); xanthosine monophosphate (XMP); uridine monophosphate (UMP); cytidine monophosphate (CMP); ribonucleic acid (RNA); deoxyribonucleic acid (DNA); monosodium glutamate (MSG); adenosine triphosphate (ATP).

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NATURE AND EXTENT OF BRAIN LESIONS IN MICE RELATED TO INGESTION OF MONOSODIUM GLUTAMATE

A Light and Electron Microscope Study

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ABSTRACT

The incidence of arcuate neuron damage following 1 to 4 mg/gm oral doses of monosdoium glutamate (MSG) to neonatal mice was studied. The first morphological manifestation of the lesion involved edematous astrocytic glia and pathological changes in neurons close to the median eminence within 15-20 minutes. The sequence of the lesion involved at least three phases: 1) neuronal edema and death, 2) multiple phagocytic cells, viz. a type of "dense" cell and an electron "lucid" cell (astrocyte), and 3) proliferation of astrocytic processes. It was noted that at 4 mg doses, the lesions occurred in other areas such as tectum, habenular nuclei, subfornical organ, dorsolateral surface of the thalamus, dentate-hippocampal gyri, cerebral cortex, and in the lower medulla, the nuclei gracilis and cuneatus and area postrema. In all these structures, the lesion was initiated superficially and radiated inward, suggesting an inflow of the deleterious agent from the cerebrospinal fluid. Comparison of our neuropathological findings with those of others, emphasized the critical aspects of species variation, developmental age, route of administration, time of examination of brain material after dosage and thoroughness of sampling methods.

INTRODUCTION

The potential toxicity of food additives for the neonate has generated great interest in recent years, especially with regard to the flavor enhancer monosodium glutamate (MSG). The early reports of Olney (21–23) regarding the susceptibility of the mouse retina and the arcuate nucleus of the hypothalamus to neuronal damage after subcutaneous administration of large doses of MSG were subsequently contradicted by a series of investigations employing animals of various species and ages, and utilizing different routes of administration (2, 27). The observation that the arcuate lesion affected primarily neurons was opposed by Arees and Mayer (4) who maintained that the target of damage was the glial cell. Confirmation of neuronal damage has been supplied by Abraham *et al* (1), who found that under certain conditions, mouse arcuate neurons were damaged following MSG administration.

Our studies are an examination of the incidence of this lesion in mouse brain as well as an attempt to elucidate some of the underlying factors contributing

to these lesions. We have identified the successive elements of the arcuate nuclear region involved in the lesion at selected time intervals following dosage. Most importantly, the spread of damaged regions in relation to various intervals after administration and to different dosage levels has been ascertained. Finally, attention has been given to whether the phenomenon of MSG-induced central nervous system lesions is restricted to the neonatal period or whether such lesions can be induced in adult animals as well. The results reported here suggest that the study of the susceptibility of brain to exogenous toxic influences for neonatal animals of various species be expanded in scope.

METHODS

1. *Animal Care.* Three strains of Swiss Albino mice were employed in this study: A/Jax (Jackson Laboratories, Bar Harbor, Maine); SCH:ARS.HA/ICR (Abrams Breeders, Chicago, Illinois); and an A/Jax-ICR hybrid mouse. Unless otherwise indicated, MSG was administered by stomach tube to neonatal mice 7 to 10 days of age. After administration of the designated dose of MSG, the mice were isolated from their dams but kept warm under a heat lamp until the end of the treatment interval (usually 3 to 4 hours). For studies involving an interval of 12 or more hours, infant mice were permitted to suckle.

2. *Acute Studies of MSG.* To study the histologic and temporal aspects of the brain lesion, neonatal mice received a single dose of MSG administered as a 20 percent solution via stomach tube at dosages of 1, 2 or 4 mg/gm of body-weight. Neonatal mice given the largest dose (4 mg/gm) became markedly quiet and ceased motion. Some animals exhibited slight tremors following administration of the amino acid. Death rate was highest for those mice receiving the largest dose (table 1). In this study MSG was most toxic for the A/Jax-ICR hybrid (table 1). These data were analyzed by means of a four-way contingency table and differences between inbred and hybrid strains were statistically significant at both the 2 and 4 mg/gm dosages.

3. *Chronic Studies of MSG.* For these studies infant hybrid mice received 2.2 mg/gm body weight of a 20 percent solution of MSG by subcutaneous injection from the second through the eleventh day of life. Littermates in equal numbers were given saline injections. All injections were given between 10:00 and 12:00 a.m. Growth and reproduction studies performed on these animals are reported elsewhere (31).

4. *Preparation of Brain for Electron Microscopy.* All mice were anesthetized with Nembutal®. The chest was opened and the whole animal perfused via a sharp cannula inserted into the left ventricle. The perfusate which consisted of 2 percent glutaraldehyde and 2 percent paraformaldehyde in phosphate buffer (pH. 7.3) was allowed to flow for 10 minutes. By this time 15 to 20 mls of perfusate had passed through the animal. Heads were removed, immersed in perfusate and refrigerated overnight before removal of the brain. Tissue for study was obtained by making 2 parasagittal cuts, approximately 1mm on either

TABLE 1

Effect of Mouse Strain on Death Rate Following Single Ingestion of Monosodium Glutamate

Dose level (mg/gm)	Inbred Strains		Hybrid Strain		P value
	Dead/Total Fed	Percentage	Dead/Total Fed	Percentage	
1	0/28	0.0	0/12	0.0	—
2	6/27	22.2	0/10	0.0	0.05
4	11/37	29.7	10/118	8.5	0.005

side of the longitudinal fissure of the cerebrum. Next, a cut close to the midline was made. The resulting two slices of tissue were trimmed anteriorly at the preoptic-anterior commissure level and posteriorly through the superior colliculus-midbrain level. These slices were post-fixed in 1 percent phosphate buffered osmium and processed through a graded series of acetones into propylene oxide and finally epon.

Sampling of epon embedded brain was done initially in the coronal plane at 2 levels through the arcuate nucleus. However, it became apparent that sampling in the parasagittal plane close to the midline was a more efficient method of visualizing the arcuate nucleus in its entire anterior-posterior extent. This became the method of choice and most control and treated mice were sampled in this plane. If initial epon sections were midventricle, further sections were cut laterally at several levels.

For study by electron microscopy, the large tissue slices were trimmed to include the arcuate nucleus, peripheral neuropil and median eminence. Ultrathin sections (1 mμ) were stained with uranyl acetate and lead citrate and examined with an RCA EMU 3H electron microscope.

5. *Preparation of Brain for Light Microscopy.* Brain fixation was achieved by immersion of fresh whole brain in 10 percent formalin. The specimens were routinely dehydrated in an alcohol series, placed in one percent celloidin in methyl benzoate for two days, then processed in benzene to paraffin. Serial sections were cut at 10 μ and stained with Cresyl Echt Violet solution.

Sampling of paraffin embedded brains consisted of examination of serial sections of brains from four 9-day-old hybrid mice treated with 4 mg/gm MSG for 3 hours. Sections were cut in the parasagittal plane from olfactory bulb to cervical spinal cord. Three 7-day-old animals given MSG at 1 mg/gm body weight were similarly studied. For optimal interpretation of the structures involved in lesions occurring in the lower medulla, the brains of 9 animals ranging from 8 to 10 days of age were serially sectioned in the coronal plane from the level of the inferior olives to the level of the cervical spinal cord. Five male mice which had been chronically treated with MSG as neonates were killed at 10 months of age and serial sections through the entire brain were made, 3 in the coronal plane and 2 in the sagittal plane. To serve as controls the brains of ten sham-injected animals ranging in age from 8 days to 3 months were serially sectioned in both coronal and sagittal planes.

RESULTS

1. *Time Course for the Arcuate Nuclear Lesion in Neonatal Mice Following a Single Oral Dose of MSG*

In order to gain more information as to the nature of the lesion that follows the ingestion of large doses of MSG by the mouse, it was of prime importance to confirm the nature of the cellular components involved in the lesion and the order of their involvement. Were the damaged structures primarily ependymal cells, capillary cells, glial cells or neurons? In what sequence, if any, did these cells manifest pathological change? To answer these questions a series of animals were given MSG (4 mg/gm) in a 20 percent solution via stomach tube and killed at 10, 15, 20 and 30 minute intervals for short-term morphological studies and at 1, 1.5, 3, 6, 12, 24, 36 and 48 hours for long-term morphological studies. No central nervous system (CNS) lesion was observable by light microscopy in mouse brains obtained 10 to 15 minutes following administration of MSG. With the electron microscope, however, there was some evidence that a small number of astrocytic somatas showed early signs of edema at 15 minutes while ependymal cells and neurons appeared normal. However, 20 minutes after

the ingestion of MSG, lesions were easily observable by either light or electron microscopic methods. With the light microscope, swollen spaces in the arcuate neuropil were the first evidence of abnormality. Electron microscopic observations again revealed that one of the first elements to appear abnormal was the astrocytic glial cell. The somatas and astrocytic processes in the central area of the lesion, but *not* the margins, appeared markedly swollen. Some large dendrites showed early evidences of abnormality, namely, the appearance of large vacuoles, and expansion of agranular reticular profiles. By 20 minutes, neuron somata exhibited abnormalities such as dispersal of ribosomes, swelling of the agranular reticulum and occasionally mitochondrial swelling. The affected areas were those closest to the median eminence. Thirty minutes after ingestion the lesion could be easily detected in plastic sections (fig. 1) since the damaged area was filled with large dilated processes, and some neurons appeared swollen with vacuolated cytoplasm. Ultrastructural changes were mainly an expansion of phenomena observed at 20 minutes. Great numbers of vastly dilated glial and dendritic profiles filled the fields examined by electron microscopy. It was not always possible to distinguish between the two cell processes since massively dilated and vacuolated dendrites could only be identified if they possessed synaptic contacts. Moreover, in neonatal material, synapses, which are just forming, are not found in great numbers. That dendritic profiles were indeed involved is shown in figure 2 in which two synapses are found on

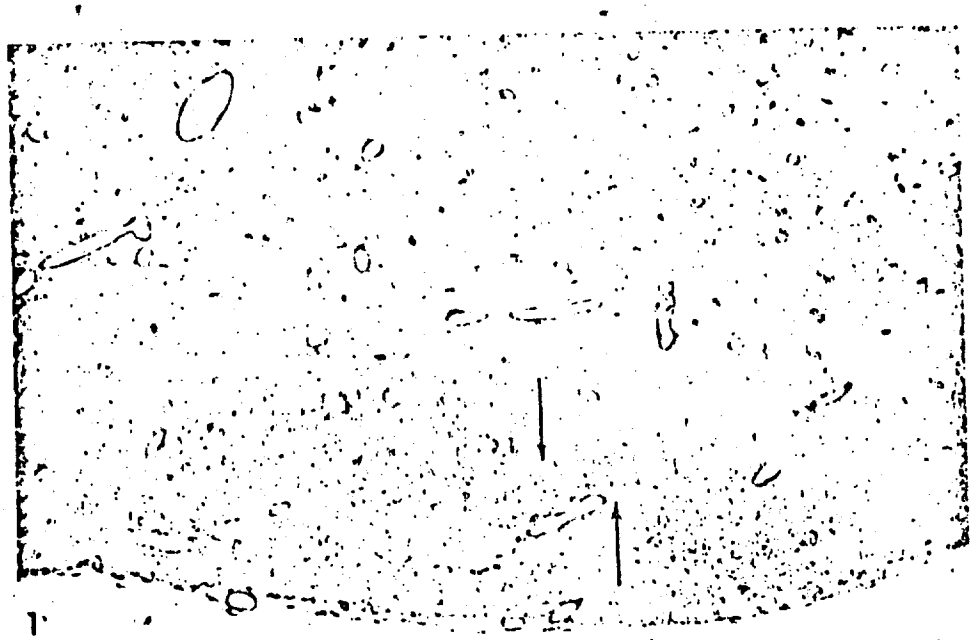


FIG. 1. Parasagittal epon section through the arcuate nucleus of an 8-day-old mouse indicating the degree of damage to the arcuate nuclear area thirty minutes after oral administration of MSG. The most obvious feature is dilated spaces in the neuropil which represent edematous astrocytic and dendritic processes. Close inspection shows neuronal somatas are swollen as well (arrows). Methylene Blue—Azure II. × 180.

FIG. 2. Electron microscopic field of arcuate nucleus from an 8-day-old mouse shown in figure 1. An astrocytic cell (A) and its processes are markedly edematous and many swollen profiles are seen in the neuropil. That at least some of these profiles are dendritic (D) is demonstrated by the presence of synapses on two of the profiles (arrows). An apparently normal neuron (N) lies to the right of the astrocyte. × 11,750.

extensively dilated dendrites. Thirty minutes after the administration of MSG, a large number of neurons are drastically affected. The cytoplasm becomes dense and the disarray of the agranular reticulum and dispersal of ribosomes throughout is marked. At this time many nuclei show clumping of chromatin along the periphery (fig. 3). In some neuronal profiles exhibiting advanced organelle disarray, large membrane-line "vacuoles" appear in the cytoplasm. In other profiles, cytoplasmic vacuolation appears without presence of internal membrane. For the first three hours after MSG administration the predominant picture is that of astrocytic and neuronal edema, vacuolation of neurons and their processes and nuclear pyknosis. All other tissue elements appear normal.

Between three and six hours after administration of MSG the second stage of the lesion, that of phagocytosis, begins. At six hours, observations by light microscopy (fig. 4a) indicate that the number of pyknotic nuclei has greatly increased. Numerous basophilic cells occur within the lesioned area and at its periphery. Electron microscopic observations indicate that the basophilic cells are phagocytic in nature and would probably best correspond to current descriptions of "microglia". Within these cells are found whorled, lamellated bodies and clumps of cytoplasmic debris (fig. 5). Typically, extremely pyknotic cells and dilated dendrites were found to be surrounded by the processes of these dark, phagocytic cells. At twelve hours the situation is essentially the same, although the frequency of lamellated membranous whorls occurring in the microglial cells may be increased, as if these represented later stages in the breakdown of ingested elements.

At twenty-four hours, the appearance of the lesion by light microscopy has changed markedly. Very few edematous processes are found within the lesion which now appears to be filled with phagocytes and nuclei in advanced stages of pyknosis. Random microscopic fields observed in corresponding ultrathin sections reveal a reduced number of dilated dendritic processes and necrotic cells. Swollen profiles of dendritic processes along with considerable numbers of "normal" appearing neurons persisted. The most striking change on the ultrastructural level was the proliferation of astrocytic processes (fig. 6a) which can be easily distinguished from those of the dark microglial cells. Phagocytic inclusions were numerous in both the somas and processes of astrocytic glial cells. At thirty-six and forty-eight hours, the number of neurons was drastically diminshed (fig. 4b) and large numbers of phagocytes were present in each section. From electron microscopic observations astrocytes and microglia seemed to be present in equal numbers, however astrocytic processes predominated. Both cell types showed continued evidence of vigorous phagocytic activity. At this time microglial processes contained many large dense bodies and vacuoles (fig. 6b).

2. *Time Course and Spread of Lesions in Neonatal Brain Following Dosing*

From observations confined to diencephalic-midbrain levels (epon sections) in all experimental situations the first areas to sustain lesions were the arcuate and/or the preoptic region of the hypothalamus. These changes were observed

FIG. 3. Electron micrograph from same animal as figure 1 and 2 showing one of the drastically affected neurons. The agranular reticulum (AGR) is vastly expanded, the ribosomes are dispersed throughout the cytoplasm, the nuclear chomatin is clumped toward the periphery of the nuclear membrane. A synapse (arrow) on the projecting dendrite unequivocably identifies this profile as a neuron. × 11,075.

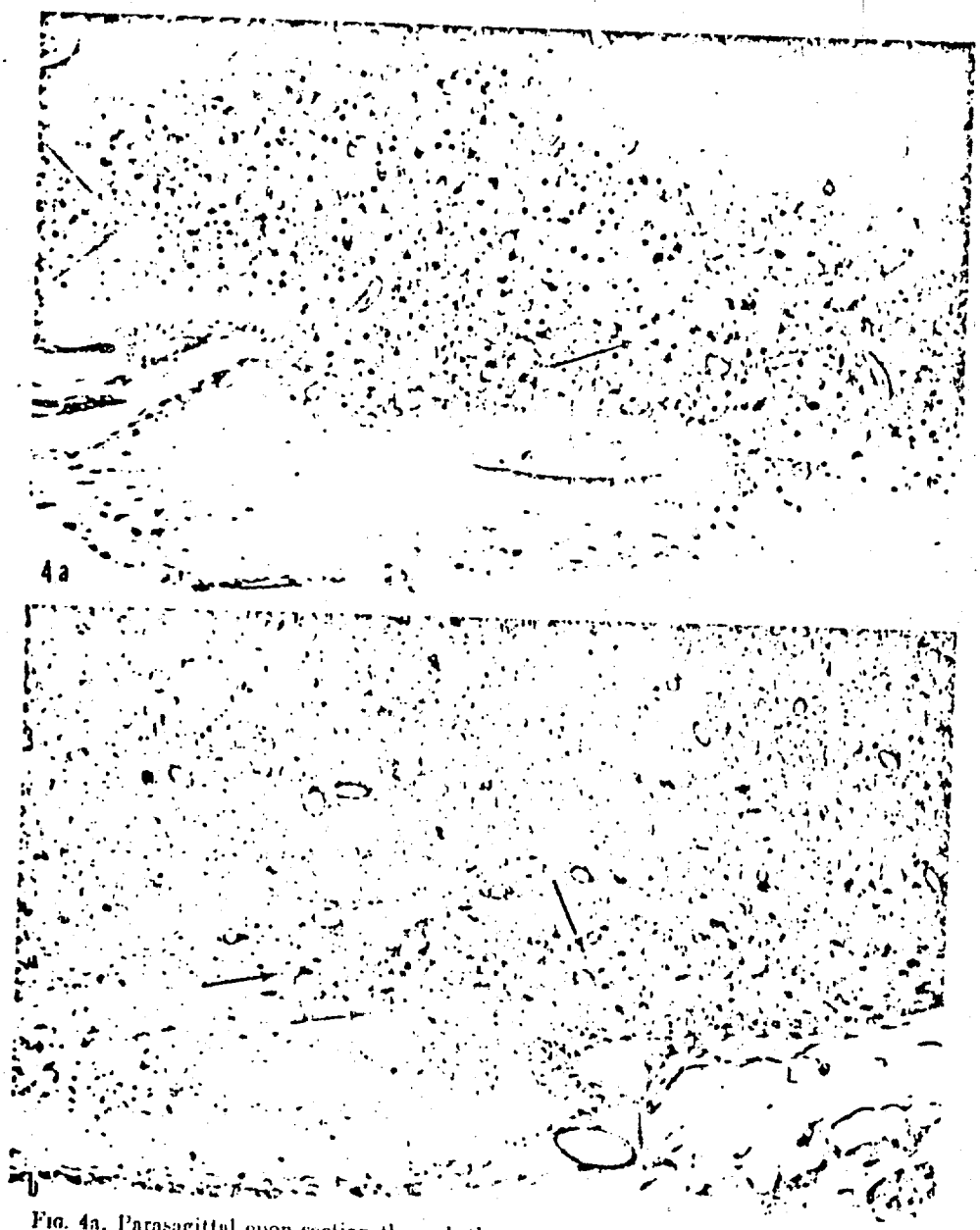


FIG. 4a. Parasagittal epon section through the arcuate nucleus of an 8-day-animal representing degree of damage six hours, after oral administration of MSG. There is a dramatic change in the number of damaged neurons which by now exhibit pyknotic nuclei anad markedly swollen somatas. Scattered throughout the neuropil are dark basophilic elements (arrows) which are probably microgha. Methylene Blue—Azure II. × 172.

FIG. 4b. Parasagittal epon section through the arcuate nucleus of a 10-day-old mouse representing the appearance of the lesioned area 48 hours after oral administration of MSG. Basophilic cells (macrophages) persist, and some neurons (arrows) persist although in vastly depleted numbers. Stain as above. × 200.

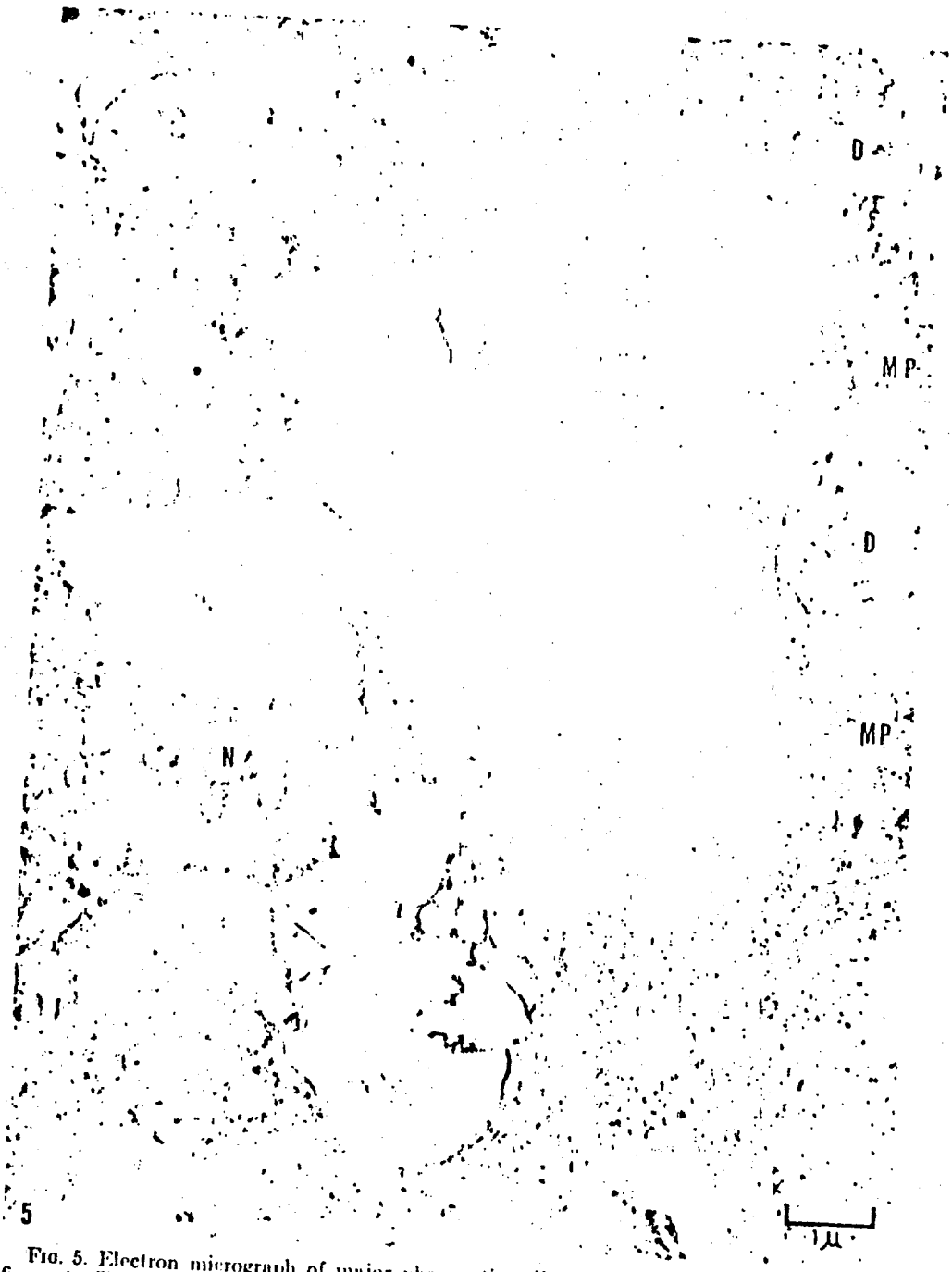


FIG. 5. Electron micrograph of major phagocytic cell from the same animal as seen in figure 4a. Totally engulfed within this phagocyte, tentatively identified as a microglial cell, are the remnants of a degenerated arcuate neuron (N), including its pyknotic nucleus (PN). The cytoplasm of some, but not all, degenerating arcuate neurons becomes totally dense as well as vacuolated as seen in this profile. Note that microglial processes (MP) at the same time surround two degenerating profiles (D). × 11,750.

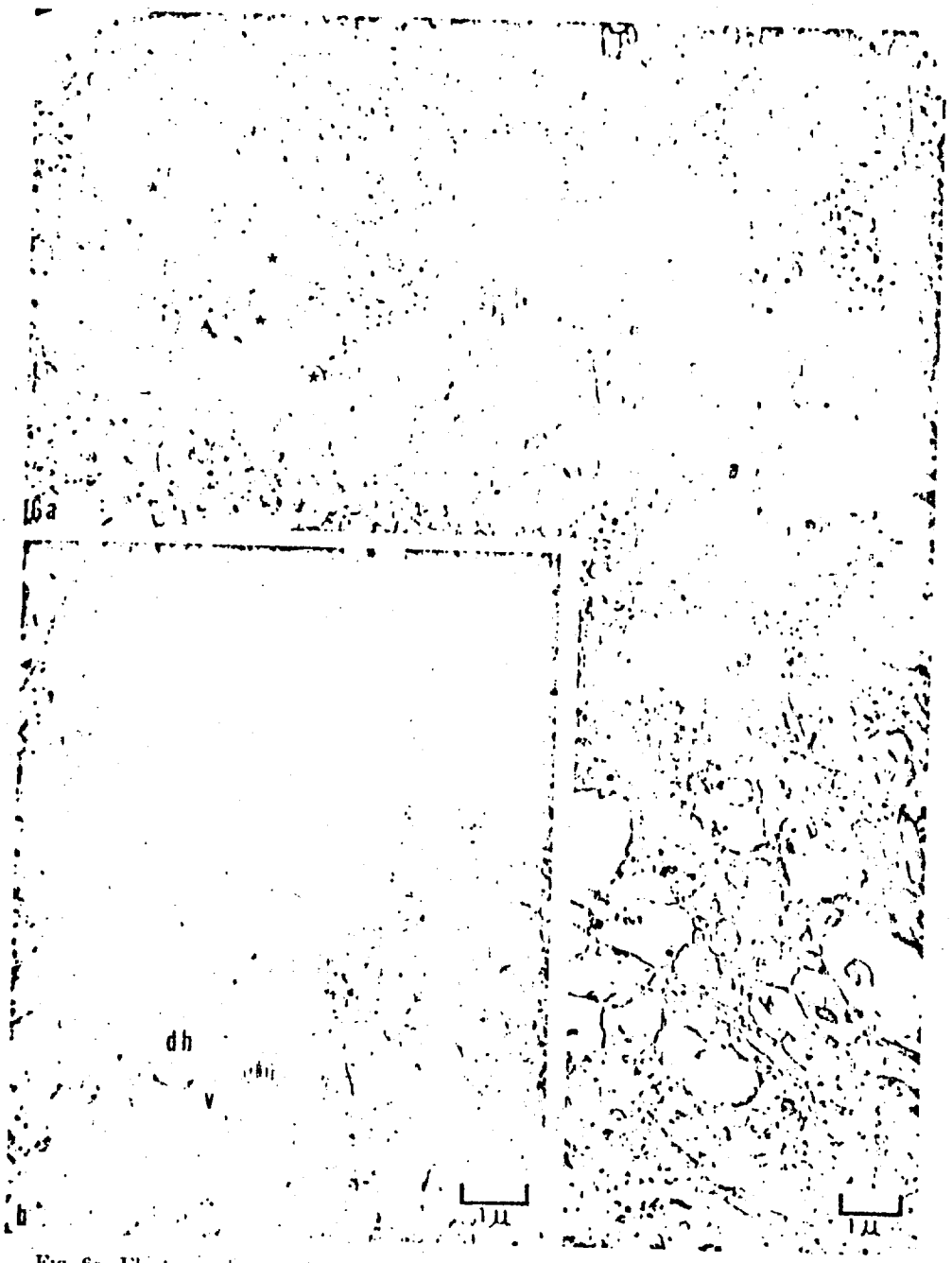


FIG. 6a. Electron micrograph of astrocyte at 24 hours. At this time, astrocytes actively participate in the phagocytic process and continue to do so for at least as long as the 48-hour sampling period. Note that both the somata and astrocytic processes are filled with debris (*) and not merely "dense bodies". × 8,172.

FIG. 6b. Electron micrograph of microglial cell at 48 hours. While some microglial cells still contain entire portions of degenerating neurons, most profiles show large numbers of vacuoles (v) and multivariegated dense bodies (db). × 8,172.

15 to 60 minutes after dosing. In some animals lesions could be detected also in the habenular nucleus and the most superficial layers of the tectum. By two hours, the lesion had spread inward from the involved outer surface of the brain. This change occurred in the tectum and habenular and arcuate nuclear regions. In this time interval the outer aspects of the dorsolateral thalamus and the subfornical organ were also damaged (fig. 7). Lesions in the tectal area were of considerable interest since the tectum is separated from cerebrospinal fluid by pial-glial membranes rather than ependymal cells. In 2 to 3 hours the tectal lesion resembles that of the arcuate nucleus in that the neuropil is filled with edematous processes and vacuolated neurons, some of which contained

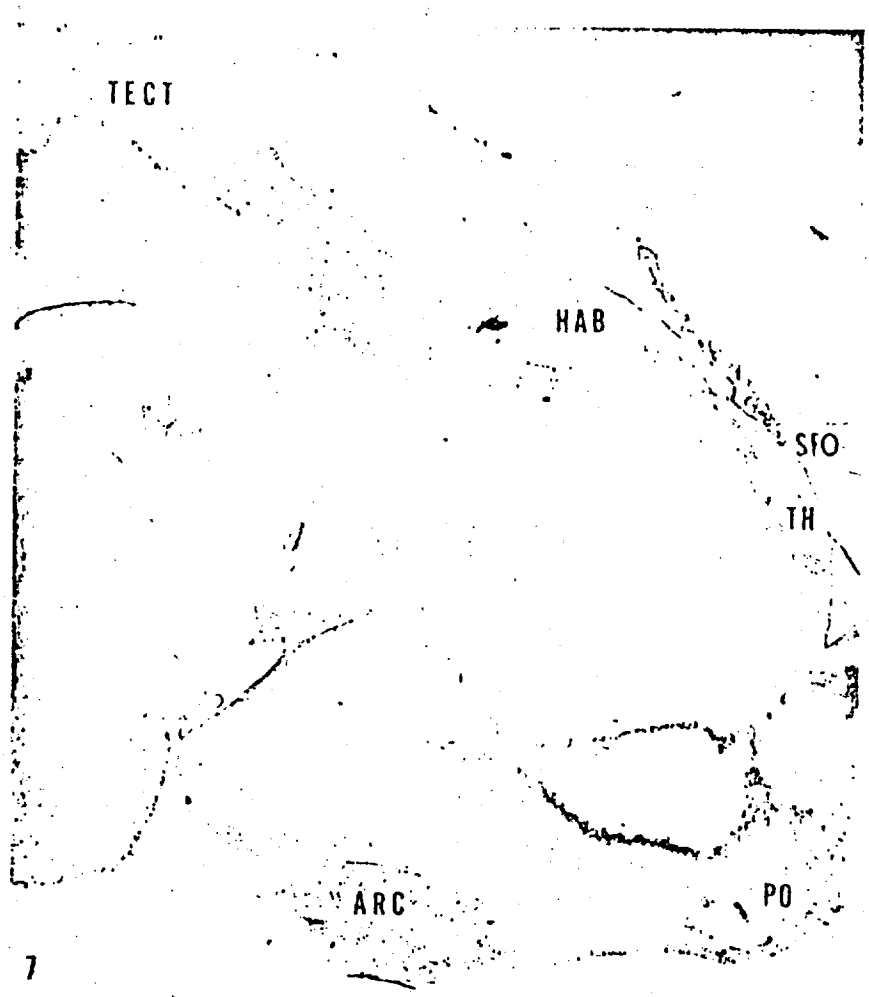


FIG. 7. Parasagittal epon section of 7-day neonatal mouse (ICR) given 4 mg/gm MSG orally and sacrificed 2 hours subsequently. It can be seen that the lesion has spread to many areas other than the "arcuate region", viz. the preoptic region (PO), the subfornical organ (SFO), the thalamus (TH), the habenular nucleus (HAB) and the tectum (TECT). Methylene Blue—Azure II. × 40.

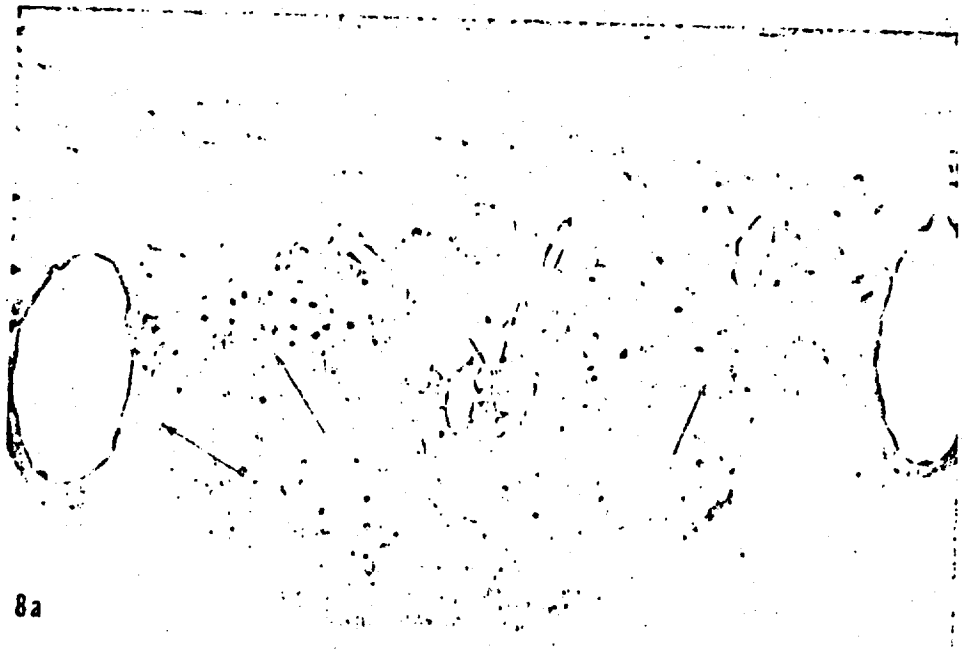
pyknotic nuclei. By 24 hours the lesion in the tectum presented an appearance never observed in the arcuate nucleus, i.e. the development of large cystic spaces almost devoid of neuronal or glial cells (Fig. 8b). These observations suggest that lesions in the inferior and superior colliculi are more substantive and potentially more capable of producing functional disabilities than lesions in the arcuate regions. Lesions in the subfornical organ observed at 3 hours were very similar to those of the arcuate nucleus (Fig. 8a). Most of the neurons appeared highly vacuolated with pyknotic nuclei. With exception of the tectum, the progress of lesions observed in other brain areas tended to resemble those of the arcuate nucleus.

3. *Incidence of Lesioned Areas in Serial Sectioned Mouse Brain*

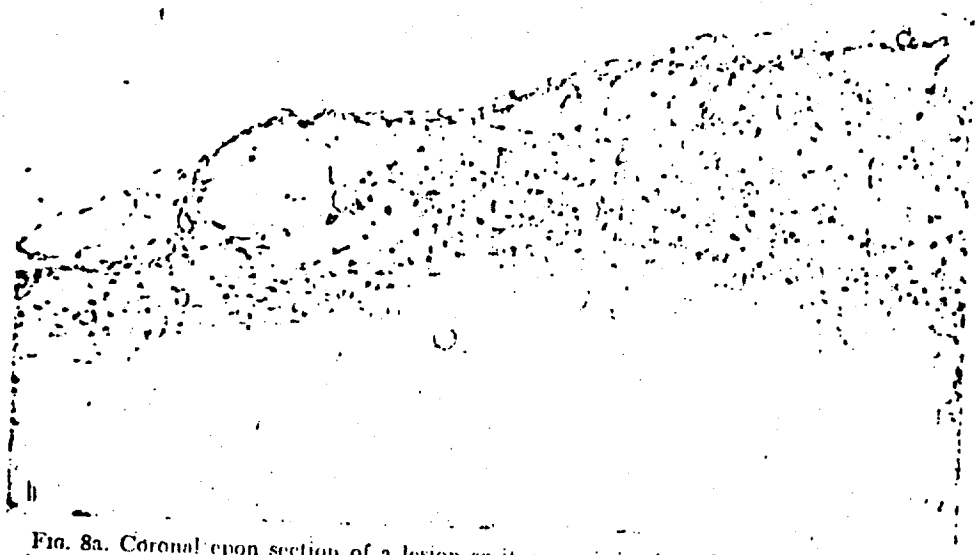
Observations made on sections embedded in epon could not be extended to include sagittal sections of whole brain. Thus a series of whole mouse brains inclusive of the olfactory bulb to the level of the cervical spinal cord were embedded in paraffin and sectioned in the sagittal plane.

The evaluation of lesions in Nissl-stained paraffin sections involved slightly different criteria than those used for plastic sections. Careful use of control sections was necessary. In paraffin sections, areas with lesions showed "bloating" or edema plus the presence of abundant pyknotic nuclei. Vacuolation of cell somas and dilation of processes in the neuropil readily discernible in ultrathin sections could not be discretely observed in thicker paraffin sections. The appearance of arcuate lesions in epon sections (fig. 1, 4a–b) can be contrasted with the same seen in paraffin in figure 9.

Examination of serial paraffin sections confirmed the presence of lesions in the diencephalic-midbrain area and permitted observation of additional areas of involvement. Figure 10a shows lesions involving the rostral and superior surfaces of the colliculi. Large areas surrounding the thalamic portion of the third ventricle also are involved. Serial sections demonstrated massive damage to dentate-hippocampal gyri surfaces abutting the ventricle, while the entire superficial dorsolateral surfaces of the thalamus and hippocampal-parahippocampal cortex were also extensively affected (fig. 10b). Figure 10d depicts the relationship of the hippocampal-cortical lesions to the lateral ventricles. Lesions could be found in the olfactory bulb (fig. 10e) but were confined to regions abutting the small finger of ventricle protruding into the core of the olfactory bulb. A large expansive lesion was observed in lower medullary levels (fig. 11a). In sagittal sections, the lesion could be traced from the spinal canal to lateral levels. Coronal sections showed that the superficial layers of the nuclei gracilis and cuneatus were edematous, containing cells with pyknotic nuclei. The area postrema varied in degree of damage from animal to animal. In some instances the damage was characterized by numerous pyknotic nuclei while in other cases by numerous edematous cells. Another region of brain frequently showing superficial lesions was the molecular layer of cerebral cortex. This part of the brain, like tectum, is separated from cerebrospinal fluid by pial-glial



8a



b

FIG. 8a. Coronal epon section of a lesion as it appears in the subfornical organ 3 hours subsequent to MSG dosage. There is marked similarity to the appearance of the damaged arcuate nucleus, viz. edematous processes in the neuropil, swollen neuronal somata with pyknotic nuclei, and infiltration of basophilic elements (macrophages) (arrows). Methylene Blue—Azure II. × 228.

FIG. 8b. Development of a lesion in the tectum of the mouse 24 hours subsequent to oral dosage. Large cystic spaces developed in this structure—a phenomenon not observed in other damaged regions of brain. Stain as above. × 99.

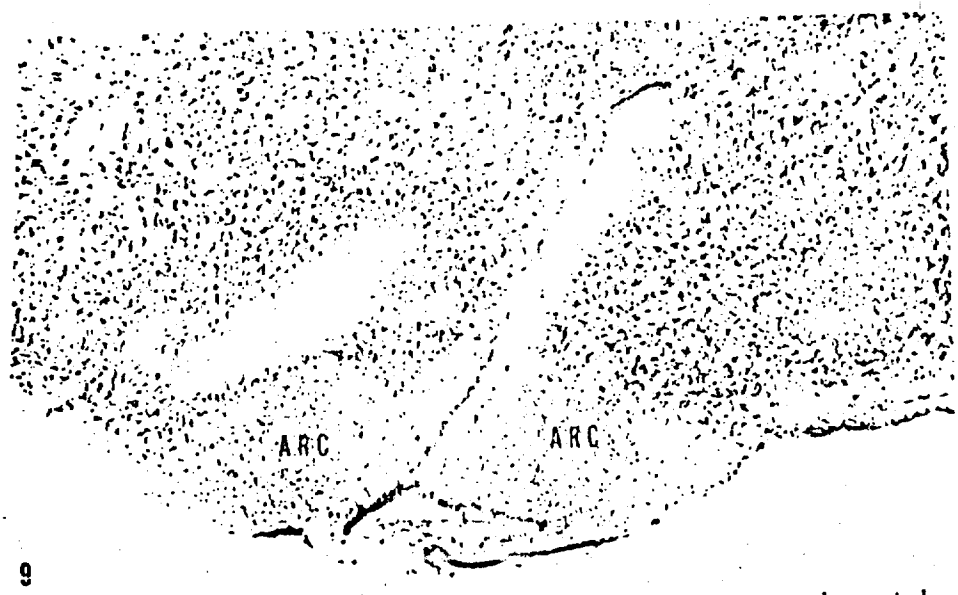


FIG. 9. Sagittal paraffin section showing appearance of lesion in arcuate nucleus cut close to midplane. Edema and the presence of large numbers of pyknotic nuclei are the chief criteria for distinguishing lesion when carefully compared to corresponding control sections. Cresyl Violet × 72.

membrane rather than by ependymal cells. Lesions here might be small focal areas as shown in figure 11b or massive ones involving almost an entire lobe.

4. *Incidence of Brain Lesion and Mouse Strain*

During the course of this study strain differences in response to MSG were suggested. At the lowest dose studied the arcuate and sometimes the preoptic area were involved in the lesion formation in all mouse strains. In the hybrids small lesions were observed in the area postrema and dentate gyrus as well. At 2 mg/gm body weight in inbred strains the lesion was confined to the arcuate nucleus although in the A/Jax strain the subfornical organ was sometimes involved. In hybrid mice at this dosage the area of lesion included the habenular nucleus and tectal areas. It is possible that lesions occurred in brain areas other than the diencephalon and midbrain at the 1 and 2 mg doses both in inbred and hybrid animals, since serial sections of entire brains were prepared only for hybrids at 1 and 4 mg/gm body weight doses. Thus, for inbred strains, only diencephalon and midbrain areas were sampled. In these preparations the thalamus always contained a lesion in the ICR mice but not in the A/Jax mice. Three points seem to emerge from our observations: 1) One of, if not the most vulnerable area to neuronal damage appears to be the arcuate region. 2) At a given dose of MSG, hybrid strains sustain lesions in more brain areas than do the inbred strains. 3) With large dosages of MSG, brain regions other than the arcuate area are susceptible to the development of substantial lesions.

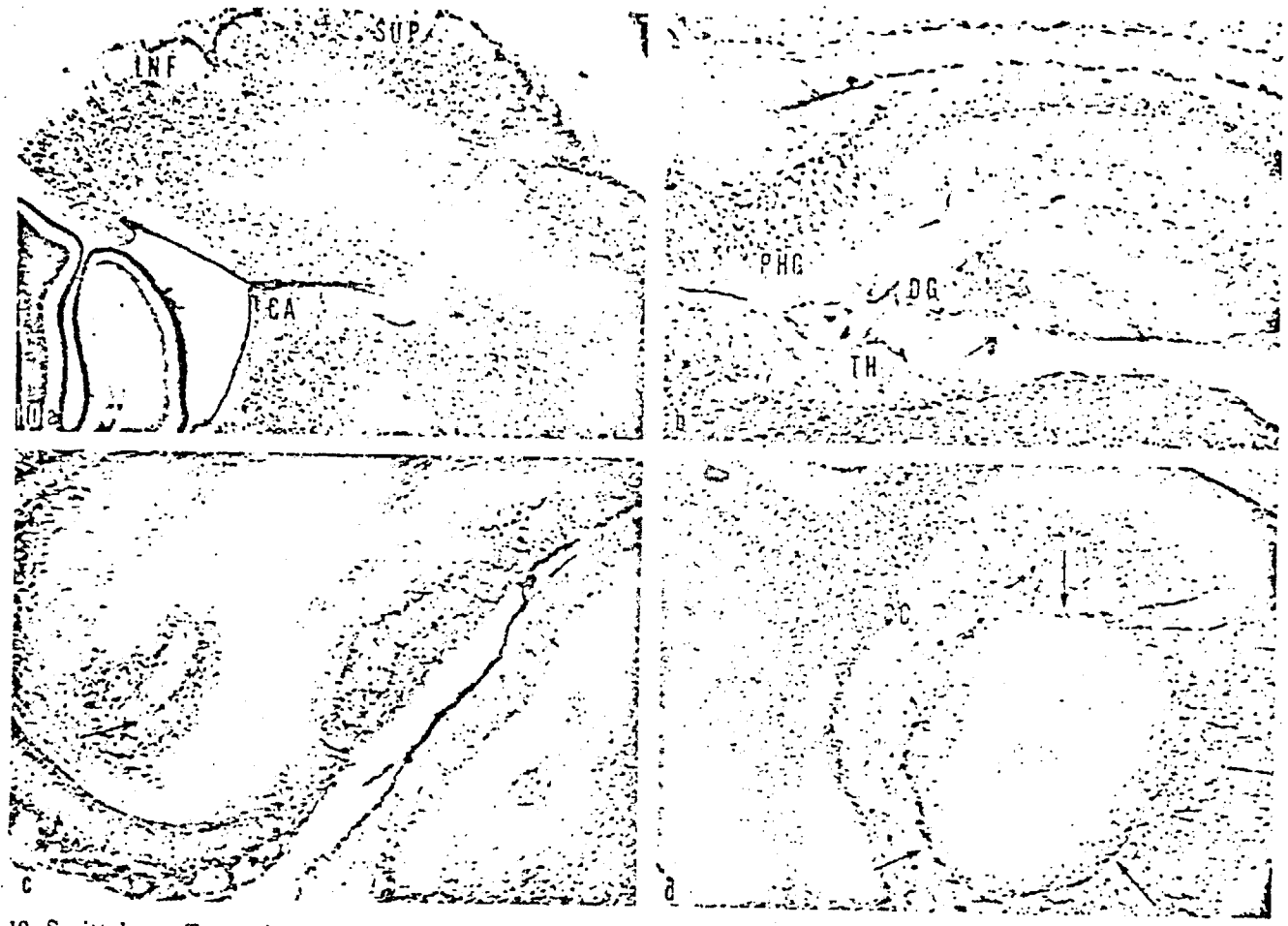


FIG. 10. Sagittal paraffin sections showing extent of damaged areas in brain other than hypothalamus after a 4 mg/gm dose of MSG. Brains were processed 3 hours after dosage. These figures and subsequent figures of paraffin sections received the same experimental treatment. All Cresyl Violet.

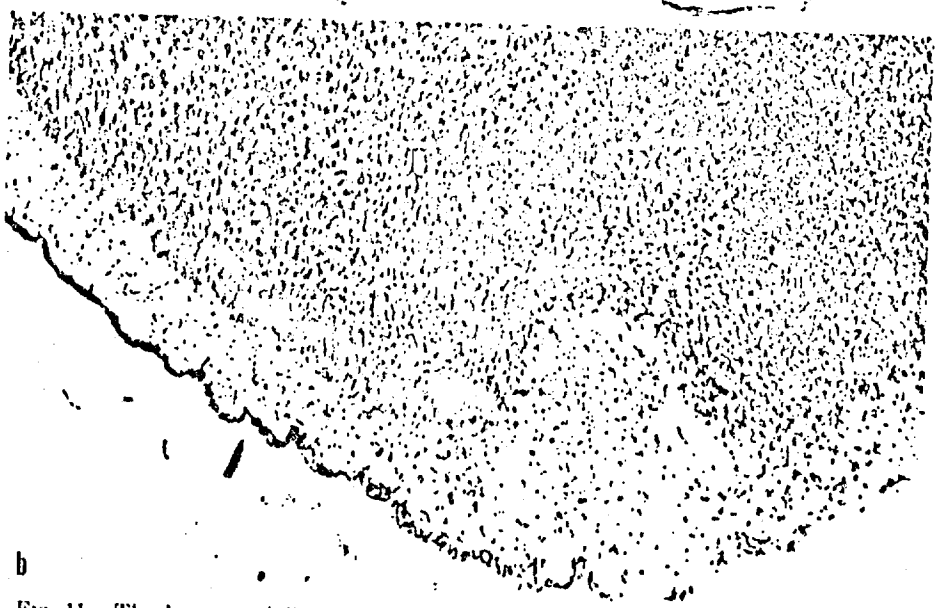
a. The external layers of both superior (SUP) and inferior (INF) colliculi are damaged as are midbrain levels surrounding the cerebral aqueduct (CA). × 35.

b. Portions of dentate gyrus (DG) abutting the ventricles as well as parahippocampal gyrus (PHG) show damage. Serial sections show that large amounts of the dorsolateral thalamus (TH) facing the third ventricle are similarly damaged. × 60.

c. Olfactory bulb lesions (arrow) are confined to small areas surrounding the extension of the ventricle. × 64.

d. Some large lesions were observed to occur in brain regions bordering the lateral ventricles (arrows). In this instance, the external surfaces of the hippocampal lobe (HIP) and cerebral cortex (CC) [illegible]

11 a



b

FIG. 11a. The lower medulla frequently manifested an expansive lesion which could be traced from spinal canal to the most lateral surface. Sagittal section showing appearance and extent of the lesion which in this case is well limited. It was observed that the lesion in other animals sometimes involved only the more superficial layers of cells bordering the fourth ventricle. Cresyl Violet. × 86.

FIG. 11b. Sagittal paraffin section of cerebral cortex from MSG-treated mouse. Focal lesion of cerebral cortex. Such lesions were found frequently in mouse brain serial sections. Stain as above. × 86.

Although the numbers were too small for statistical validity, it was interesting to note that the 8 animals 10 or more days old (hybrids and inbreds) given a 1 mg/gm body weight dose, failed to sustain lesions. The lesion rate for 6- to 8-day-old animals given this dose was 80 percent. Three of four 10-day mice treated with 2 mg/gm body weight dose sustained a lesion; the lesion rate mice mice 7 to 10 days old given this dose was 88 percent. All animals given MSG at 4 mg/gm body weight exhibited a lesion. Thus, there is an indication that slightly older animals (at the lowest dosage employed) may be less susceptible to MSG.

5. *The Effect of Monosodium Glutamate on Adult Mice*

Almost all work completed thus far has focused on the toxicity of monosodium glutamate for the neonate. The two reports (4, 22) suggesting that arcuate lesions can be induced in adult mice at the grossly high dosages of 5 to 10 mg/gm body weight have been largely ignored and unconfirmed. Thus, we decided to administer MSG to a series of adult inbred mice at the following doses: 2, 4, 5 and 7 mg/gm body weight. Sampling was limited to epon sections of the diencephalic-midbrain levels. In every animal an arcuate nuclear lesion developed within the 3 to 5 hour dosage period (Fig. 12) though it was never as acute or expansive in appearance as the lesion observed in the neonate. Furthermore, the lesion was not observed in other nearby brain regions, such as preoptic nucleus, habenular nucleus or tectum.

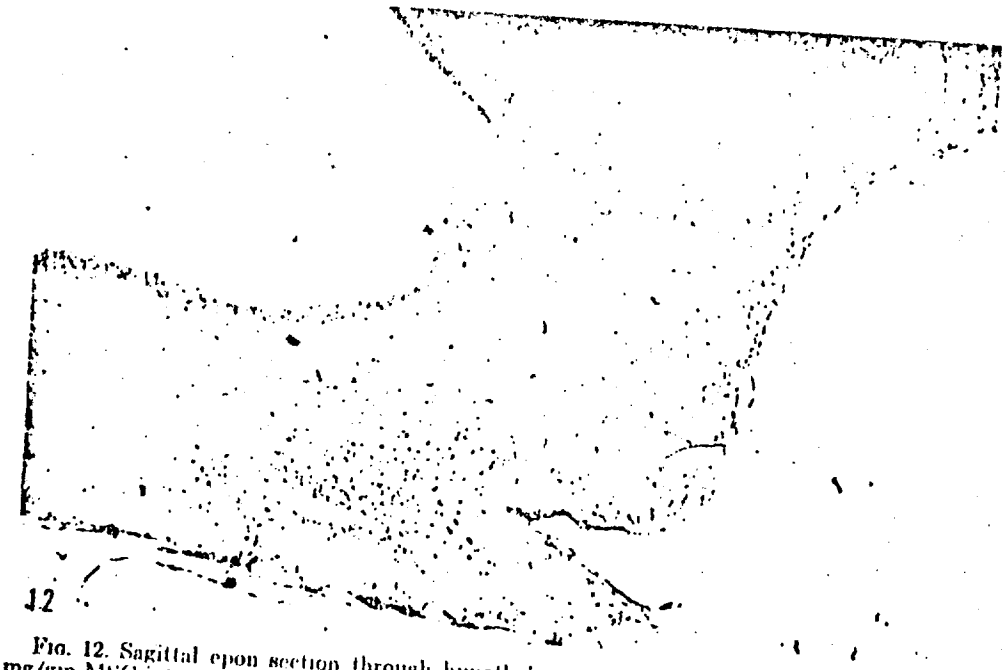


FIG. 12. Sagittal epon section through hypothalamus of an adult mouse treated with 2 mg/gm MSG intraperitoneally and sacrificed within 3 hours after dosage.
Low power view showing less extensive lesion in adult which may be contrasted to the expansive lesion found in neonates. Methylene Blue—Azure II. × 85.

DISCUSSION

1. *Pathological Changes Involving Neurons and Glia in the Arcuate Nucleus*

Although there have been general descriptions of the progress of the lesion in the retina (21) and in the arcuate nucleus (23) the phagocytic elements involved in the process have not been identified.

At the electron microscope level our observations confirm the involvement of *at least two distinct types of glial cells* in the evolution of the lesion. One of these is a "dense" cell, which can be correlated with the large densely staining basophilic cell observed by light microscopy. The second is a cell with electron lucid cytoplasm. While identification of the latter cell type as astrocyte is not difficult, easy identification of the "dense" cell is more controversial. A detailed analysis of this phagocytic process will be reported elsewhere (16).

Pathological changes in the arcuate nucleus were found to occur in three distinct phases: 1) neuronal degeneration and astrocytic edema, 2) phagocytosis first by dense cells that might be microglia or a neuroglial type similar to that of Vaughn and Peters (39), and 3) many hours later phagocytosis by astrocytes which simultaneously proliferated to fill the spaces left by degenerating neurons and their processes.

2. *The Incidence of Damage to Other Areas of Mouse Brain*

The CNS lesion induced by MSG was not confined to the arcuate nucleus but spread with time to other areas of the brain. In every instance, the damaged area occurred in a region of the CNS which was in close proximity to circulating cerebrospinal fluid and damage was initiated in that portion of a structure bordering CSF. At high doses, the lesion was found to be non-specific for distinct nuclear groups, spreading instead as a broadly diffuse or radiating band. In no instance was a lesion seen to begin within the geometric center of a given structure and radiate outward in a circular fashion, nor could the lesion ever be correlated with the pattern of distribution of blood vessels.

With time, the lesion radiates steadily inward from those areas adjacent to the CSF. Observations of lesions in the inferior and superior colliculi best illustrate this. Lesions first appear on the outer surface but with time they are observed to envelop deeper and deeper collicular layers (Fig. 7).

The same pattern occurs in the arcuate nucleus where thirty to 60 minutes following a 4 mg/gm body weight dose of MSG, the lesion is more or less contained in the nuclear area bordering the infundibular recess. After 2 to 5 hours the lesion has spread dorsally to encompass portions of the ventromedial nucleus (fig. 7).

These observations suggest that one of the access routes to central neurons, if not *the* route of entry of the deleterious agent is via cerebrospinal fluid. We cannot rule out entry from some blood vessels since in the well-documented lesions occurring in the retina (17, 21) direct access to CSF is lacking. Nor can the possibility that the deleterious agent enters from capillary systems in which the blood brain barrier is modified be ruled out as a possible contributory

access route. The presence of fenestrated capillaries in the median eminence-neurohypophysis (34), adjacent to the arcuate nucleus, as well as in the subfornical organ and area postrema (41) might be involved in facilitating entry. It was disconcerting to note, however, that cells of the subfornical organ and area postrema in many instances did not sustain a lesion, while arcuate cells were consistently found damaged.

3. *Nature of the Toxic agent and Arcuate Neuron Susceptibility*

The observation that the arcuate nuclear region is particularly susceptible to neuronal damage following ingestion of MSG raises two questions of prime importance. *First*, following administration of MSG either by the subcutaneous route or the oral route, what is the final molecular moiety which enters the brain substance and causes the death of neurons, and *second*, why are cells in the arcuate nuclear region so susceptible to this agent?

Most workers have found that the oral or parenteral administration of glutamic acid has no effect on total brain glutamate levels in the adult rat (9, 12, 19, 20, 29, 30, 33). In one report of 24 hour old neonatal mice receiving MSG by injection, there was no change in brain glutamate levels at 30 minutes and a small increase after 3 hours (38). But in another report involving 24 hour old rats, brain glutamate levels were found to increase 30 minutes after injection of MSG (10). Rats fed a diet containing up to 20 percent for 16 weeks experienced no alteration in brain glutamate levels (29), but mice fed 1.25 gm/kg of MSG for 8 weeks were reported to have doubled their brain content of glutamic acid (13).

From microchemical assay for glutamic acid of tissue samples obtained by microdissection of the arcuate nuclear region, Perez and Olney (28) reported increases in glutamic acid for this region of mouse brain following subcutaneous administration of MSG. They found the glutamate level doubled in 15 minutes, which is about the time we observed the first evidence of a lesion. Their study infers that a localized increase in glutamic acid accompanies the development of the lesion found in the arcuate area after subcutaneous dosage of MSG. However, proof that glutamic acid is the actual substance entering the arcuate nuclear area (rather than being secondarily induced) and still further, that glutamate is the final molecule reacting with neuronal and/or glial membranes is unanswered. Just what substance does enter the mouse brain after oral administration of MSG?

A more recent method employed to resolve the question of what molecules enter the brain following MSG administration has involved the use of radioisotopes. Lajtha *et al* (15) showed no net transfer of ^{14}C-glutamic acid into whole brain in adult mice and rats although small amounts of labeled glutamate quickly reached the brain suggesting that there is rapid exchange between blood and brain glutamic acid pools. Studies in our laboratories have shown that neonatal or adult mice injected with ^{14}C-L-glutamate rapidly convert large quantities into glucose, acetoacetate, a-ketoglutarate and lactate, with

glucose and acetoacetate predominating (5, 32). Contrastingly, glucose and lactate predominate after administration of ^{14}C-glutamate to the neonatal pig or monkey, and little if any acetoacetate and a-ketoglutarate are noted (36, 37). Interestingly, acetoacetate is the major ninhydrin-negative metabolite found in whole mouse brain extracts following glutamate injection (5).

The majority of published studies support the notion that glutamic acid enters the brain in small amounts representing exchange between glutamate in plasma and brain tissue. High circulating levels of glutamic acid are seemingly not followed by accretion of brain glutamate.

Glutamic acid and its metabolite, alpha-ketoglutaric acid, are putative neurotransmitter substances (8, 14). The concentration of glutamine plus glutamic acid within mammalian brain far exceeds that of any other amino acid (18). Further, glutamic acid is one of the amino acids whose increase in concentration during rapid brain growth provides an index of brain maturation (3, 11). The fact that high peripheral levels of glutamic acid are not reflected in brain levels of the substance strongly suggest that most glutamate found in the brain is synthesized there. Local brain regulation of glutamic acid levels and relative impermeability of the blood-brain barrier to glutamic acid are probably mandates for the role of a neurotransmitter substance. Some evidence points toward a metabolite of glutamic acid, perhaps acetoacetate, as being the deleterious agent responsible for the lesion rather than glutamic acid itself.

The second question of why the neurons in the arcuate region appear to be initially the most susceptible to damage is even more difficult to answer. It would seem that a combination of factors may be at work: a) variations in cell metabolism and/or b) local concentration gradients of the toxic substance be it glutamic acid or a metabolite. It has been observed that those cells in close proximity to cerebrospinal fluid are the cells which are damaged (retinal ganglion cells being the only exception), but that not all brain regions next to cerebrospinal fluid are equally susceptible. Perhaps neonatal arcuate neurons are least able to survive large local increments of glutamic acid or its metabolites when these molecules rapidly permeate the neuropil. It is possible that arcuate nuclear susceptibility is related to the fact that ependymal cells in the arcuate nuclear region are different from those in other parts of the CNS. Brawer (6) has observed that arcuate tanycytes possess zonulae adherentes instead of maculae occludentes, meaning that the extracellular space between adjacent ependymal cells may extend uninterrupted into the substance of the arcuate nucleus. However, a molecule as small as glutamic acid should have free passage from cerebrospinal fluid into brain extracellular space at any ependymal site. Perhaps we should reemphasize here the proximity of arcuate neurons to fenestrated capillaries. Ready passage of many substances, including glutamic acid, might be expected through fenestrated blood vessels such as occur in the external layers of the median eminence and the neurohypophysis and this factor might contribute to a faster rate of influx here than in other areas. It should be repeated that arcuate lesions initiate in regions closest to the

median eminence. The fact that neurons of the subfornical organ and area postrema are similarly affected might point to this route, although these cells do not seem as vulnerable as arcuate neurons.

4. *MSG Toxicity in Neonates vs. Adult Mice*

The problem of whether MSG toxicity is limited to the neonate and if so, to what extent, deserves consideration. A major finding in this study was the observation that the *adult* mouse brain arcuate nucleus sustains a readily detectable lesion after a dosage as low as 2 mg/gm body weight (the lowest dosage employed). While neurons were damaged unequivocally in the adult, the number of involved cells and areas of damage at this dose were markedly less than those of any of the neonates given an equivalent oral dose. These observations are supported by the fact that at 1 mg/gm body weight older neonates (10 to 12 days of age) sometimes fail to sustain an observable lesion. It appears that susceptibility of neurons to damage does change with maturation, but the threshold of susceptibility is not an absolute one at large doses.

With regard to MSG toxicity, it must be remembered that doses in the 1 to 4 mg/gm body weight range are exceedingly high. At levels of 1 mg/gm body weight a lesion is not always found. Thus, the MSG lesion in the mouse is a phenomenon of "unphysiologic" or very high doses. In our laboratory systematic sampling studies at lower dose levels (0.1 to 0.5 mg/gm body weight) are underway. The potential value of MSG as a selective agent for inducing chemical lesions in arcuate neurons is still to be exploited.

5. *Interpretation of Neuropathological Results Following MSG Administration*

Many of the various investigators studying the neuropathologic changes following the administration of monosodium glutamate (1, 2, 4, 7, 22–25, 27) have overlooked or underemphasized one extremely important variable, viz., the route of administration. Many of these studies have involved subcutaneous routes (2, 4, 21–23, 25, 28). This method while having certain experimental advantages, ignores relevance to nutrition. The interpretation of lesions following subcutaneous or intraperitoneal versus oral administration have often failed to take into account differences in brain susceptibility derived from route of administration. Abraham *et al* (1) did find oral administration of MSG less profound in its effects upon the arcuate nucleus in mice than subcutaneous dosing. Investigators employing procedures involving oral dosing must consider such variables as maturation of the intestinal mucosa and rate of intestinal absorption. There is some evidence that the nutritive state of man or animal influences glutamic acid absorption by the intestinal mucosa (35, 36, 40). Studies involving all routes of administration should consider factors affecting liver metabolism of glutamic acid such as maturation of enzyme systems. All of these factors influence the rate of passage of glutamate into the circulation, and ultimately the amount of glutamic acid or its metabolic by-products available to enter the brain.

Another major consideration in evaluating and comparing reports dealing with MSG-induced lesions is tissue sampling methods. The extent of sampling and levels of brain examined have usually been very sketchily described. It would seem to us that the method of choice for sampling of any given region of brain is by serial sections of plastic embedded material. This method permits observation of the most minute lesions, since these sections readily show edematous neurons and dendrites, even at early stages. However, this method is extremely tedious for even small regions of the brain and of doubtful feasibility for surveying damage throughout the entire brain of a large series of experimental animals. It is also difficult to apply to whole brain whose dimensions exceed that of neonatal mouse. An alternate method of sampling is serial paraffin sections throughout entire neonatal brains. Experience in our laboratory has shown that with frequent referral to control material, paraffin sections are adequate for showing large to medium-sized lesions. Serial paraffin sections can quickly and easily demonstrate the full extent of well-developed lesions, and of course permit easy sampling of an entire brain. However, we do think caution must be exerted when paraffin sections are used for "all or none" detection of brain lesions.

A "microlesion" consisting of but 2 to 3 cells in a single plane of section and observable only in serial 1 μ plastic sections has been reported (26). This "microlesion" was found only in the subependymal portion of the infundibular nucleus of neonatal monkeys receiving large oral doses of MSG, in contrast to the massive lesions sustained in the lateral hypothalamic extensions of rodent arcuate nucleus. This interesting finding, if confirmed, raises the further consideration of the clinical significance of 50 to 90 lesioned cells. Numbers of lesioned cells approaching this order of magnitude must be compared with the ongoing rate of cell death due to excessive neuronal proliferation characteristic of developing brains. However, if such microlesions should give evidence of clinical significance, revisions would be necessary in sampling methods in order to detect the presence of brain lesions in circumscribed brain areas.

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