REFERENCES

Booman, K. A. & Niemann, C. (1956). *J. Amer. chem. Soc.* **78**, 3642.
Briggs, G. E. & Haldane, J. B. S. (1925). *Biochem. J.* **19**, 338.
Dixon, M. (1953). *Biochem. J.* **55**, 171.
Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627; cf. Hawk, P. B., Oser, B. L. & Summerson, W. H. (1947). *Practical Physiological Chemistry*, p. 879. London: J. and A. Churchill.
Hall, A. N., Hollingshead, S. & Rydon, H. N. (1961). *J. chem. Soc.* p. 4290.
Hall, A. N., Leeson, J. A., Rydon, H. N. & Tweddle, J. C. (1960). *Biochem. J.* **74**, 209.
Halvorson, H. & Elias, E. (1958). *Biochim. biophys. Acta*, **30**, 28.
Hammett, L. P. (1940). *Physical Organic Chemistry*, p. 186. New York: McGraw-Hill.
Hollingshead, S. (1958). Ph.D. Thesis: Manchester University.
Jaffé, H. H. (1953). *Chem. Rev.* **53**, 191.
Jsaiev, V. J. (1926). *J. Inst. Brewing*, **32**, 552.
Laidler, K. J. (1958*a*). *The Chemical Kinetics of Enzyme Action*, p. 60. Oxford: Clarendon Press.
Laidler, K. J. (1958*b*). *The Chemical Kinetics of Enzyme Action*, pp. 188–192. Oxford: Clarendon Press.
Larner, J. (1960). In *The Enzymes*, vol. 4, pp. 369–378. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
Larner, J. & Gillespie, R. E. (1956). *J. biol. Chem.* **223**, 709.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Mamalis, P. & Rydon, H. N. (1955). *J. chem. Soc.* p. 1049.
Michaelis, L. & Menten, M. L. (1913). *Biochem. Z.* **49**, 333.
Nath, R. L. & Rydon, H. N. (1954). *Biochem. J.* **57**, 1.
Rydon, H. N. (1948). *Symp. biochem. Soc.* **1**, 55.
Van Slyke, D. D. & Cullen, G. E. (1914). *J. biol. Chem.* **19**, 141.
Willstätter, R. & Bamann, E. (1926). *Hoppe-Seyl. Z.* **151**, 273.

Biochem. J. (1962) **84**, 394

Effects of Ouabain on Cerebral Metabolism and Transport Mechanisms *in vitro*

BY O. GONDA AND J. H. QUASTEL
McGill–Montreal General Hospital Research Institute, 3619 *University Street, Montreal, Canada*

(*Received* 5 *February* 1962)

This work is part of a study of the effects of metabolic inhibitors and neurotropic agents on the formation of amino acids from glucose in the isolated rat-brain cortex, some results of which have been reported (Gonda & Quastel, 1961). We have examined the action of ouabain, a drug well known for its inhibitory effects on electrolyte transport at the cell membrane (Schatzmann, 1953; Kahn & Acheson, 1955; Glynn, 1957; Caldwell & Keynes, 1959). This substance has remarkable effects on the transformation of glucose into amino acids in isolated rat-brain slices and on amino acid and creatine transport into brain *in vitro*. This paper describes results that have been obtained and their possible interpretation.

MATERIALS AND METHODS

The method for the study of the conversion of radioactive glucose into radioactive amino acids was based on that described by Kini & Quastel (1959). The Warburg manometric apparatus was used for incubation of brain-cortex slices in oxygen at 37°. Brain-cortex slices, from adult hooded rats of about 150 g., were placed in ice-cold Krebs–Ringer phosphate medium of the following composition: NaCl, 128 mM; KCl, 5 mM; $CaCl_2$, 3·6 mM; $MgSO_4$, 1·3 mM; Na_2HPO_4, 10 mM (brought to pH 7·4 with N-HCl). For study of the effects of high K^+ ion concentrations, the KCl concentration in the medium was increased to 105 mM, the concentrations of the other ions remaining the same. The slices were prepared with a Stadie–Riggs microtome, care being taken that the slices were not more than 0·3 mm. thick, but not so thin that they tended to disintegrate when shaken in a Warburg manometric apparatus. The slices (average wet wt. 80–90 mg.) were weighed at once on a torsion balance and suspended in the incubation medium in chilled manometric vessels. The dry wt. of the slices prepared under these conditions was approximately 16% of the wet wt. The final volume of medium in each Warburg manometric flask was 1 ml. and each flask contained 5 mM-glucose uniformly labelled with ^{14}C. Corrections were not made for swelling of the tissue. This was variable and did not exceed 10%.

After aerobic incubation for 1 hr. at 37°, the flasks were chilled in crushed ice, and the slices were removed, rinsed with a small quantity of Krebs–Ringer phosphate medium, suspended in 5 ml. of 80% (v/v) ethanol and homogenized in a Potter–Elvehjem homogenizer. The homogenizer was washed with small portions of 80% ethanol and the washings were added to the original homogenate. The homogenate was kept at 5° for 2 hr., then centrifuged; the ethanolic extract was collected and the insoluble residue washed twice with 1·5 ml. portions of 80% ethanol and finally with

1 ml. of water. The pooled ethanolic extract was evaporated to dryness at room temperature in a current of air and the dried extract was dissolved in a small amount of 80% ethanol and 'spotted' with a micropipette on Whatman no. 1 filter paper for two-dimensional chromatography. The solvents used for chromatography were: phase I, butan-2-ol–90% (v/v) formic acid–water (70:11:17, by vol.); phase II, 90% (w/v) phenol–water–aq. NH_3 soln. (sp.gr. 0·88) (960:110:3, by vol.). The incorporation of ^{14}C into amino acids from [$^{14}C_6$]glucose was calculated as μmg.atoms of carbon from glucose incorporated into amino acids/100 mg. of wet tissue (Gonda & Quastel, 1962). The radioactivity, A, of the individual amino acids on the filter paper was corrected by a factor due to the decrease in the counting efficiency on the paper. This factor was determined by measuring the radioactivity, R_1, of a sample (100 μl.) of the ethanolic tissue extract on an aluminium planchet and also that, R_2, of another sample (100 μl.) spotted on filter paper. The result for each labelled amino acid on the filter paper (measured as counts/min.) was multiplied by R_1/R_2 (which was about 3·4–3·6 and was determined after each experiment).

The corrected value of counts/min. of amino acids, $A_{corr.}$, was transformed to μmg.atoms of carbon by multiplying by a factor, F, given by: [6 × amount of glucose (μm-moles) in the incubation medium]/[radioactivity of glucose (counts/min.) in the incubation medium]. The amount of glucose in each experiment was 5000 μm-moles (30 000 μmg.atoms) and the labelling was 200 000 counts/min. Therefore F equals 0·15. Finally the term expressing μmg.atoms of carbon was corrected for the weight of the tissue and expressed/100 mg. wet wt. of tissue. The μmg.-atoms of carbon derived from the glucose incorporated into amino acids in 100 mg. wet wt. of tissue are given, therefore, by: ($A_{corr.} \times F \times 100$)/(wet wt. of tissue).

Amino acids in the incubation medium were also determined. For this purpose the sugars and salts were separated from the amino acids by a method similar to that described by Moore & Stein (1954). After removal of the slices, the medium was passed through a column (0·8 cm. × 4 cm.) of Dowex 50W (X4; 200–400 mesh) resin. The column was washed with water, and the adsorbed amino acids were then eluted with 50 ml. of aq. N-NH_3 soln. and the eluate was evaporated at room temperature in a rotating vacuum evaporator. The residues were dissolved in a small amount of 80% ethanol and chromatographed two-dimensionally on Whatman no. 1 filter paper as described above. The values for the amino acids obtained by the column chromatography were corrected for the loss of amino acids during the procedure. For control purposes, labelled amino acids were used. These were extracted from brain-cortex slices after incubation with [$^{14}C_6$]glucose.

After two-dimensional chromatography the paper was thoroughly dried and placed in contact with Kodak 'No Screen' Medical X-ray film for 3 days. After the films had been developed the radioactive spots were located on the paper. The radioactivities of the spots on the paper were quantitatively measured with a Tracerlab counter with a mica window (diam. 28 mm., thickness 1·5–1·8 mg./cm.^{2}). The spots were divided into sizes to suit the diameter of the counter window. Activities were corrected for background.

Incorporation of ^{32}P *into the total nucleotide fraction.* The incorporation of labelled phosphate into the total nucleotides was also examined, the method used being based on the procedure described by Crane & Lipmann (1953). After incubation for 1 hr. at 37°, the slices were removed from the medium, rinsed with cold Krebs–Ringer phosphate medium and were homogenized in the presence of 10 ml. of 10% (w/v) trichloroacetic acid in a Potter–Elvehjem homogenizer. After removal of the proteins an aqueous suspension of acid-washed Norit A was added (equivalent to approx. 25 mg. dry wt.) to the supernatant. The charcoal was sedimented by centrifuging, washed once with 10 ml. of 5% (w/v) trichloroacetic acid and twice with 10 ml. of water, and resuspended in a small volume of ethanol. The ethanol suspension of the charcoal was transferred quantitatively to planchets and dried under an infrared lamp. The radioactivity of nucleotides adsorbed on the charcoal was measured with a Tracerlab counter. Corrections for self-absorption were not found to be necessary.

Influx of creatine and formation of creatine phosphate in rat-brain-cortex slices. The influx of creatine and its incorporation into creatine phosphate were examined as follows. After incubation of rat-brain-cortex slices with glucose and [1-^{14}C]creatine, for 1 hr. at 37°, 5 ml. of ice-cold 12% (w/v) trichloroacetic acid (containing unlabelled mM-creatine phosphate) was added to the vessel containing the slices. The vessels were then immersed in acetone–solid carbon dioxide mixture. After 5 min. the contents of the vessels were thawed and homogenized in a Potter–Elvehjem homogenizer. The protein was sedimented by centrifuging and re-extracted in the homogenizer with a further 4 ml. of 5% trichloroacetic acid. The two supernatants were then combined, sufficient 5N-NaOH was added to bring the pH to about 9·2 and 2 ml. of M-$BaCl_2$ was added. The mixture was kept for 30 min. in an ice bath to ensure the precipitation of the barium salts of inorganic and organic phosphates and was then centrifuged. The precipitate was discarded and 4 vol. of ice-cold ethanol (previously adjusted to pH 9·2) was added to the supernatant which was then kept overnight at −10°. The water-soluble 80% ethanol-insoluble barium salt was then sedimented by centrifuging and the supernatant discarded. The barium salts were washed twice with 5 ml. of 80% ethanol (previously adjusted to pH 9·2), kept at −10° for 30 min. and then centrifuged. The suspension was plated on tared aluminium planchets, dried under an infrared lamp, weighed and counted with a Tracerlab counter with a mica window (diam. 28 mm., thickness 1·5–1·8 mg./cm.^{2}). Radioactivities were corrected for background and self-absorption.

The creatine phosphate in some experiments was identified by paper chromatography. The water-soluble 80% ethanol-insoluble barium salt was dissolved in a minimum volume of N-HCl at 0°. Any insoluble material was sedimented by centrifuging and sufficient N-NaOH was added to the clear solution to bring the pH to about neutral (phenolphthalein was added as indicator). The solvent used for chromatography was 90% phenol–water–aq. NH_3 soln. (sp.gr. 0·88) (960:110:3, by vol.). After paper chromatography the paper was thoroughly dried and placed in contact with Kodak Medical 'No Screen' X-ray film for 3 days. After the films had been developed the radioactive spots were located on the paper. The R_F of the water-soluble 80% ethanol-insoluble compound was about 0·15, and that of the control creatine was about 0·8. Hydrolysis of the 80% ethanol-insoluble barium salt in N-HCl for 7 min. resulted after paper chromatography in the disappearance of the spots with R_F 0·15 and appearance of

radioactive spots with R_F 0·8, identical with that of the control creatine.

Preparation of glutamine synthetase. A procedure similar to that of Elliott (1951) was used. Acetone-dried grey matter of rat-brain-cortex slices was extracted with 10 vol. of water for 10 min. with very gentle stirring. The extract was centrifuged, and the supernatant was filtered through cotton wool, cooled to 4° and mixed with 0·2 vol. of 0·1 M-sodium acetate buffer, pH 4·2. The precipitate was collected by centrifuging, washed twice by suspension in cold water and centrifuging, and finally redissolved in water (half the original volume of extract) by adjusting the pH to 6·8 with a little NaOH. The enzyme preparation was incubated aerobically in the presence of uniformly labelled glutamate, ATP and NH_4Cl for 1 hr. at 37°. The composition of the incubation medium was: L-[$^{14}C_5$]glutamate, 10 mM; Na_2HPO_4 (brought to pH 7·4 with N-HCl), 2 mM; ATP, 10 mM; $MgSO_4$, 10 mM; NH_4Cl, 5 mM; total vol. 1 ml. The enzymic reaction was stopped by adding 4 vol. of 96% (v/v) ethanol. The proteins were sedimented by centrifuging and the labelled glutamine was separated from the glutamic acid by two-dimensional paper chromatography and detected by radioautography.

Measurements of uptake of L-[$^{14}C_5$]*glutamate and* [1-^{14}C]-*creatine by rat-brain-cortex slices.* After aerobic incubation for 1 hr. at 37° in an appropriate medium the slices were removed, weighed and rinsed in a Krebs–Ringer phosphate medium and homogenized in 10 ml. of 80% ethanol. The homogenate was centrifuged, and a portion of the supernatant was plated and counted. Details of the procedure are given by Vardanis & Quastel (1961).

Reagents. [$^{14}C_6$]Glucose and L-[$^{14}C_5$]glutamate were purchased from Merck and Co., Montreal. Ouabain was supplied by Nutritional Biochemicals Corp. Dowex 50W (X4) resin was obtained from Dow Chemical Co., Montreal. All other chemicals were of reagent grade and were used without further purification.

RESULTS

Ouabain and rat-brain-cortex respiration. Ouabain at low concentrations (e.g. 10 μM) has no effect within experimental error on the rate of oxygen uptake of rat-brain-cortex slices incubated at 37° in glucose/Krebs–Ringer phosphate medium. When the concentration of ouabain is increased inhibitory effects are observed. These are small when the incubation is carried out in a medium containing 5 m-equiv. of K^+ ion/l. but relatively large in the presence of 105 m-equiv. of K^+ ion/l. Table 1 shows that the stimulation of respiration due to added (100 m-equiv./l.) K^+ ions is almost completely suppressed by mM-ouabain. Closer examination of these results shows that ouabain, at the concentrations tested, inhibits the total respiration to a roughly similar extent in the presence of either 5 or 105 m-equiv. of K^+ ion/l. For example, with 0·1 mM-ouabain, the percentage inhibitions are 17 and 26 respectively. Thus ouabain inhibits brain-cortex respiration *in vitro*, the inhibition increasing with its concentration presumably by its well-known suppression of the transport mechanisms responsible for K^+ ion uptake and Na^+ ion extrusion. On the other hand, the addition of K^+ ions stimulates brain-cortex respiration, presumably through its intermediate effect on cation displacements at the cell membrane. Thus ouabain and K^+ ions may be considered to exert antagonistic effects on brain-cell respiration.

Effects of ouabain on the incorporation of ^{32}P *into the nucleotide pyrophosphate fraction in rat-brain-cortex slices.* Although it is known (Kunz & Sulser, 1957; Furchgott & de Gubareff, 1958; Lee, Schwartz & Burstein, 1960; Cotton & Moran, 1961) that ouabain, at the low concentrations affecting cation transport, does not affect cell ATP concentrations, experiments were carried out to observe whether 10 μM-ouabain would affect the labelling of nucleotide pyrophosphate in rat-brain-cortex slices. Results are shown (Table 2) in which the effects of incubation with 2,4-dinitrophenol are also given as a contrast with those obtained with ouabain. Ouabain (10 μM) does not affect, within the experimental range of variation, the labelling of '7 min. hydrolysable' nucleotide pyrophosphate in the presence of either 5 or 105 m-equiv. of K^+ ion/l. The marked suppressing effect of dinitrophenol (Terner, Eggleston & Krebs, 1950) and the lesser effect of increased K^+ ion concentration on the nucleotide pyrophosphate labelling are also shown in Table 2. The latter result might be expected from the decreased concentration of brain creatine phosphate caused by increased extracellular K^+ ion concentrations (Gore & McIlwain, 1952; McIlwain,

Table 1. *Effects of ouabain at various concentrations on rat-brain-cortex respiration in glucose/Krebs–Ringer phosphate medium after incubation for* 1 *hr. at* 37°

Rat-brain-cortex slices from adult hooded rats of about 150 g. were placed in ice-cold Krebs–Ringer phosphate medium of the following composition: NaCl, 128 mM; $CaCl_2$, 3·6 mM; $MgSO_4$, 1·3 mM; Na_2HPO_4, 10 mM (brought to pH 7·4 with N-HCl). KCl and ouabain were added to the main vessel at the start of the experiment. The average wet wt. of slices was 80–90 mg. The final volume of medium in each Warburg manometric flask was 1 ml., and each flask contained 5 mM-glucose. The temperature of incubation was 37°. Incubations were carried out in oxygen for 1 hr. The results are means ± S.D. of six independent determinations.

	Q_{O_2}	
[K^+] (m-equiv./l.) ...	5	105
Concn. of ouabain (mM)		
0	8·8 ± 0·4	12·9 ± 0·5
0·01	8·8 ± 0·4	12·0 ± 0·4
0·1	7·3 ± 0·3	9·6 ± 0·3
1	6·3 ± 0·3	6·7 ± 0·3

1952; Heald, 1954; Rossiter, 1957) and the operation of creatine phosphokinase.

Ouabain and glucose–amino acid interrelations. Table 3 shows the effects of 10 μM- and μM-ouabain on the amounts of radioactive amino acids found in rat-brain-cortex slices after incubation with radioactive glucose (5 mM) for 1 hr. at 37° in Krebs–Ringer phosphate medium containing either 5 or 105 m-equiv. of K^+ ion/l. Amounts found in the absence of Ca^{2+} ions are also noted. The addition of more K^+ ions increases the yields of labelled glutamine, γ-aminobutyrate and glutamate, in confirmation of the results of Kini & Quastel (1959).

The increased yields of labelled glutamine and glutamate found in the presence of high concentrations of K^+ ions (or in the absence of Ca^{2+} ions) are attributed to a more rapid rate of turnover of the citric acid cycle in the brain mitochondria, so that more α-oxoglutarate is converted by transamination into glutamate. Increased availability of ATP in the mitochondria causes an increased yield of glutamine.

The most noteworthy effect of the addition of ouabain is its suppression of the formation of labelled glutamine in the brain tissue, the effect being greater in the presence of 105 than in that of 5 m-equiv. of K^+ ion/l. A large suppressing effect also occurs with alanine, though this seems to be less dependent on the concentration of K^+ ions. There is no depressing effect on the yield of labelled glutamate and relatively little on that of aspartate or of γ-aminobutyrate. The absence of Ca^{2+} ions (in the presence of 5 m-equiv. of K^+ ion/l.), like the addition of K^+ ions, increases the inhibitory effect of ouabain on the formation of labelled glutamine.

An examination was made of the effects of ouabain at different concentrations not only on the radioactive amino acid content of the rat-brain-cortex slices at the end of the incubation period but on that in the incubating medium. Normally there is a small leakage of amino acids from rat-brain-cortex slices incubated aerobically in a glucose medium, the amounts being greatest (relative to those in the tissue) with alanine and least with glutamate (Table 4). The presence of μM-ouabain increases the leakage of amino acids from the tissue, the greatest amounts (relative to those remaining in the tissue) occurring with glutamine and alanine. With 10 μM-ouabain no radioactive glutamine is to be found either in the medium or in the tissue, but increased quantities of labelled alanine, γ-aminobutyrate, aspartate and glutamate are found in the medium. The tissue content of glutamate is slightly increased, and that of γ-aminobutyrate is slightly diminished.

Hence the presence of ouabain, even at a concentration of 1 μM, exercises a considerable influence on the efflux of amino acids from the brain tissue into the surrounding medium, an influence that cannot be ascribed to any appreciable changes in

Table 2. *Effects of ouabain and 2,4-dinitrophenol on the incorporation of* ^{32}P *into the total nucleotide fraction (adenosine triphosphate and adenosine diphosphate) in rat-brain-cortex slices* in vitro *after incubation for 1 hr. at 37°*

Conditions were as in Table 1 except that labelled phosphate (10^6 counts/min.) was added to each vessel. The radioactivities of the total nucleotides were determined as described in the Materials and Methods section. The results are means ± S.D. of five independent determinations.

	Labelling of total nucleotide fraction (ATP and ADP) (counts/min./100 mg. wet wt. of tissue/hr.)	
$[K^+]$ (m-equiv./l.) ...	5	105
Addition		
Nil	3100±90	2700±50
2,4-Dinitrophenol (50 μM)	1395±40	675±40
Ouabain (10 μM)	3150±120	2740±65

Table 3. *Rates of formation of radioactive amino acids from radioactive glucose by rat-brain-cortex slices in the presence of ouabain and* [$^{14}C_6$]*glucose*

Rat-brain-cortex slices were incubated in Krebs–Ringer phosphate medium (composition indicated in Table 1) for 1 hr. in oxygen at 37° in the presence of [$^{14}C_6$]glucose (5 mM; 200 000 counts/min.). The final volume was 1·0 ml. The results are given as means ± S.D. of six independent determinations.

	Amount of amino acid (incorporated μmg.atoms of carbon derived from glucose/hr./100 mg. wet wt. of tissue)							
Concn. of ouabain (μM)	0	0	1	1	0	10	10	10
Concn. of KCl (mM)	5	105	5	105	5	5	5	105
Concn. of $CaCl_2$ (mM)	3·6	3·6	3·6	3·6	0	0	3·6	3·6
Amino acid								
Glutamate	865±52	965±60	920±61	1030±70	1120±60	810±35	887±42	1101±60
Glutamine	192±8	341±17	95±5	60±5	250±17	35±5	50±5	Nil
γ-Aminobutyrate	217±10	325±25	220±11	280±15	170±7	100±5	225±10	260±12
Aspartate	261±12	264±15	270±10	282±12	290±18	210±11	155±7	163±12
Alanine	152±7	162±10	98±7	105±8	80±5	60±5	80±8	92±7

respiratory rates. Moreover, the biosynthesis of glutamine from glucose is abolished by 10 μM-ouabain in the presence of a high K^+ ion concentration. Such a concentration of K^+ ions shows almost its normal stimulating effect on brain respiration in the presence of the ouabain (Table 1).

Effects of ouabain on amino acid formation in rat-brain-cortex slices from [$^{14}C_6$]glucose in the presence of L-*glutamate.* The ouabain suppression of glutamine biosynthesis from glucose is greatly diminished by the addition of L-glutamate to the glucose/Krebs–Ringer phosphate medium in which the rat-brain-cortex slices are incubated. The findings (Tables 5 and 7) show that addition of L-glutamate to the [$^{14}C_6$]glucose medium markedly reduces the labelling of glutamine. This is probably due to isotopic dilution of labelled glutamate, derived from [$^{14}C_6$]glucose, by unlabelled glutamate transferred from the medium.

The addition of 10 μM-ouabain to the glucose medium abolishes glutamine formation and reduces the alanine content of the tissue (see also Table 4). Addition of 10 μM-ouabain, however, to the glucose–glutamate medium diminishes but little the radioactive glutamine concentration found without ouabain and has no appreciable effect on the radioactive alanine concentration. Decreased labelling of aspartate, by addition of unlabelled glutamate, is doubtless partly due to isotopic dilution [as glutamate is converted into aspartate (see Tables 7 and 8)].

These results would be consistent with the conclusion that the retention of brain-cell K^+ ions by a mixture of glucose and glutamate (Terner *et al.* 1950; Krebs, Eggleston & Terner, 1951; Pappius & Elliott, 1956) diminishes the loss of K^+ ions induced by ouabain (Gardos, 1960; Yoshida, Nukada & Fujisawa, 1961), and that the amino acid changes induced by ouabain are a consequence of its suppression of membrane cation transfer. It is necessary, however, to explain how such a suppression by 10 μM-ouabain can cause abolition of glutamine biosynthesis in rat-brain-cortex slices.

Effects of ammonium chloride on amino acid formation in rat-brain-cortex slices from [$^{14}C_6$]*glucose in the presence of ouabain.* Experiments were carried out to observe the effects of NH_4^+ ions on ouabain inhibition of glutamine formation from [$^{14}C_6$]-

Table 4. *Effects of ouabain on radioactive-amino acid formation from radioactive glucose by rat-brain-cortex slices in the presence of* 105 *m-equiv. of* K^+ *ion/l.*

Rat-brain-cortex slices were incubated in Krebs–Ringer phosphate medium (see Table 1) for 1 hr. in oxygen at 37° in the presence of [$^{14}C_6$]glucose (5 mM), and ouabain was added where indicated. After 1 hr. the slices were removed from the incubation medium and the labelling of amino acids was determined in the tissue and medium as described in the Materials and Methods section. Results are means ± S.D. of four independent determinations.

	Amount of amino acid (incorporated μmg.atoms of carbon derived from glucose/hr.)							
Concn. of ouabain (μM) ...	0		0·1		1		10	
Amino acid	Tissue (100 mg.)	Medium (1 ml.)	Tissue (100 mg.)	Medium (1 ml.)	Tissue (100 mg.)	Medium (1 ml.)	Tissue (100 mg.)	Medium (1 ml.)
Glutamate	965 ± 62	35 ± 15	960 ± 57	38 ± 12	1030 ± 72	163 ± 12	1101 ± 62	234 ± 23
Glutamine	341 ± 17	40 ± 23	350 ± 15	40 ± 18	60 ± 5	105 ± 17	0	0
γ-Aminobutyrate	325 ± 25	15 ± 9	320 ± 21	15 ± 6	280 ± 15	90 ± 8	260 ± 12	117 ± 9
Aspartate	264 ± 15	15 ± 11	270 ± 13	22 ± 9	282 ± 12	40 ± 6	163 ± 12	80 ± 9
Alanine	162 ± 12	38 ± 15	170 ± 15	50 ± 11	105 ± 8	86 ± 9	92 ± 7	110 ± 12

Table 5. *Effects of ouabain on amino acid formation in rat-brain-cortex slices from* [$^{14}C_6$]*glucose in the presence and absence of* L-*glutamate*

Experimental conditions were as in Table 1. Each vessel contained [$^{14}C_6$]glucose (5 mM; 200 000 counts/min.); ouabain (when present) was 10 μM, and L-glutamate (when present) was 10 mM; KCl was 105 mM. Incubation was carried out in oxygen for 1 hr. at 37°. Values are means ± S.D. of six independent determinations.

	Amount of amino acid (incorporated μmg.atoms of carbon derived from glucose/hr./100 mg. wet wt. of tissue)			
Additions	None	L-Glutamate	Ouabain	L-Glutamate + ouabain
Amino acid				
Glutamate	965 ± 60	905 ± 67	1101 ± 60	709 ± 70
Glutamine	341 ± 17	110 ± 8	0	77 ± 7
γ-Aminobutyrate	325 ± 25	270 ± 12	260 ± 12	220 ± 10
Aspartate	264 ± 15	220 ± 13	163 ± 12	110 ± 10
Alanine	162 ± 10	175 ± 15	92 ± 7	198 ± 14

glucose in the presence of rat-brain-cortex slices. Results are shown in Table 6.

The effect of the presence of 10 mM-ammonium chloride on the content of labelled amino acids derived from [$^{14}C_6$]glucose in a Krebs–Ringer phosphate medium containing 5 mM-potassium chloride is to increase greatly the tissue concentration of labelled glutamine, in confirmation of findings (Weil-Malherbe, 1936; Tower, 1958) that brain glutamine concentrations are increased in the presence of added NH_4^+ ions. The concentration of labelled glutamate is but little affected.

The ouabain suppression of labelled-glutamine formation is greatly diminished by the addition of 10 mM-ammonium chloride. This is also seen when the incubation is carried out in Krebs–Ringer phosphate medium containing 105 mM-potassium chloride, where the suppression of glutamine biosynthesis by 10 μM-ouabain is greatly reduced by the addition of 10 mM-ammonium chloride.

The increased rate of formation of labelled glutamine by the addition of 10 mM-ammonium chloride in a medium containing a low concentration (5 m-equiv./l.) of K^+ ions is probably partly, if not wholly, explained by the fact that increased respiration occurs on the addition of NH_4^+ ions (Annau, 1934; Weil-Malherbe, 1938). The increased mitochondrial respiration secures, through an increased rate of mitochondrial ATP formation, a higher rate of glutamine biosynthesis. The phenomenon is, in fact, similar to that occurring when respiring brain-cortex slices in a glucose medium are exposed to a high K^+ ion concentration. It is presumably for this reason that addition of NH_4^+ ions has much less effect in enhancing glutamine formation in brain cortex in a medium with a high K^+ ion concentration than in one containing a low K^+ ion concentration.

The results with ouabain would be explained if ouabain suppresses transport of NH_4^+ ions from the cytoplasm into the brain mitochondria or microsomes which are principal sites of glutamine synthesis (Waelsch, 1958). If this is so, it is to be expected that addition of NH_4^+ ions would overcome the suppression.

The fact that the inhibition of glutamine biosynthesis by ouabain is greater in the presence of 105 than in that of 5 m-equiv. of K^+ ion/l. could be explained if the carrier sites required for the transport of NH_4^+ ions at the mitochondrial membrane are also capable of taking up K^+ ions, so that fewer NH_4^+ ions are transferred when the cell K^+ ion concentration is high (as with a high external K^+ ion concentration) than when it is diminished (as with a low external K^+ ion concentration).

Lack of effect of ouabain on glutamine synthetase. Although 10 μM-ouabain cannot directly inhibit (to any appreciable extent) the activity of glutamine synthetase (Tables 5 and 6), experiments were carried out with the isolated enzyme system. The results show that 10 μM-ouabain has no inhibitory effect on glutamine synthetase under the given experimental conditions. After incubation of 10 mM-L-[$^{14}C_5$]glutamate for 1 hr. at 37° under aerobic conditions in the presence of ATP, ammonium chloride, Mg^{2+} ions and a preparation of glutamine synthetase from brain-cortex slices (see Materials and Methods section), the amount of glutamine formed, expressed as μmg.atoms of carbon derived from glutamate, was 1980 ± 105; in the presence of 10 μM-ouabain the value was 2010 ± 112, the results being means $\pm$ S.D. of four experiments.

Radioactive-amino acid formation from radioactive glutamate and glucose in the presence of rat-brain-cortex slices. Table 7 shows the pattern of amino acids formed from L-[$^{14}C_5$]glutamate, [$^{14}C_6$]glucose and mixtures of labelled glutamate and glucose. The results are similar to those described by Takagaki, Hirano & Nagata (1959). The labelled

Table 6. *Effects of ammonium chloride on amino acid formation in rat-brain-cortex slices from* [$^{14}C_6$]*glucose in the presence and absence of ouabain*

Composition of the incubation medium was as indicated in Table 1. Each vessel contained [$^{14}C_6$]glucose (5 mM; 200 000 counts/min.). Ouabain (when present) was 10 μM; NH_4Cl (when present) was 10 mM. Incubations were carried out in oxygen for 1 hr. at 37°. The results are means $\pm$ S.D. of four independent determinations.

	Amount of amino acid (incorporated μmg.atoms of carbon derived from glucose/hr./100 mg. wet wt. of tissue)							
Concn. of KCl (mM)	5				105			
Additions ...	None	Ouabain	NH_4Cl	Ouabain + NH_4Cl	None	Ouabain	NH_4Cl	Ouabain + NH_4Cl
Amino acid								
Glutamate	865±52	887±42	830±51	730±40	965±60	1101±60	924±70	730±30
Glutamine	192±8	50±5	340±17	280±12	341±17	0	395±20	155±7
γ-Aminobutyrate	217±10	225±10	170±8	190±10	325±25	260±12	366±15	205±10
Aspartate	261±12	155±7	278±15	182±7	264±15	163±12	256±13	230±15
Alanine	152±7	80±8	190±10	60±6	162±10	92±7	180±10	145±7

amino acids derived from labelled glutamate are aspartate, glutamine, and γ-aminobutyrate. No labelled alanine is observed (within the experimental error). This result would be expected if the labelled glutamate is converted into α-oxoglutarate and thence into oxaloacetate, which is then transaminated to give labelled aspartate. Presumably the labelled pyruvate, derived from oxaloacetate, is converted into acetyl-CoA before any appreciable transamination to give alanine can take place.

The addition of unlabelled glucose to L-[$^{14}C_5$]-glutamate greatly increases the labelling of glutamine, γ-aminobutyrate and aspartate. This is attributed to the marked increase of uptake of glutamate into the brain cells in the presence of glucose.

Effects of ouabain on radioactive amino acids derived from radioactive glutamate in rat-brain-cortex slices. Experiments were carried out to observe whether 10 μM-ouabain affects the rates of formation of amino acids from L-[$^{14}C_5$]glutamate in rat-brain-cortex slices respiring in glucose/Krebs–Ringer phosphate medium containing 5 m-equiv. of K^+ ion/l. Results are shown in Table 8.

In the presence of ouabain and mM-L-[$^{14}C_5$]-glutamate the yield of labelled glutamine is almost completely suppressed, with no diminution of the yields of labelled aspartate and γ-aminobutyrate. Such a result is consistent with the inhibitory effect on glutamine biosynthesis from glucose by ouabain at low concentrations (10 μM) (Table 4). When the concentration of labelled glutamate in the medium is increased, the percentage (but not the absolute) inhibition by ouabain is diminished. As the external glutamate concentration is increased, more labelled glutamine is formed, the amount being proportional to the amount of glutamate. This is probably due to the fact that the glutamate concentration in the cell is not sufficient to saturate the synthesizing enzyme and that the

Table 7. *Metabolism of rat-brain-cortex slices in the presence of glucose,* L-*glutamate, or a mixture of glucose and* L-*glutamate*

Composition of the incubation medium was as indicated in Table 1 except that [$^{14}C_6$]glucose and L-[$^{14}C_5$]-glutamate were added where indicated. The final vol. was 1 ml. and incubation was carried out in oxygen for 1 hr. at 37°. KCl was 5mM. The results are means ± S.D. of five independent determinations.

		Amount of CO_2 or amino acid (incorporated μmg.atoms of carbon derived from glutamate or glucose/100 mg. wet wt. of tissue)					
Additions	Q_{O_2}	$^{14}CO_2$	Glutamate	Glutamine	γ-Aminobutyrate	Alanine	Aspartate
[$^{14}C_6$]Glucose (5 mM; 200 000 counts/min./vessel)	8·8 ± 0·4	2583 ± 175	850 ± 48	197 ± 10	222 ± 11	160 ± 7	258 ± 9
L-[$^{14}C_5$]Glutamate (10 mM; 90 000 counts/min./vessel)	9·4 ± 0·4	3519 ± 133	5950 ± 350	1670 ± 105	520 ± 25	0	4100 ± 290
[$^{14}C_6$]Glucose (5 mM; 200 000 counts/min./vessel) + L-glutamate (10 mM; unlabelled)	10·3 ± 0·3	2119 ± 105	575 ± 45	90 ± 10	190 ± 15	155 ± 13	232 ± 18
L-[$^{14}C]_5$Glutamate (10 mM; 90 000 counts/min./vessel) + glucose (5 mM; unlabelled)	10·3 ± 0·4	3400 ± 155	13000 ± 500	3878 ± 91	870 ± 20	0	4300 ± 75

Table 8. *Rates of formation of radioactive amino acids from radioactive glutamate by rat-brain-cortex slices in the presence of ouabain*

Conditions were as in Table 1. Glucose (5 mM) was present throughout; L-[$^{14}C_5$]glutamate (90 000 counts/min.) was added to each vessel at the concentrations indicated. The final vol. was 1 ml. and incubation was carried out in oxygen for 1 hr. at 37°. KCl was 5mM. The results are means ± S.D. of four independent determinations.

	Amount of amino acid (incorporated μmg.atoms of carbon derived from glutamate/hr./100 mg. wet wt. of tissue)					
	Without ouabain			With ouabain (10 μM)		
Concn. of L-[$^{14}C_5$]glutamate added (mM)	1	5	10	1	5	10
Amino acid						
Glutamate	1512 ± 107	8100 ± 170	13000 ± 50	1890 ± 110	7000 ± 120	9300 ± 130
Glutamine	423 ± 23	2000 ± 60	3878 ± 91	30 ± 10	620 ± 15	1000 ± 30
γ-Aminobutyrate	114 ± 7	530 ± 20	870 ± 20	157 ± 8	600 ± 10	850 ± 15
Aspartate	667 ± 28	2850 ± 50	4300 ± 75	739 ± 35	2250 ± 50	3550 ± 75
Alanine	0	0	0	0	0	0

Table 9. *Effects of ammonium chloride on amino acid formation in rat-brain-cortex slices from* L-[$^{14}C_5$]*glutamate in the presence and absence of ouabain*

Conditions were as in Table 1. Each vessel contained glucose (5 mM), L-[$^{14}C_5$]glutamate (90 000 counts/min.); ouabain (when present) was 10 μM, and NH_4Cl (when present) was 10 mM. Incubation was carried out at 37° for 1 hr. in oxygen. KCl was 5mM. The final vol. was 1 ml. The results are means ± S.D. of four independent determinations.

Additions	Amount of amino acid (incorporated μmg.atoms of carbon derived from glutamate/hr./100 mg. wet wt. of tissue)			
	None	NH_4Cl	Ouabain	NH_4Cl + ouabain
Amino acid				
Glutamate	1512 ± 107	1530 ± 92	1890 ± 112	1368 ± 58
Glutamine	423 ± 23	428 ± 20	30 ± 10	167 ± 6
γ-Aminobutyrate	114 ± 7	125 ± 10	157 ± 8	87 ± 11
Aspartate	626 ± 28	645 ± 17	739 ± 35	560 ± 27
Alanine	0	0	0	0

amount of condensation with ammonia increases with the amount of glutamate available.

The addition of 10 m-equiv. of NH_4^+ ion/l. to a glucose–mM-L-[$^{14}C_5$]glutamate medium greatly reduces the ouabain inhibition of glutamine formation from glutamate, but has little effect on the glutamine formation in absence of ouabain (Table 9). In the presence of added glutamate, or with the increased intracellular glutamate formation from glucose such as occurs in presence of a high K^+ ion concentration, the concentration of NH_4^+ ions formed in the cell is sufficient to ensure optimum glutamine biosynthesis, for added NH_4^+ ions have but little enhancing effect on labelled glutamine formation under these conditions (Tables 6 and 9). In the presence of ouabain, NH_4^+ ions have a rate-limiting effect on glutamine biosynthesis in rat-brain-cortex slices, possibly owing to a diminished mitochondrial concentration of NH_4^+ ion, either because of loss of NH_4^+ ions from the brain mitochondria or because of suppression of NH_4^+ ion transport into the mitochondria.

Effects of ouabain on glutamate influx into rat-brain-cortex slices. The presence of 10 μM-ouabain retards considerably the influx of glutamate into rat-brain-cortex slices incubated aerobically in a glucose–Krebs–Ringer phosphate medium in the presence of relatively high concentrations of either glutamate (5 mM) or of K^+ ions (105 m-equiv./l.). This result could have been expected from the fact that 10 μM-ouabain accelerates the leakage of glutamate, as well as that of other amino acids, from brain-cortex slices (Table 4).

Table 10 shows that 10 μM-ouabain has little or no effect on the influx of L-glutamate (mM) in the absence of K^+ ions or in presence of 5 m-equiv. of K^+ ion/l. in the incubating medium. In the presence of 105 m-equiv. of K^+ ion/l., the diminution of the influx of glutamate (mM) into the rat-brain-cortex slices amounts to over 50%. Increased concentration of extracellular K^+ ions itself reduces the amino acid uptake, a phenomenon which occurs in isolated brain with L-glutamate (Vardanis & Quastel, 1961), D-glutamate (Takagaki *et al.* 1959), and tyrosine (Guroff, King & Udenfriend, 1961). When the glutamate concentration in the medium containing 5 m-equiv. of K^+ ion/l. is increased to 5 or 10 mM, the influx of the amino acid into the tissue is correspondingly increased. At the same time, the presence of 10 μM-ouabain causes an inhibition of influx which increases with increase of concentration of the amino acid (Table 10).

The results of Table 10 give the total radioactivity of ethanol-soluble material of the brain-cortex slices after incubation in the glucose–L-[$^{14}C_5$]glutamate media. The values are largely measures of the total radioactive amino acids, formed from glutamate, present in the tissue. The largest inhibitory effect on glutamate influx by ouabain occurs in presence of mM-glutamate and 105 m-equiv. of K^+ ion/l.

This phenomenon may be accounted for as follows. Amino acid transport into brain-cortex slices is energy-dependent (Stern, Eggleston, Hems & Krebs, 1949; Elliott & van Gelder, 1958; Tsukada, Nagata & Hirano, 1960; Schanberg & Giarman, 1960). Abadom & Scholefield (1961) reported that the rates of transport of some amino acids in the presence of glucose are proportional to the ATP concentrations in the brain. Also the concentration of brain ATP is decreased by the presence of a high concentration of K^+ ions in the incubation medium (Rossiter, 1957), so that a diminution of the rate of amino acid transport by a high K^+ ion concentration in the medium may be expected. The diminution in the rate of transport of glutamate (mM) in a glucose medium by increase of the extracellular K^+ ion concentration from 5 to 105 m-equiv./l. is only about 7% (Table 9), but amounts to 50% with 20 mM-glutamate under similar experimental conditions (Vardanis & Quastel, 1961).

The increased inhibition of influx by high K^+ ion concentrations, obtained with the higher concen-

tration of glutamate, may result from a further decrease of the concentration of brain ATP, as glutamate may itself decrease the ATP concentration (Acs, Balazs & Straub, 1952, 1953) by operation of creatine phosphokinase (Narayanaswami, 1952) after its large depression of brain creatine phosphate concentrations (Rossiter, 1957; Woodman & McIlwain, 1961); but it would be simpler to understand this phenomenon if the carrier responsible for the transport of glutamate has also an affinity for K^+ ions. If this were true, then with high concentrations of K^+ ions there would be fewer carrier sites available for the amino acid than with lower concentrations of K^+ ions. Whereas the diminished number of carrier sites would allow a normal, or almost normal, rate of transport with a small concentration of glutamate, it would be insufficient to allow the normal rate of transport with an increased concentration of glutamate; thus the percentage diminution of amino acid influx by high extracellular K^+ ion concentrations would be greater with a high concentration of glutamate than with a low concentration.

The same hypothesis makes it possible to understand the larger inhibition by 10 μM-ouabain of the transport of glutamate at high concentrations than that of glutamate at low concentrations. Combination of glutamate-carrier sites with ouabain would diminish the influx of glutamate, so that the rate of transport of a large quantity of glutamate would be more affected by ouabain than that of a small quantity of glutamate. Moreover, a combination of ouabain and high K^+ ion concentrations would clearly give the largest inhibitory effect of glutamate transport. These phenomena are all shown in Table 10.

Also, in the presence of 10 mM-ammonium chloride, 10 μM-ouabain inhibits glutamate (mM) transport more than in its absence (Table 10). The possibility that this may be due largely to decreased cell ATP concentrations (known to be brought about in brain by the addition of NH_4^+ ions) is unlikely, as the rate of transport of mM-glutamate with 10 mM-ammonium chloride present is approximately the same as that in its absence. This phenomenon would be readily explained if NH_4^+ ions are also taken up by the carrier or carriers responsible for K^+ ion and glutamate transport, the additional presence of ouabain leaving fewer carrier sites available for the transport of the amino acid. Such a view would be consistent with the conclusion given above, that ouabain suppresses NH_4^+ ion transport into brain mitochondria, thus accounting for its suppression of glutamine biosynthesis there.

The values of influx of glutamate (Table 10) have not been corrected for the values of the passive diffusion rates. Such corrections will not affect the conclusions that have been made, as subtraction of passive diffusion rates from the observed values of influx only increases the percentage inhibitions of active transport of the amino acid brought about by ouabain.

Effects of ouabain on creatine uptake by and formation of creatine phosphate in the brain in vitro. Experiments were carried out to observe whether

Table 10. *Effects of* 10 μM-*ouabain on aerobic uptake at* 37° *of* L-[$^{14}C_5$]*glutamate by rat-brain-cortex slices in the presence of various concentrations of* K^+ *and of* NH_4^+ *ions*

Rat-brain-cortex slices were incubated in Krebs–Ringer phosphate medium (see Table 1) for 1 hr. in oxygen at 37° in the presence of L-[$^{14}C_5$]glutamate and unlabelled glucose (5 mM). After incubation the slices were removed from the medium and the radioactivities of the slices and the medium were determined as described in the Materials and Methods section. The results are means ± S.D. of four independent determinations.

Concn. of NH_4Cl (mM)	Concn. of KCl (mM)	Concn. of L-[$^{14}C_5$]-glutamate (mM)	Concn. of ouabain (μM)	Amount of uptake (incorporated μmg.atoms of carbon derived from glutamate/hr.)	
				Tissue (100 mg.)	Medium (1 ml.)
0	0	1	0	2843 ± 71	880 ± 21
0	0	1	10	2 882 ± 67	957 ± 30
0	5	1	0	2 711 ± 105	845 ± 23
0	5	1	10	2760 ± 96	860 ± 31
0	5	5	0	13 400 ± 265	9 650 ± 125
0	5	5	10	10 780 ± 189	13 100 ± 276
0	5	10	0	21 300 ± 310	22 300 ± 511
0	5	10	10	15 500 ± 300	27 400 ± 603
0	105	1	0	2 520 ± 58	1 150 ± 34
0	105	1	10	1 160 ± 34	2 360 ± 59
10	5	1	0	2 730 ± 81	850 ± 24
10	5	1	10	2 280 ± 64	1 540 ± 37

Table 11. *Effects of 10 µM-ouabain on aerobic uptake at 37° of [1-^{14}C]creatine by rat-brain-cortex slices in the presence of different concentrations of potassium chloride*

The composition of the incubation medium was as indicated in Table 1. Each vessel contained glucose (5 mM) and [1-^{14}C]creatine (0·25 mM; 110 000 counts/min./vessel). Radioactivities of creatine and creatine phosphate in the slices and in the medium were determined as described in the Materials and Methods section. The results are means ± S.D. of six independent determinations.

	Amount of uptake (incorporated µmg.atoms of carbon derived from creatine/hr.)			
	Tissue (100 mg. wet wt. of tissue)			
Additions	Creatine	Creatine phosphate	Total creatine uptake corrected for diffusion	Medium (1 ml.) creatine
K^+ ions (5 m-equiv./l.)	66 ± 2·8	18 ± 1·2	59	144 ± 5
K^+ ions (5 m-equiv./l.) + ouabain (10 µM)	27 ± 1·1	18 ± 0·7	20	185 ± 4
K^+ ions (5 m-equiv./l.) + 2,4-dinitrophenol (0·1 mM)	36 ± 0·7	1 ± 0·5	12	198 ± 6
K^+ ions (105 m-equiv./l.)	33 ± 0·2	3 ± 0·3	11	195 ± 5
K^+ ions (105 m-equiv./l.) + ouabain (10 µM)	29 ± 0·1	3 ± 0·5	7	196 ± 4

10 µM-ouabain affects the uptake of labelled creatine or the formation of labelled creatine phosphate when rat-brain-cortex slices are incubated in a glucose–Krebs–Ringer phosphate medium in the presence of [1-^{14}C]creatine. Thomas (1956) showed that creatine will accumulate in brain against a concentration gradient during aerobic incubation in the presence of glucose.

Table 11 shows that 10 µM-ouabain in glucose–Krebs–Ringer phosphate medium greatly diminishes the influx of creatine (0·25 mM) with no effect (within experimental error) on the labelling of creatine phosphate.

The presence of a high concentration (105 m-equiv./l.) of K^+ ions in the incubation medium brings about both a large fall in the uptake of creatine and in the formation of labelled creatine phosphate. Creatine phosphate concentrations in brain fall on exposure of the tissue to high K^+ ion concentrations (McIlwain, 1952; Kratzing & Narayanaswami, 1953; Heald, 1954; Rossiter, 1957), presumably as a consequence of the fall of intracellular ATP concentrations and rise in ADP concentrations. The diminished transport of creatine may be attributed to the lowered ATP concentration. The suppression due to the presence of dinitrophenol is shown in Table 11. It is, however, possible that there is competition between K^+ ions and creatine for a carrier site in the brain-cell membrane.

On the addition of 10 µM-ouabain to the medium containing 105 m-equiv. of K^+ ion/l., there is no change, within experimental error, in the labelling of the creatine phosphate and only a small further diminution of the influx of creatine. Examination of the medium showed increased quantities of labelled creatine but no labelled creatine phosphate in the presence of ouabain.

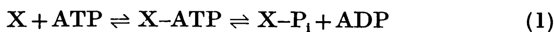
The inhibitory effect of 10 µM-ouabain on the creatine transport is not as large as that due to 105 m-equiv. of K^+ ion/l., as the total creatine uptake must be measured by the sum of the labelled creatine and creatine phosphate found in the tissue. Though the latter is relatively high in the presence of ouabain, it is low in presence of the high concentration of K^+ ions. The value of total creatine uptake [i.e. the sum of creatine and phosphocreatine derived from [1-^{14}C]creatine (Table 11)] should be corrected for the creatine that enters by passive diffusion. The maximum value for this process is 25 µm-moles (or 25 µmg.atoms of carbon derived from the labelled creatine)/100 mg. wet wt. of tissue. After correction of the values of creatine uptake for this amount, the results of Table 11 show that the combination of 10 µM-ouabain and 105 m-equiv. of K^+ ion/l. almost completely suppresses the active transport of creatine. The facts are consistent with the conclusion that a carrier exists in the brain-cell membrane for the transport of creatine and that the activity of this carrier depends on the concentration of cell ATP and is suppressed by ouabain.

DISCUSSION

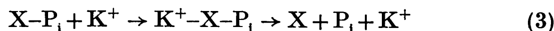
Since the cardiac glycosides inhibit cationic fluxes without depressing respiration or glycolysis (Schatzmann, 1953; Kunz & Sulser, 1957; Maizels, Remington & Truscoe, 1958; Yoshida *et al.* 1961), they must act by means other than those bringing about energetic changes. However, Wollenberger (1947) described an inhibitory effect (after an initial stimulation) on guinea-pig-brain-cortex respiration by low concentrations of ouabain. Moreover, Whittam (1961) showed that 5 µM-ouabain reduces the oxygen uptake of rabbit-brain-

cortex slices incubated in a glucose medium which was apparently devoid of Ca^{2+} ions and which would therefore correspond to a stimulating medium with a high K^+ ion concentration. Ouabain at high concentrations (mM or 0·1 mM) inhibits rat-brain-cortex respiration *in vitro*, particularly K^+ ion-stimulated respiration, but at the low concentrations (10 μM or 1 μM) where it inhibits uptake of glutamate or of creatine into the brain-cortex slices it has little or no inhibitory effect on brain respiration. Therefore reasons other than those based on changes of cell energetics are needed to explain the following effects of ouabain on brain-cortex metabolism described above: (1) suppression of K^+ ion-stimulated brain respiration; (2) inhibition of formation of labelled glutamine from labelled glucose or labelled glutamate and the suppression of this inhibition by addition of NH_4^+ ions; (3) increased efflux of amino acids from brain slices into the glucose–Krebs–Ringer phosphate medium in which the tissue is respiring; (4) diminished influx of glutamate into brain slices from the medium, the percentage diminution increasing with increase of glutamate concentration; (5) diminished influx of creatine from the medium; (6) increased inhibition of influx of glutamate by ouabain by addition of increased concentrations of K^+ or NH_4^+ ions. In considering these phenomena, the inhibitory effects of ouabain on adenosine triphosphatase (Dunham & Glynn, 1961; Post & Albright, 1961; Post, Merritt, Kinsolving & Albright, 1960; Skou, 1957, 1960, 1961) and their bearing on cation movements at the cell membrane must be taken into account.

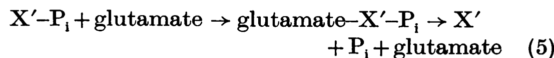
The following series of reactions, in which X, X′ and X″ represent membrane carriers, constitutes a scheme which will account for the facts described above:

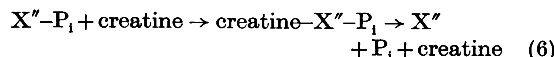

$$X + ATP \rightleftharpoons X\text{–}ATP \rightleftharpoons X\text{–}P_i + ADP \quad (1)$$

$$X + \text{ouabain} \rightarrow X\text{–ouabain} \quad (2)$$


$$X\text{–}P_i + K^+ \rightarrow K^+\text{–}X\text{–}P_i \rightarrow X + P_i + K^+ \quad (3)$$

$$X\text{–}P_i + NH_4^+ \rightarrow NH_4^+\text{–}X\text{–}P_i \rightarrow X + P_i + NH_4^+ \quad (4)$$


$$X'\text{–}P_i + \text{glutamate} \rightarrow \text{glutamate–}X'\text{–}P_i \rightarrow X' + P_i + \text{glutamate} \quad (5)$$


$$X''\text{–}P_i + \text{creatine} \rightarrow \text{creatine–}X''\text{–}P_i \rightarrow X'' + P_i + \text{creatine} \quad (6)$$

Reactions (1)–(3) can account for the inhibitory effects of ouabain on both membrane adenosine triphosphatase and K^+ ion transport and the antagonism between ouabain and K^+ ions (Dunham & Glynn, 1961) on adenosine triphosphatase. Similarly, the antagonism between K^+ ions and ouabain on brain-cortex-slice respiration receives an explanation. Reaction (1) is presumably associated with both Na^+ and Mg^{2+} ions (Skou, 1961; Järnefelt, 1961). Reactions (3)–(6) may be regarded as analogous to those postulated by Skou (1961), except that the phosphorylated carrier is held responsible for the transport of cations, glutamate or creatine. The hypothesis that NH_4^+ ions behave similarly to K^+ ions is shown in reaction (4) and is supported by the observations of Post & Albright (1961) on the effects of these ions on adenosine-triphosphatase activity.

The antagonistic action of NH_4^+ ions and of glutamate on ouabain suppression of glutamine biosynthesis can be understood by operation of reactions (4) and (5). The effects, described above, of the mutual effects of K^+, NH_4^+ and glutamate ions at different concentrations are explicable by the affinities of these ions for the carrier sites.

However, although the addition of K^+ ions depresses the influx of glutamate, the reverse does not occur, i.e. the addition of glutamate increases the influx of K^+ ions into the brain tissue. This leads to the conclusion that the carrier site for glutamate, though having an affinity for K^+ ions, is not identical with that which is responsible for K^+ ion transport. For this reason it is necessary to assume the existence of several carrier sites for transport, which may, however, resemble each other in their association with ATP and their combination with ouabain as described in reactions (1)–(6).

Little can be said, from the above results, about the chemical nature of the postulated carrier sites. However, tetraethyl-lead resembles ouabain in its ability to suppress the influx of glutamate (and glycine) into rat-brain-cortex slices, at concentrations that do not affect brain-slice respiration nor, apparently, the ATP concentration (Vardanis & Quastel, 1961), and the evidence obtained with phospholipase A indicates that phospholipid groups are probably involved in the amino acid and cation transport. It is possible also that the participation of phosphatidic acids in cation transport, suggested by Hokin & Hokin (1961), may represent mechanisms additional to those of reactions (1)–(6). If so, the inhibitory action of ouabain (reaction 2) would result in ATP being diverted from reaction (1) to its increased involvement in phosphatidic acid synthesis. There should, therefore, result an increased rate of incorporation of labelled phosphate into phosphatidic acid, as reported by Yoshida *et al.* (1961).

SUMMARY

1. Ouabain inhibits rat-brain-cortex respiration *in vitro* at concentrations greater than 10 μM, the absolute inhibition being greater in glucose–Krebs–Ringer phosphate medium containing a high (105 m-equiv./l.) concentration of K^+ ions than in

one containing 5 m-equiv. of K^+ ions/l. The percentage inhibitions exercised by ouabain are of the same order in both media.

2. Ouabain at a concentration of 10 μM does not affect the labelling of nucleotide pyrophosphate in rat-brain-cortex slices incubated in a glucose–Krebs–Ringer medium containing labelled phosphate.

3. Ouabain at the low concentrations that have little or no effect, within experimental error, on rates of respiration of rat-brain-cortex slices brings about the following phenomena: (*a*) inhibition of formation of labelled glutamine from either labelled glucose or labelled glutamate, this inhibition being diminished by the addition of NH_4^+ ions or of glutamate; (*b*) increased efflux of amino acids (glutamine, glutamate, alanine, γ-aminobutyrate and aspartate) from the brain-cortex slices into the glucose–Krebs–Ringer phosphate medium in which the tissue is respiring; (*c*) diminished influx of glutamate into brain slices from the medium, the percentage diminution increasing with increase of glutamate concentration; (*d*) diminished influx of creatine from the medium, though labelled-creatine phosphate formation in the brain is unaffected by ouabain; (*e*) increased inhibition of influx of glutamate on addition of an increased concentration of K^+ and NH_4^+ ions.

4. It is suggested that ouabain affects the transport of NH_4^+ ions into mitochondria (or microsomes). Such an effect would account for its action on glutamine synthesis in the brain slices. Ouabain has no effect on glutamine synthetase isolated from brain tissue.

5. It is postulated that ouabain combines with carrier sites for K^+ (or NH_4^+) ions, for glutamate and for creatine, such sites requiring ATP for their activities. This hypothesis makes it possible to link adenosine-triphosphatase activities with transport phenomena.

We acknowledge with gratitude financial assistance from the Medical Research Council of Canada and the National Science Foundation (U.S.).

REFERENCES

Abadom, P. N. & Scholefield, P. G. (1961). *Proc. Canad. Fed. biol. Soc.* **4**, 56.
Acs, G., Balazs, R. & Straub, F. B. (1952). *Biokhem. Zh.* **25**, 17.
Acs, G., Balazs, R. & Straub, F. B. (1953). *Kisérl. Orvostud.* **5**, 466; cited in *Chem. Abstr.* (1954) **48**, 8923.
Annau, E. (1934). *Hoppe-Seyl. Z.* **224**, 141.
Caldwell, P. C. & Keynes, R. D. (1959). *J. Physiol.* **148**, 8P.
Cotton, M. de V. & Moran, N. C. (1961). *Annu. Rev. Pharmacol.* **1**, 261.
Crane, R. K. & Lipmann, F. (1953). *J. biol. Chem.* **201**, 235.
Dunham, E. T. & Glynn, I. M. (1961). *J. Physiol.* **156**, 274.
Elliott, K. A. C. & van Gelder, N. N. (1958). *J. Neurochem.* **3**, 28.
Elliott, W. H. (1951). *Biochem. J.* **49**, 106.
Furchgott, R. F. & de Gubareff, T. (1958). *J. Pharmacol.* **124**, 203.
Gárdos, G. (1960). *J. Neurochem.* **5**, 199.
Glynn, I. M. (1957). *J. Physiol.* **136**, 148.
Gonda, O. & Quastel, J. H. (1961). *Proc. Canad. Fed. biol. Soc.* **4**, 26.
Gonda, O. & Quastel, J. H. (1962). *Nature, Lond.*, **193**, 137.
Gore, M. B. R. & McIlwain, H. (1952). *J. Physiol.* **117**, 471.
Guroff, G., King, W. & Udenfriend, S. (1961). *J. biol. Chem.* **236**, 1773.
Heald, P. J. (1954). *Biochem. J.* **57**, 673.
Hokin, L. E. & Hokin, M. R. (1961). In *Symp. CSAV: Membrane Transport and Metabolism*, p. 204. Ed. by Kleinzeller, A. & Kotyk, A. Prague: Publishing House of the Czechoslovak Academy of Science.
Järnefelt, J. (1961). *Biochem. biophys. Res. Commun.* **6**, 237.
Kahn, J. B. & Acheson, G. H. (1955). *J. Pharmacol.* **115**, 305.
Kini, M. M. & Quastel, J. H. (1959). *Nature, Lond.*, **184**, 252.
Kratzing, C. C. & Narayanaswami, A. (1953). *Biochem. J.* **54**, 317.
Krebs, H. A., Eggleston, L. V. & Terner, C. (1951). *Biochem. J.* **48**, 530.
Kunz, H. A. & Sulser, F. (1957). *Experientia*, **13**, 365.
Lee, K. S., Schwartz, A. & Burstein, R. (1960). *J. Pharmacol.* **129**, 123.
McIlwain, H. (1952). *Biochem. J.* **52**, 289.
Maizels, M., Remington, M. & Truscoe, R. (1958). *J. Physiol.* **140**, 61.
Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 893.
Narayanaswami, A. (1952). *Biochem. J.* **52**, 295.
Pappius, H. M. & Elliott, K. A. C. (1956). *Canad. J. Biochem. Physiol.* **34**, 1053.
Post, R. L. & Albright, C. D. (1961). In *Symp. CSAV: Membrane Transport and Metabolism*, p. 219. Ed. by Kleinzeller, A. & Kotyk, A. Prague: Publishing House of the Czechoslovak Academy of Science.
Post, R. L., Merritt, C. R., Kinsolving, C. R. & Albright, C. D. (1960). *J. biol. Chem.* **235**, 1796.
Rossiter, R. J. (1957). In *Metabolism of the Nervous System*, p. 355. Ed. by Richter, D. London: Pergamon Press.
Schanberg, S. & Giarman, N. J. (1960). *Biochim. biophys. Acta*, **41**, 556.
Schatzmann, H. J. (1953). *Helv. physiol. Acta*, **11**, 346.
Skou, J. C. (1957). *Biochim. biophys. Acta*, **23**, 394.
Skou, J. C. (1960). *Biochim. biophys. Acta*, **42**, 6.
Skou, J. C. (1961). *Symp. CSAV: Membrane Transport and Metabolism*, p. 238. Ed. by Kleinzeller, A. & Kotyk, A. Prague: Publishing House of the Czechoslovak Academy of Science.
Stern, J. R., Eggleston, L. V., Hems, R. & Krebs, H. A. (1949). *Biochem. J.* **44**, 410.
Takagaki, G., Hirano, S. & Nagata, Y. (1959). *J. Neurochem.* **4**, 124.
Terner, C., Eggleston, L. V. & Krebs, H. A. (1950). *Biochem. J.* **47**, 139.
Thomas, J. (1956). *Biochem. J.* **64**, 335.

Tower, D. B. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **3**, 213.
Tsukada, Y., Nagata, Y. & Hirano, S. (1960). *Nature, Lond.*, **186**, 474.
Vardanis, A. & Quastel, J. H. (1961). *Canad. J. Biochem. Physiol.* **39**, 1811.
Waelsch, H. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **3**, 36.
Weil-Malherbe, H. (1936). *Biochem. J.* **30**, 665.
Weil-Malherbe, H. (1938). *Biochem. J.* **32**, 2257.
Whittam, R. (1961). *Nature, Lond.*, **191**, 603.
Wollenberger, A. (1947). *J. Pharmacol.* **91**, 39.
Woodman, R. J. & McIlwain, H. (1961). *Biochem. J.* **81**, 83.
Yoshida, H., Nukada, T. & Fujisawa, H. (1961). *Biochim. biophys. Acta*, **48**, 614.

Biochem. J. (1962) **84**, 406

The Role of the Pentose Phosphate Pathway in the Reduction of Methaemoglobin in Human Erythrocytes

By J. H. STRÖMME* and L. ELDJARN
Institute of Clinical Biochemistry, Rikshospitalet, University of Oslo, Oslo, Norway

(*Received* 11 *January* 1962)

The precise mechanism by which methaemoglobin is reduced to haemoglobin in intact erythrocytes is still unknown, though several hypotheses have been put forward. Reduced glutathione (Morrison & Williams, 1938), ascorbic acid and a few other naturally occurring compounds (Kiese, 1944) spontaneously reduce methaemoglobin *in vitro*, but the physiological role of these reductions is uncertain. The normal process of methaemoglobin reduction is associated with glucose metabolism (Wendel, 1930; Kiese, 1944), and seems to depend on the oxidation of either $NADPH_2$ or $NADH_2$ (Gutmann, Jandorf & Bodansky, 1947).

There are a number of indications that a $NADPH_2$-dependent methaemoglobin reductase is present in erythrocytes. Kiese (1944) partially purified an enzyme from horse erythrocytes which catalyses the reduction of methaemoglobin by $NADPH_2$, and to a lesser extent also by $NADH_2$. The addition of a cofactor, such as methylene blue, greatly increased the reduction rate. On further purification of the same enzyme from human erythrocytes, it became inactive unless a cofactor were added (Kiese, Schneider & Waller, 1957). Huennekens, Caffrey, Basford & Gabrio (1957) isolated from human erythrocytes a similar methaemoglobin reductase which also required a cofactor such as methylene blue or, under certain conditions, Fe^{3+} ions (Huennekens, Caffrey & Gabrio, 1958). It was suggested that a physiological cofactor might have been lost during purification.

Indirect evidence for a $NADH_2$-dependent methaemoglobin reductase in human erythrocytes was presented by Gibson (1948). Recently such an enzyme was partially purified; it was also found to be lacking in patients with hereditary methaemoglobinaemia (Scott & Griffith, 1959; Scott, 1960). Some authors believe that the $NADH_2$-dependent reduction of methaemoglobin accounts for the main part of the physiological reduction of this pigment (Gibson, 1948; Waller & Löhr, 1961; Jaffé, 1959).

Thus earlier observations indicate that the reduction of methaemoglobin may be associated both with the Embden–Meyerhof pathway (regeneration of $NADH_2$) and with the pentose phosphate pathway (regeneration of $NADPH_2$). However, the relative significance of these systems in the physiological reduction of methaemoglobin has not as yet been established. The experiments presented in this paper show that in human erythrocytes the utilization of glucose by the pentose phosphate pathway accounts for only a small part of the total methaemoglobin reduction. The main part, evidently, is reduced by the Embden–Meyerhof pathway. The former mechanism involves a direct reduction of methaemoglobin by reduced glutathione. The oxidized glutathione is in turn reduced by $NADPH_2$.

* Research Fellow, Norwegian Cancer Society, Oslo, Norway.

MATERIALS AND METHODS

Uniformly labelled [^{14}C]glucose (specific activity 3·77 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. Before use, it was diluted approximately 65-fold with inactive glucose. Scintillators and M-hyamine in methanol were purchased from Packard Instrument Co. Inc., La Grange, Ill., U.S.A. Glucose 6-phosphate was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. GSH, GSSG and NADP were obtained from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.