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Glutamic Acid, other Amino Acids and Related Compounds as Substrates for Cerebral Tissues: Their Effects on Tissue Phosphates

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This investigation was prompted by the apparently opposed actions shown by glutamic acid in cell-free and in cell-containing systems derived from mammalian brain.

When cell-containing cerebral tissues respire in glucose media, phosphocreatine is resynthesized from creatine already present in the tissue; addition of glutamic acid then increases respiratory rate (Weil-Malherbe, 1936) but decreases phosphocreatine (McIlwain, Buchel & Cheshire, 1951; McIlwain, 1952). When added as only oxidizable substrate, glutamic acid again supports relatively high respiratory rates but does not lead to resynthesis of phosphocreatine. Nevertheless, with ground cerebral tissues or with mitochondrial preparations from them, glutamic acid supports oxidative phosphorylation (Brody & Bain, 1952), and thus would be expected to lead to the accumulation of energy-rich phosphates including phosphocreatine.

One interpretation of these findings is that they indicate the occurrence of additional, energy-consuming processes in the cell-containing tissue, processes which are diminished with loss of cell structure or not represented in the mitochondrial systems. Thus when it was observed that the addition of glutamic acid to tissues respiring in glucose increased the quantity of potassium salts associated with a given weight of tissue (Terner, Eggleston & Krebs, 1950), the assimilation of potassium salts appeared likely to represent the additional energy-consuming process. Pappius & Elliott (1956) have since shown that the additional potassium under these conditions is associated with increased volume, the concentration of potassium salts falling or remaining little changed.

In the present study, other processes which might lead to loss of adenosine triphosphate or phosphocreatine in the presence of glutamate have been examined. These include the synthesis of glutamine, noted by Krebs (1935) and Weil-Malherbe (1936), the appearance of inorganic pyrophosphate from reactions such as those described with amino acids by Moldave, Castelfranco & Meister (1959) and the displacement of creatine from the tissue. In addition, evidence has been sought by comparing the action of glutamic acid on tissue phosphates with the actions of other amino acids and with related keto and guanido derivatives.

EXPERIMENTAL

Tissues and media

Three different preparations of tissues were made from the brains of young and adult rats, and of guinea pigs.

(*a*) For tissue frozen *in situ*, young rats of 50–100 g. were dropped into liquid N_2 in a vacuum flask and the brain of each was chiselled out with cold tools as described by Richter & Dawson (1948) and McIlwain *et al.* (1951). (*b*) Tissue slices, unless otherwise stated, were prepared from adult guinea pigs, only the cerebral cortex (and not subcortical white matter), removed from the animal within 90 sec. of death, being used. Three slices were cut successively from each hemisphere with a blade and guide (McIlwain, 1961) lubricated with the medium subsequently used for incubation. Values for the slices cut at different depths in the cortex are given separately only in Table 1. After cutting, the slices were floated from the blade or guide into a shallow dish of medium and trimmed to a chosen weight, usually 80–100 mg. (*c*) For experiments of Table 6 only, slices were cut with a blade and guide not lubricated with medium, and were picked from the guide with a bent wire rider. (*d*) Ground cerebral tissues were prepared from blocks of cerebral cortex which included both grey and white matter. About 0·5 g., weighed on a torsion balance, was added to 5 ml. of 0·9% NaCl at 0° in a test-tube homogenizer and ground for 15 sec.

The inorganic constituents of the fluid media used with tissue slices were based on previous practice except that phosphates were not used as buffers because to do so involved phosphate concentrations greater than those that exist *in vivo*, and the present studies concerned tissue phosphates. The glycylglycine medium contained (mM): NaCl, 128; KCl, 5; KH_2PO_4 and $MgSO_4$, 1·3; $CaCl_2$, 2·8; glycylglycine, brought to pH 7·4 by NaOH, 30. Glutamic acid affects similarly the phosphates of tissues incubated in $NaHCO_3$-buffered and glycylglycine-buffered media (McIlwain, 1952), and to examine concomitant effects on respiration glycylglycine buffer has been used in the present experiments. Glucose was normally employed as substrate at 10 mM, and was present throughout the experiment unless stated otherwise.

L-Aspartic acid, L-asparagine, L-leucine, L-proline, L-hydroxyproline, L-lysine hydrochloride, L-histidine hydrochloride, L-tryptophan, L-tyrosine, D-glutamic acid and α-oxoglutaric acid were from L. Light and Co. Ltd.; L-alanine, β-alanine, L-isoleucine, L-methionine, L-threonine, L-serine, L-valine and L-glutamine were from British Drug Houses Ltd.; L-citrulline and L-phenylalanine were from Roche Products Ltd., L-glutamic acid and L-ornithine from Merck Corp. (U.S.A.), oxaloacetic acid from the Biochemical Corp. (Calif., U.S.A.) and sodium pyruvate from C. F. Boehringer und Soehne. γ-Aminobutyric acid, chromatographically pure, was kindly given by Dr D. B. Tower, National Institute of Neurological Diseases and Blindness, U.S. Public Health Service, and γ-guanidobutyric acid was prepared from it with cyanamide as Dubnoff (1957) described for the preparation of glycocyamine. The product melted at 182° and was identical chromatographically with an authentic specimen given by Dr I. Smith, Courtauld Institute of Biochemistry.

Analytical methods

Oxygen uptake was measured manometrically by conventional methods. Unless stated otherwise, slices of 80–100 mg. were employed in 3·5 ml. of fluid in a vessel of 15–20 ml. In a few instances slices totalling 300–500 mg. in weight were examined in 15 ml. of fluid in vessels of 100 ml. For analysis of tissue constituents the tissue specimen was removed promptly from the fluid at the end of the experiments with a mounted bent wire and in different instances certain of the determinations of the following paragraphs were carried out. Lactic acid formation was measured in the fluid by the method of Barker & Summerson (1941).

Weight. Slices were drained from fluid by repeated contact with a glass surface, while held on the wire rider of a torsion balance. They were weighed wet and then placed in small tared crucibles and dried to constant weight at 100° (1·5 hr.).

Orthophosphate and phosphocreatine in slices. The tissues were collected with a mounted bent wire, drained for 2 sec. only on the side of the vessels from which they were being removed, and placed in 1·5 ml. of 0·6M-trichloroacetic acid, ice-cold in a centrifuge tube. They were immediately ground in the acid with a homogenizer pestle which fitted the tube, and the pestle was washed with 1 ml. of the trichloroacetic acid. After centrifuging, 2 ml. of supernatant was taken to another centrifuge tube for determinations based on the methods of Berenblum & Chain (1938), Long (1943) and Heald (1954). The supernatants from a number of slices, together with appropriate standards, in separate tubes, were brought to pH 8·4 at 0° with 5N-NaOH (about 0·25 ml.), 0·1 ml. of 0·1M-$NaHCO_3$ and 0·25 ml. of 25% (w/v) barium acetate were added, and the tube was shaken and after 20 min. centrifuged. The supernatant (i) was transferred to a further tube, the residue suspended in 1 ml. of a solution 50 mM in both barium acetate and NaOH, centrifuged, and this supernatant (ii) added to supernatant (i).

For orthophosphate determination, the washed residue was dissolved in 1 ml. of 0·2N-HCl, the solution made to 4 ml. with water and 0·5 ml. of 10N-H_2SO_4 added. Ammonium molybdate [1 ml. of 5% soln. (w/v; 40 mM)] was added, the mixture shaken, 4 ml. of *iso*butanol added and the tubes were stoppered and shaken vigorously for 15 sec. The aqueous layer was removed with a Pasteur pipette and the *iso*butanol layer was shaken with 2 ml. of N-H_2SO_4; this was also removed, and 2 ml. of 35 mM-stannous chloride in N-H_2SO_4 was added. The tubes were shaken, the aqueous layer was removed, the *iso*butanol was made to 10 ml. with ethanol and shaken and centrifuged to remove traces of $BaSO_4$, and E was measured at 700 mμ.

For phosphocreatine, to the supernatants (i) and (ii) (combined volume 3·5 ml.) were added 19 ml. of ethanol and 0·1 ml. of 0·1N-$NaHCO_3$, the tubes were shaken, left at 0° for 1 hr. and centrifuged. The supernatant was removed, the residue was dissolved in 0·2N-HCl and treated as described in the preceding paragraph, except that after addition of the ammonium molybdate the tubes were incubated at 30° for 30 min. before *iso*butanol extraction. This residue contains a small proportion of the tissue's adenosine triphosphate, which does not, however, yield inorganic phosphate under these conditions (Ennor & Stocken, 1948).

To find whether the alkaline conditions of the phosphate separation just described caused change in tissue phos

phates, the following determinations, which avoid alkali, were also carried out. For inorganic phosphate the method of Lowry & Lopez (1946), with 0·2 mM-$CuSO_4$ as indicated by Peel, Fox & Elsden (1955), was applied to samples of the trichloroacetic acid extracts of slices, prepared as described above. Phosphocreatine plus inorganic phosphate was determined in further samples by exposing them, after molybdate addition, to 30° for 30 min. and then completing the determination as described in the preceding section.

Phosphates in media and reaction mixtures. For inorganic phosphate of media from which slices had been removed, 0·2–3 ml. was taken according to the expected phosphate content, brought to 5 ml. and made N in H_2SO_4 by addition of water and 10N-H_2SO_4; molybdate was added and the determination completed as described above.

In reaction mixtures containing ground tissue and added phosphocreatine, precipitation of a salt of phosphocreatine was not necessary. The mixture (0·5 ml.) was added to 1·5 ml. of trichloroacetic acid at 0°, centrifuged and 0·5 ml. of the supernatant was taken and inorganic phosphate precipitated as described above. The supernatant was then treated directly with H_2SO_4 and molybdate at 30° for 30 min., to determine phosphocreatine.

Creatine. The free creatine of tissues was determined by transferring them immediately after incubation to 1·5 ml. of 5% (w/v) sulphosalicylic acid in a centrifuge tube, grinding with a pestle and centrifuging. In portions of the supernatant the diacetyl method was applied as described by Thomas (1956).

Inorganic pyrophosphate. Method (i). The following method is based on that of Flynn, Jones & Lipmann (1955). Tissue (about 100 mg.) was ground in 2·5 ml. of 10% (w/v) trichloroacetic acid, and, after centrifuging, 2 ml. of the supernatant was adjusted to pH 7 with 5N-NaOH. Barium acetate (0·25 ml. of 25% soln., w/v) and 0·1 ml. of 0·1N-$NaHCO_3$ were added and after 30 min. at 0° the precipitate was collected by centrifuging and washed with 1 ml. of 50 mM-barium acetate, pH 8·4. It was then suspended in 4 ml. of water, 0·1N-HCl was added to give a solution and the pH brought to 7. Exactly 0·5 ml. of 10N-H_2SO_4 and 1 ml. of 2·5% ammonium molybdate were added, and the mixture was shaken with *iso*butanol. The lower layer was washed twice with 2 ml. of benzene, 0·1 ml. of 10 mM-$CuSO_4$ was added, followed by the 1:2:4-aminonaphtholsulphonic acid reagent (0·4 ml.; containing 0·25 g. of the acid, 7·5 g. of sodium metabisulphite and 1·5 g. of $Na_2SO_3,7H_2O$ in 100 ml.). Cysteine (1 ml. of 0·1M) was added, the mixture made to 10 ml., centrifuged and *E* at 700 mμ read 90 min. later. In this modification of the method, extraction with *iso*butanol prevents orthophosphate from interfering with the reaction and enables it to be determined separately. Washing with benzene was found necessary to remove the *iso*butanol, which yielded an extraneous colour. The copper increased *E* from a given amount of pyrophosphate, enabling 0·02 μmole to be determined. Satisfactory values and recovery of pyrophosphate were obtained from incubated cerebral tissue, but not from the quickly frozen tissue. In this situation method (ii), based on observations of Crane & Lipmann (1953) and Umbreit, Burris & Stauffer (1957), was used.

Method (ii). Tissue (about 100 mg.) was treated as in method (i) to yield the water-insoluble barium salts, and these were dissolved in 2 ml. of 0·05N-trichloroacetic acid. Norit (40 mg.; prepared from SX grade by boiling with 2N-HCl for 20 min. and washing on a Buchner funnel with water until the washings were neutral: see Hawk, Oser & Summerson, 1947) was added and the tubes were shaken for 15 min., centrifuged, the supernatant was transferred to a 10 ml. stoppered tube and the charcoal washed twice with 1 ml. of 0·2M-sodium acetate, pH 4·5. To the combined supernatant and washings 0·5 ml. of 10N-H_2SO_4 was added and the volume made to 5 ml. Ammonium molybdate (0·3 ml., 40 mM) and 4 ml. of *iso*butanol were added, the tube was shaken, the upper layer removed and the lower aqueous layer re-extracted with 2 ml. of *iso*butanol and this was again removed. The tube with the aqueous phase was then placed in a boiling-water bath for 15 min., rapidly brought to room temperature and a further 0·5 ml. of 40 mM-ammonium molybdate added. Extraction with 4 ml. of *iso*butanol now gave the phosphate derived from pyrophosphate, and this was determined after treatment with stannous chloride as described above.

Ammonia and glutamine. At chosen times during incubation, slices were rapidly removed from manometric vessels and ground in 1·5 ml. of ice-cold trichloroacetic acid (10%, w/v). The mixture was centrifuged, the supernatant collected, the residue ground with a further 1 ml. of trichloroacetic acid, the supernatants were combined and samples (1 ml.) made pH 5–6 with 10N-NaOH. Ammonia was measured in the samples in Conway units, with 1 ml. of sat. K_2CO_3 in the outer ring and 10 mM-HCl used to trap the NH_3; colorimetric estimation according to Russell (1944) was followed. For glutamine, a further sample (1 ml.) before neutralizing was placed in a water bath at 70° for 75 min. (see Harris, 1943), cooled, taken to pH 5–6 and NH_3 was determined as described above; the NH_3 formed during hydrolysis was taken as a measure of the glutamine. L-Glutamine itself was found to yield 4% of its amide N as NH_3 during the exposure to K_2CO_3; no correction has been applied for this.

RESULTS

Specificity of action of glutamic acid on phosphocreatine

Incubation of cerebral cortical slices in the standard medium with glucose and oxygen allowed resynthesis of phosphocreatine to concentrations of 1·6–1·7 μmoles/g. (Table 1). Similar values were found after periods of 30–60 min. incubation, and for investigating the effects of added substances a period of 40 min. was chosen. If glucose was not present during this period, respiratory rate, lactic acid accumulation and phosphocreatine were all lower than in its presence and inorganic phosphate was greater.

Actions of 20 amino acids. Table 2 shows, first, the previously described action of L-glutamic acid in depleting the tissue's phosphocreatine and increasing its inorganic phosphate. This property is now shown to be shared by D-glutamic acid and by L-aspartic acid. Glutamine and asparagine are markedly less active, as also are L-alanine and (of little effect) β-alanine. The many substances which are inactive at concentrations up to 29 mM include valine, leucine, isoleucine, lysine, ornithine,

Table. 1 *Water, inorganic phosphate and phosphocreatine of slices of guinea-pig cerebral cortex*

The slice cut first from the outer convexity of a cerebral hemisphere is described as an 'outer' slice and the two cut subsequently as 'inner' slices. The medium, unless stated otherwise, was glycylglycine-buffered, oxygenated and with glucose, and incubation was for 40 min. at 37°. Values for the phosphates refer to the moist tissue drained free from adhering fluid. Results are followed by S.D. and, in parentheses, the number of determinations.

Tissue preparation	Treatment before analysis	Water (%)	Inorganic phosphate (μmoles/g.)	Phosphocreatine (μmoles/g.)
Block	None	81·0±0·5 (7)	—	—
Slices*	In medium 2–4 min., room temp.	85·1±0·8 (24)	—	—
Slices*	In medium 8–10 min., room temp.	87·5±0·8 (18)	5·52±0·60 (4)	0·94±0·28 (4)
Outer slices†	Incubated in medium	89·0±0·8 (8)	3·60±0·25 (42)	1·71±0·13 (42)
Inner slices†	Incubated in medium	88·8±1·1 (16)	3·35±0·38 (66)	1·58±0·26 (68)
Inner slices‡	Incubated in medium without glucose	—	6·7±1·1 (11)	0·37±0·18 (11)

* Values for inner and outer slices did not differ significantly.
† Respiratory rate: 62±3 (40) μmoles of O_2/g./hr.; lactic acid accumulating: 38±5 (30) μmoles/g./expt.
‡ Respiratory rate: 38±5 (21) μmoles of O_2/g./hr.; lactic acid accumulating: 6·7 (3) μmoles/g./expt.

proline, hydroxyproline and threonine. There is thus a certain but fairly wide specificity in the group glutamic acids–aspartic acid–alanine in bringing about loss of phosphocreatine.

The action of L-glutamic acid on phosphocreatine is associated with increase in inorganic phosphate and respiration. These properties are associated also in L-aspartic acid, but not in the other amino acids which cause loss of phosphocreatine. Increased accumulation of lactic acid, which is also a feature of the action of L-glutamic acid, again does not always occur with these other amino acids.

Two amino acids were found to increase the phosphocreatine resynthesized by cerebral tissues: these were methionine and phenylalanine. In methionine this action was not associated with change in respiration, but in phenylalanine it was accompanied by decrease in both respiration and glycolysis. Tryptophan showed these latter effects without change in phosphocreatine but with diminution in tissue orthophosphate. A large measure of independent variation can thus take place in the four metabolic properties recorded in Table 2.

Actions of further amino, keto and guanido acids. Knowledge of metabolic routes in which glutamic acid and aspartic acid and alanine are involved in cerebral tissues led to an examination of the effects of the compounds of Table 3. Considering first the corresponding keto acids, it is seen that their actions in relation to phosphocreatine differed from those of the amino acids. Each compound, alone, brought about resynthesis of the tissue's phosphocreatinine. When present together with glucose, none lowered the phosphocreatine yielded by glucose. Actions of the keto acids on respiratory rate, however, paralleled those of the corresponding amino acids. Thus α-oxoglutarate and oxaloacetate increased respiration in the presence of glucose whereas pyruvic acid (like alanine) did not. The hydroxy acids tested, lactic acid and malic acid, supported phosphocreatine resynthesis and moderate respiratory rates.

γ-Aminobutyric acid, readily produced from glutamic acid by cerebral preparations (Roberts & Frankel, 1950, 1951), proved similar to glutamic acid in its relationship to phosphocreatine: it did not itself support resynthesis and depressed phosphocreatine resynthesis in the presence of glucose. The depression was not as great as that caused by glutamate. γ-Guanidobutyric acid, formed from the amino compound by cerebral preparations (Pisano & Udenfriend, 1958), was without effect on any of the properties measured, as also were arginine and citrulline; some depression of phosphocreatine was caused by histidine. None of these actions seemed of sufficient magnitude to contribute to understanding the action of glutamic acid on phosphocreatine.

Basis for the action of glutamic acid on phosphocreatine

(i) Glutamic acid might displace tissue creatine and thus diminish phosphocreatine. With and without added creatine, a gradient in free creatine is maintained in favour of the tissue when glucose and oxygen are available (Thomas, 1957). Results of Table 4 show, however, that glutamic acid did not decrease tissue creatine, whether or not additional creatine was provided; nor did aspartate or β-alanine.

(ii) Evidence has been given (Moldave *et al.* 1959) of interactions with adenosine triphosphate to yield enzyme-bound amino acid acylates and inorganic pyrophosphate. In the belief that such reaction might be part of a system leading to loss of energy-rich phosphate on addition of glutamate, determination of cerebral pyrophosphate was carried out. Values for the content of this

Table 2. *Metabolic effects of 20 amino acids on guinea-pig cerebral cortex respiring with glucose*

Tissues were fixed for analysis after 40 min. at 37·5°, some in the presence and some in the absence of the added compound. Values with the added compounds are quoted, followed by their difference from those of the tissues without the addition but in the same experiment. Values with added compounds may also be compared with mean values for normal tissues throughout the study, quoted in Table 1. Substances leading to no significant change in inorganic orthophosphate, phosphocreatine, respiration or glycolysis (i.e. of <0·3, 0·3, 5 or 5 units respectively) were: 20 mM-threonine, 5 and 25 mM-leucine, 20 mM-isoleucine, 20 mM-lysine, 20 mM-ornithine, 20 mM-proline, 20 mM-hydroxyproline and 20 mM-valine.

	Inorganic phosphate (μmoles/g.)		Phosphocreatine (μmoles/g.)		Respiration (μmoles of O_2/g./hr.)		Lactic acid accumulation (μmoles/g./expt.)	
Compound added (mM)	With addition	Change caused by addition	With addition	Change caused by addition	With addition	Change caused by addition	With addition	Change caused by addition
L-Glutamic acid 5	4·10±0·15 (4)	+0·25	0·48±0·05 (4)	−0·98	83±6 (5)	+24	—	—
25	4·27±0·6 (10)	+0·82	0·34±0·13 (10)	−1·11	83±7 (20)	+24	46 (2)	+11
D-Glutamic acid 5	4·47±0·3 (6)	+0·98	0·69±0·37 (6)	−1·04	60±1 (6)	0	—	—
15	4·33 (2)	+0·94	0·4 (2)	−1·35	59	2	—	—
L-Glutamine 5	2·88 (2)	−0·06	1·01 (2)	−0·01	60 (2)	−1	—	—
20	3·58±0·52 (11)	−0·09	1·11±0·3 (11)	−0·23	60±3 (10)	+2	—	—
L-Aspartic acid 7	3·56 (2)	+0·04	0·95 (2)	−0·63	—	—	—	—
20	4·35±0·3 (6)	+0·41	0·72±0·04 (6)	−0·83	75±11 (10)	+13	43	+4
36	3·65 (2)	+0·13	0·27 (2)	−1·30	—	—	—	—
L-Asparagine 5	3·44±0·3 (8)	+0·04	1·11±0·2 (8)	−0·35	63±10 (7)	+5	31 (3)	−3
20	3·63±0·2 (6)	+0·15	0·91±0·17 (6)	−0·55	63 (4)	+5	38 (3)	+2
L-Alanine 5	3·63 (2)	+0·06	0·88 (2)	−0·59	64 (2)	+2	—	—
25	3·59±0·28 (5)	+0·02	0·95±0·15 (5)	−0·52	—	—	—	—
β-Alanine 5	2·95 (2)	−0·31	1·23 (2)	−0·24	71 (2)	+3	—	—
20	3·38±0·22 (5)	−0·09	0·68±0·05 (5)	−0·79	71±3 (5)	+5	—	—
L-Serine 20	3·24±0·17 (6)	−0·43	1·29±0·17 (6)	−0·33	61±3 (6)	−1	—	—
L-Methionine 20	3·19±0·15 (5)	−0·29	1·97±0·2 (5)	+0·52	59±3 (7)	−2	—	—
L-Phenylalanine 20	3·14±0·17 (8)	−0·33	1·77±0·17 (8)	+0·38	47±3 (13)	−14	26 (3)	−9
L-Tryptophan 20	2·63 (3)	−0·73	1·53 (3)	+0·01	42±3 (6)	−17	20 (3)	−16
L-Tyrosine 20	3·4±0·17 (6)	−0·12	1·55±0·44 (6)	−0·13	46±3 (6)	−17	35 (3)	−7

Table 3. *Metabolic effects of amino, keto and guanido acids*

Tissues were incubated for 40 min. at 37·5°, during which respiration was measured. They were then fixed for determination of phosphates and samples of media taken for determination of lactic acid. +, Glucose added at 10 mM. Respiration falls to zero between 30 and 40 min. No significant changes were caused by 20 mM-citrulline, 5 or 20 mM-arginine, or 5 or 25 mM-γ-guanidobutyric acid in the presence of glucose.

Glucose	Acid added (mM)	Inorganic phosphate (μmoles/g.)	Phosphocreatine (μmoles/g.)	Respiration (μmoles of O_2/g./hr.)	Lactic acid accumulation (μmoles/g./expt.)
0	α-Oxoglutaric, 20	5·25±0·48 (7)	0·96±0·05 (7)	54±8 (14)	—
+	α-Oxoglutaric, 5	3·87 (2)	1·38 (2)	59	—
+	α-Oxoglutaric, 20	3·47±0·38 (6)	1·42±0·03 (6)	70±5 (6)	26 (4)
0	Oxaloacetic, 5	5·19±0·72 (4)	1·26±0·33 (4)	82±6 (4)	10
0	Oxaloacetic, 25	5·95±0·56 (10)	1·26±0·33 (4)	121±5 (10)	14
+	Oxaloacetic, 5	4·51	1·71	90, 80*	—
+	Oxaloacetic, 20	4·44 (3)	1·94 (3)	120, 80	—
0	Pyruvic, 0·5	5·34 (3)	0·97 (3)	52 (3)	10
0	Pyruvic, 5	4·0 (2)	1·39 (2)	51 (2)	—
0	Pyruvic, 25	3·92±0·10 (4)	1·27±0·19 (4)	55±8 (4)	—
0	Malic, 20	6·5±0·30 (4)	1·12±0·25 (4)	51±6 (4)	18±7·5 (4)
0	Malic, 20; pyruvic, 0·5	5·24±0·50 (4)	1·34±0·22 (4)	74±6 (4)	20±5 (4)
0	Lactic, 20	4·25±0·44 (5)	1·64±0·28 (5)	62±3 (5)	—
+	Lactic, 10	3·22 (3)	1·88 (3)	58 (3)	—
0	L-Glutamic, 5	5·64±0·43 (4)	0·23±0·02 (4)	64±1 (4)	—
0	L-Glutamic, 20	5·35±0·25 (4)	0·4±0·26 (4)	70±7 (6)	—
0	D-Glutamic, 5	6·54±0·55 (4)	0·23±0·06 (4)	24±6 (4)	—
0	D-Glutamic, 20	5·53±0·39 (4)	0·20±0·05 (4)	22±7 (4)	—
0	Aspartic, 5	7·73 (3)	0·32 (3)	27±6 (10)	—
0	Aspartic, 20	7·65±0·46 (4)	0·40±0·09 (4)	29±3 (4)	5·5 (1)
0	Asparagine, 5	7·28±0·69 (4)	0·36±0·05 (4)	36±7 (6)	2·5 (1)
0	Asparagine, 20	6·56 (3)	0·68 (3)	33±4 (6)	5·0 (2)
0	γ-Aminobutyric, 20	4·69 (2)	0·23 (2)	36±4 (6)	6·0 (2)
+	γ-Aminobutyric, 5	2·92 (2)	0·92 (2)	65 (2)	—
+	γ-Aminobutyric, 25	3·37±0·9 (5)	1·08±0·25 (5)	74±10 (9)	—
+	Histidine, 20	3·81±0·45 (6)	1·02±1·0 (6)	72±6 (9)	—

* Respiratory rate was unstable, commencing at the value quoted and falling during the experiment.

Table 4. *Amino acids and tissue creatine*

Slices of guinea-pig cerebral cortex were incubated as described in Table 2 and after 40 min. creatine was extracted and determined as described in the Experimental section. Results on each line give mean values from three or four slices from the same animal, under each of the conditions *A* and *B*.

Additions (mM)		Free creatine of tissue (μmoles/g. wet wt.)	
To vessels *A* and *B*	To vessels *B* only	Vessels *A*	Vessels *B*
0	Glutamate, 20	2·02	2·0
Creatine, 1	Glutamate, 20	2·4	2·8
Creatine, 1	Aspartate, 20	3·4	3·9
Creatine, 1	β-Alanine, 20	2·16	2·14

substance in the brain do not appear to be available and it was therefore first determined in the brain fixed *in situ* to provide an indication of normal values (Table 5). The pyrophosphate so determined was largely lost from the tissue when it was prepared at ordinary temperatures for metabolic studies, but reappeared after incubation in media with glucose as the only oxidizable substrate. Addition of glutamate then caused loss of the pyrophosphate. There is thus no indication of its accumulating as a product of reactions involving glutamate.

(iii) The increase caused in the inorganic phosphate of cerebral slices by addition of glutamate raised the question of whether a change was being caused in the distribution of inorganic phosphate between the slice and the ambient medium; the experiments of Table 6 were carried out to answer this. They indicated that the inorganic phosphate of the medium also, increased in the presence of glutamate. This occurred whether or not phosphate was initially present in the medium; the increase in the glycylglycine medium was markedly greater than in a sodium hydrogen carbonate medium. These together with earlier observations (e.g. Table 3) show that glutamic acid causes a net increase in the inorganic phosphate of the incubation mixture and is not, for example, leading only to assimilation of inorganic phosphate from the medium to the tissue.

Table 5. *Inorganic pyrophosphate of cerebral tissues*

For methods of preparation and determination, see Experimental section. Values are either from duplicate determinations on single specimens, or when followed by S.D. are from the number of specimens given in parenthesis.

Preparation	Inorganic pyrophosphate (μmole/g. of tissue) Method (i)	Method (ii)
(*a*) Whole brain fixed *in situ* with liquid N_2	(Not applicable)	0·38±0·07 (4)
(*b*) Whole brain removed at room temperature	<0·01	0·004 (4)
(*c*) Cerebral cortex incubated for 40 min. in glucose–glycylglycine media	0·31, 0·35	0·27±0·03 (4)
(*d*) As (*c*), with glucose and 20 mM-glutamate	0, 0·10	0·02±0·02 (4)

Table 6. *Glutamate and the inorganic phosphate of the incubation medium*

Slices of guinea-pig cerebral cortex (80–100 mg.) were cut without contact with media and were placed in 3·5 ml. of the fluids named, which were either the ordinary oxygenated glucose media described in the Experimental section or differed from them only by the omission of KH_2PO_4 or addition of glutamate. After incubation for 40 min. tissues were removed and the media sampled for inorganic phosphate as described in the Experimental section; values quoted are the means of triplicate results, agreeing closely.

Buffer and initial inorganic phosphate (mM)	Inorganic phosphate gained by medium (μmoles/g. of tissue) Medium without addition	Medium with 20 mM-L-glutamate
Sodium hydrogen carbonate, 0	9·68	10·73
Sodium hydrogen carbonate, 1·3	12·0	13·1
Glycylglycine, 0	8·53	11·27
Glycylglycine, 1·3	12·1	17·1

(iv) Previous study showed phosphocreatine to be relatively stable in the presence of ground cerebral tissue, considerable loss occurring only on adding adenosine diphosphate or a substance which could yield it, when the creatine-phosphokinase reaction occurred (Narayanaswami, 1952; Ennor & Rosenberg, 1954). However, a reaction involving glutamate had not specifically been sought, and this possibility was therefore examined. With ground guinea-pig cerebral cortex at 5–20 mg./ml. in glycylglycine-buffered solutions at pH 7·4 with mM-$MgSO_4$ and 2·5 mM-phosphocreatine, glutamate at 20 mM caused no change in phosphocreatine loss. This remained true when the following additions (mM) were made: adenosine monophosphate, 0·3; inosine, guanosine and cytosine monophosphates, 0·5 and 5; NH_4Cl, 20; KCl, 100. Phosphocreatine synthesis was examined in mixtures containing (mM): glycine buffer, pH 8·9, 100; $MgSO_4$, 20; creatine, 7. With tissue at 20–40 mg./ml., 20 mM-glutamate did not affect the phosphocreatine synthesized or the inorganic phosphate released; synthesis proceeded at about 270 μmoles/g./hr. with tissue at 20 mg./ml.

Ammonia, glutamate and the speed of change in tissue phosphocreatine

The findings described emphasize glutamine synthesis as being the major known reaction which consumes energy-rich phosphate and in which glutamate participates. To appraise the adequacy of this reaction as a basis for the observed changes in phosphocreatine, an important characteristic is the speed at which loss of phosphocreatine occurs on addition of glutamate. This has been determined by incubating slices of guinea-pig cerebral cortex in media which initially contained only glucose as substrate. After phosphocreatine had reached a stable value, the media then received glutamate, ammonia or both, and tissues were removed for analysis at brief intervals after the additions. Fig. 1 shows the resulting changes in phosphocreatine and inorganic phosphate to be largely complete in 2 min. By fixing tissues at intervals of 30 sec., addition of either glutamate or ammonia was found to cause loss of about 25% of the tissue's phosphocreatine in 1 min. With both substrates, over half the phosphocreatine was lost in this time, corresponding to a change of 56 μmoles/g. of tissue/hr. Although ammonia accelerated the loss of phosphocreatine caused by glutamate, the final extent of the loss, after 10 min., was not appreciably altered. This is consistent with previous findings (Krebs, 1935; Weil-Malherbe, 1936) that glutamate can itself provide ammonia for glutamine synthesis.

Cocaine affects ion movements in neural tissues (Shanes, 1958) and at 20 μM inhibits the metabolic response of cerebral tissues to electrical pulses (Bollard & McIlwain, 1959). Because of the suggestions mentioned at the beginning of this paper that glutamic acid might affect tissue phosphates secondarily to an action on ion movement, experi-

ments analogous to those of Fig. 1 were carried out in the presence of cocaine. Phosphocreatine breakdown was not affected by 20 μM-cocaine; 75 μM-cocaine caused a 30% depression. These experiments therefore supported the view that glutamate–phosphate interactions were at a metabolic level.

Rate of glutamine formation. Previous studies of glutamine synthesis by cerebral tissues (Krebs, 1935; Weil-Malherbe, 1936) employed experimental periods of 20 min. to 4 hr. and concerned the total glutamine content of the tissue plus incubation fluid. The synthesis has since been examined in briefer periods with ground tissues or enzyme systems (Elliott, 1951; Krishnaswamy, Pamiljans & Meister, 1960), but data on glutamine production in intact tissues while the phosphate changes of Fig. 1 are taking place do not appear to be available. Such determinations have therefore been carried out and results are shown in Fig. 2. In these experiments tissues were preincubated for 30 min. to allow resynthesis of phosphocreatine. Addition of glutamate was then followed by production of glutamine. Negligible synthesis occurred in the first minute; during the second, 0·9 μmole of glutamine formed/g. of tissue, corresponding to synthesis at 54 μmoles/g./hr. The rate in the next 2 min. corresponded to 18 μmoles/g./hr. Thus the time-course and maximum observed rate of glutamine formation closely paralleled those of the loss of phosphocreatine (Fig. 1), except for the initial lag in glutamine production. Fall in tissue ammonia was smaller than the concomitant gain in glutamine, which is consistent with the formation of ammonia from glutamic acid, referred to above.

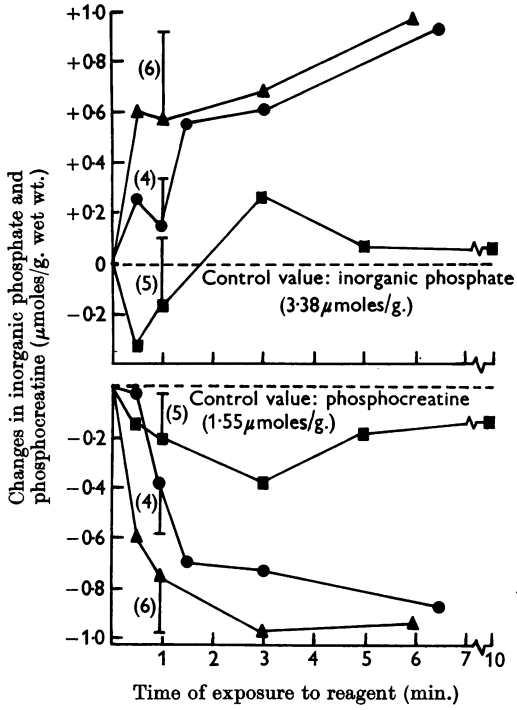

Fig. 1. Rapid changes in phosphates of guinea-pig cerebral cortex on addition of glutamates and ammonium salts. Tissues were incubated for 30 min. in glycylglycine media at 37°, and some removed for analysis at this time (zero time on the Figure). To others were added sodium glutamate, to make the solution 20 mM (●), ammonium chloride to 5 mM (■), or both (▲). After addition, at the intervals indicated, tissues were removed for analysis. Points give the mean of two or three values except when indicated by a number near them, when a line extends from the point, giving the S.D.

DISCUSSION

Phosphocreatine and glutamine synthesis

The relationship between Figs. 1 and 2 gives good evidence for the action of glutamic acid on tissue phosphates being due to the synthesis of glutamine (Elliott, 1951) according to (I):

(I) Glutamic acid + ammonia + ATP
→ glutamine + ADP + P

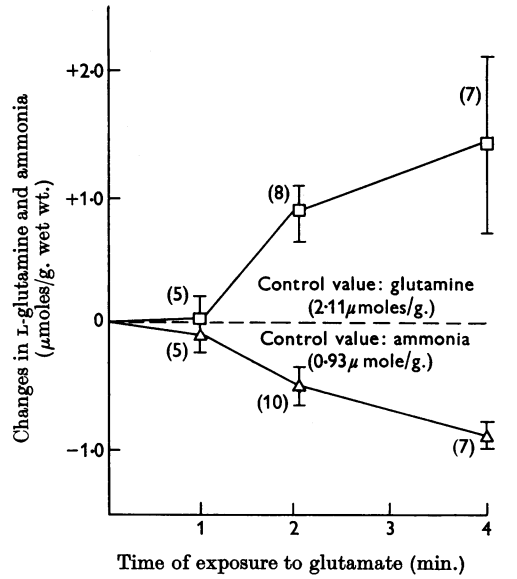

Fig. 2. Course of change in ammonia and glutamine on addition of glutamate to cerebral cortex. Slices of guinea-pig cortex of 90–110 mg. were incubated as described in Fig. 1, in vessels which contained 0·2 ml. of 0·5 M-sodium L-glutamate in side arms. After 30 min., the glutamate was tipped in; slices were removed at the intervals indicated and ground in trichloroacetic acid (see Experimental section) for analysis.

which, in association with creatine phosphokinase (of cerebral tissues: Narayanaswami, 1952), accounts for the change observed (II; Cr: creatine):

(II) Glutamic acid + ammonia + CrP → glutamine + Cr + P

A previous measurement (Achs, Balasz & Straub, 1952) of formation of glutamine and loss of adenosine triphosphate in cerebral tissues allowed the reaction to proceed for 40 min., or 10 times the period now found sufficient, and did not measure phosphocreatine; stoicheiometric relationship between glutamine and phosphate reacting were not found.

The glutamine-synthesizing enzyme of sheep brain has recently been purified and its action involves as intermediate an enzyme-bound, γ-carboxyl-activated glutamic acid (Krishnaswamy *et al.* 1960). Intermediates, formed from enzyme, glutamic acid, adenosine triphosphate and magnesium salts, were yielded by both D- and L-glutamic acid but that from the L-acid reacted more effectively with ammonia. In the present experiments D-glutamic acid also depleted tissue phosphocreatine, but did so rather less effectively than the L-acid (Table 2). Present observations on L-aspartic acid, which also depletes phosphocreatine, may receive explanation in terms of the very potent glutamic–aspartic transaminase of cerebral tissues, which can proceed at up to 2000 μmoles/g./hr. (Cohen & Hekhius, 1941; McArdle, Thompson & Webster, 1960), and thus provide glutamate from endogenous α-oxoglutarate and the aspartate supplied. By contrast, transamination between α-oxoglutarate and alanine or γ-aminobutyrate, though occurring in cerebral tissues, are markedly slower (of γ-aminobutyrate, about 10 μmoles/g./hr.: Baxter & Roberts, 1958) and the depletion now observed in phosphocreatine is much less (Table 3). β-Alanine has also been noted as possibly reacting with α-oxoglutarate, at a still lower rate; its effect in lowering phosphocreatine in the present experiments was the smallest of the compounds which so acted.

If the increase in phosphocreatine observed to follow incubation of cerebral tissues with phenylalanine is related to the findings of the preceding paragraph, inhibition of the glutamine-synthesizing system is suggested, but has not been examined.

Respiration with amino and keto acids

The actions of related keto and amino acids on tissue respiration and phosphates can be compared. The diminution in phosphocreatine which occurred when L- or D-glutamic acid, or aspartic acid or alanine was added to glucose-containing media did not take place with addition of the corresponding keto acids: α-oxoglutaric, oxaloacetic or pyruvic. On the other hand, α-oxoglutaric acid and oxaloacetic acid increased respiration of tissues in glucose media, as did L-glutamic acid and aspartic acid; D-glutamic acid and alanine did not. This suggests that the ability of the amino acids to act as oxidizable substrates depends on their conversion into the keto acids and is independent of their effects on phosphocreatine.

L-Glutamic acid is the only amino acid appreciably increasing respiration in the absence of glucose (Weil-Malherbe, 1936; Table 3), and the formation of glutamine may play an essential part in this. Reaction (III) can be regarded as including reaction (I), glutamic dehydrogenase and oxidative phosphorylation:

(III) 2 Glutamic acid + [O] → α-oxoglutarate + glutamine

It provides a source of oxoglutarate independently of glucose or its intermediates.

D-Glutamic acid, as noted, is less capable of participating in reaction (I) and may for this reason or through stereospecificity in glutamic dehydrogenase support a lesser respiratory rate. If aspartic acid, used as substrate in the presence of glucose, undergoes transamination with α-oxoglutarate derived from glucose, this may result in increased respiratory rate because oxaloacetate at lower concentrations (5 mM in Table 3) affords higher respiratory rates than does an equimolar concentration of α-oxoglutarate. The higher respiratory rate with oxaloacetate occurs in the presence or absence of glucose; the potency of the glutamic–aspartic transaminase makes understandable the formation (and preferential oxidation) of oxaloacetate. By contrast, transamination with alanine is less active, and pyruvate as only substrate does not afford respiratory rates as high as oxaloacetate: presumably for these reasons alanine does not have the effect on respiration which is given by aspartic acid.

Relation to ion movements

The additional potassium observed by Terner *et al.* (1950) to become associated with cerebral tissues on addition of glutamate to media in which they were respiring with glucose as substrate may receive explanation in terms of the foregoing results. Considering reaction (III) in relation to the ions involved, and supposing glutamic acid to enter the cell with K^+ ion 'as its cationic equivalent' (Terner *et al.* 1950), it is there converted into α-oxoglutarate, which does not greatly increase in amount in the tissue or medium and hence is assumed to be completely oxidized, and glutamine, which does increase in tissue and medium but which is not acidic. The cation could thus accumu-

late and possibly its uptake leads secondarily to the observed uptake of fluid, for the result of the ingress of both is to leave potassium salts at nearly their normal intracellular concentration (Pappius & Elliott, 1956).

This increase of tissue potassium is not, however, envisaged as making a major contribution, as such, to transport in the present tissue. This judgement is based on (1) the abnormalities which accompany the uptake of potassium promoted by glutamate: (*a*) the fluid uptake, (*b*) the fall in phosphocreatine and (*c*) the fall in membrane potential. Also (2) a mechanism based on reaction (III) is not sufficiently rapid. Cummins & McIlwain (1961) observed cerebral tissues to be capable of reassimilating potassium salts at the rate of 600 μequiv./g. of tissue/hr. The greatest increase in rate of K^+ ion assimilation observed (Pappius & Elliott, 1956) to be brought about by glutamate approximated to 15–20 μequiv./g./hr., a rate understandable if the assimilation is by reaction (III) and this is limited by reaction (I) to about 50 μmoles/g./hr. (see above). Further (3) the mechanism based on (III) requires uptake of glutamate from the environment of the tissue and the extrusion of glutamine. This does not occur in the brain *in vivo* at appreciable rates; those observed by Adams, Harper, Gordan, Hutchin & Bentinck (1955) corresponded to 2 μmoles/g. of brain/hr. An aspect of these findings which does appear significant in relation to normal ion transport is that the cation which accumulates on addition of glutamic acid is the K^+ ion. This takes place in spite of the preponderance of Na^+ ions in the tissue environment, and thus requires a specific explanation.

SUMMARY

1. Slices of mammalian cerebral cortex resynthesized phosphocreatine on incubation in media containing (5–25 mM) glucose, or lactic acid, or pyruvic acid, or malic acid or oxaloacetic acid. A lesser resynthesis occurred with α-oxoglutarate, and little or none with glutamic acid, aspartic acid and γ-aminobutyric acid as the only oxidizable substrates.

2. The concentrations of phosphocreatine produced in the tissue in the presence of glucose were diminished by the further addition of 5–25 mM-D- or L-glutamic acid, L-aspartic acid or γ-aminobutyric acid, asparagine, alanine or β-alanine, but were slightly increased by the further addition of 25 mM-L-methionine or L-phenylalanine.

3. Respiratory rates were increased, in the presence of glucose, by α-oxoglutarate and oxaloacetate but were diminished by the further addition of L-phenylalanine or L-tryptophan.

4. The depression of phosphocreatine by L-glutamate was not prevented by the addition of creatine to the medium. Also creatine assimilation was not affected by L-glutamate, L-aspartate or β-alanine.

5. Tissue inorganic pyrophosphate, normally at 0·2 μmole/g., was diminished by addition of L-glutamate.

6. Creatine-phosphokinase activity in cerebral homogenates was unaffected by the addition of glutamate or several nucleotide phosphates, when observed with phosphocreatine and adenosine diphosphate as initial reactants. Phosphocreatine synthesis by cerebral homogenates was also unaffected by the addition of L-glutamate.

7. The rate of phosphocreatine breakdown in cerebral slices on the addition of 20 mM-L-glutamate was about 20 μmoles/g./hr. and was not lowered by the addition of 20 μM-cocaine. Simultaneous addition of 5 mM-NH_4^+ ion and L-glutamate more than doubled this rate.

8. The rates of increase of tissue glutamine and loss of ammonia on addition of 20 mM-glutamate were similar to those for the loss of phosphocreatine and increase in inorganic phosphate, indicating that these changes were due to glutamine synthesis.

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An Electrophoretic Analysis of Protein Extracts from Normal and Dystrophic Ovine Muscle

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Various extraction techniques have allowed the study of specific proteins found in muscle tissue, and the early work of Jacob (1947) demonstrated the usefulness of electrophoretic analysis in the study of these proteins. Such methods have been used both in fundamental studies of muscle proteins (see reviews by Perry, 1960; Szent-Gyorgyi, 1960) and in studies of pathological conditions of muscle (Azzone & Aloisi, 1958).

A degenerative myopathy affecting young lambs has been recognized for many years and referred to as nutritional muscular dystrophy, 'white muscle disease' or 'stiff lamb disease'. This disease is distinguished from infantile forms of muscular dystrophy in man by its non-progressive character, the capacity for complete regeneration of affected muscles, the absence of any evidence of familial predisposition and an apparent direct association of the ovine disease with maternal dietary factors. Defects in vitamin E metabolism have previously been recognized as important causative factors in the ovine disease (see review by Blaxter, 1955), but more recent evidence suggests that selenium metabolism may also be involved (Muth, Oldfield, Remmert & Schubert, 1958). There is also some evidence suggesting that a succinoxidase inhibitor in certain feeds may be of importance in the pathogenesis of this disease (Cartan & Swingle, 1959).

In order to characterize the changes occurring in the protein constituents of muscles in this disease, an electrophoretic analysis of extracts from affected and normal muscles has been made. This report describes specific changes in the electrophoresis patterns of these protein extracts that are shown to occur consistently in this disease.

MATERIALS AND METHODS

A high incidence of muscular dystrophy can be produced in lambs by feeding certain types of hay to their dams during pregnancy and immediately after parturition (Young, Hawkins & Swingle, 1961). Tissues for the observations described in this report were obtained from lambs affected with this experimentally induced muscular dystrophy and from normal lambs whose dams had received a hay known not to produce the disease.

Muscular dystrophy was detected in affected lambs by elevations in the quantity of glutamic–oxaloacetic transaminase occurring in their serum. For this purpose, serum samples were drawn from all lambs at 7, 14 and 21 days of age, and at the time of slaughter, and subjected to the test procedure described by Cabaud, Leeper & Wroblewski (1956). The close correlation that exists between increases in serum glutamic–oxaloacetic transaminase and the early changes of muscular dystrophy in lambs has previously been reported (Blincoe & Dye, 1958; Swingle, Young & Dang, 1959). In this experiment all affected lambs had elevations of this enzyme above 2000 units between the age of