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ETHANOL AND THE METABOLIC INTERRELATIONS OF CARBOHYDRATES AND AMINO ACIDS IN BRAIN PREPARATIONS

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Abstract—Certain fractions of brain homogenate prepared from ethanol-treated rats consumed glucose and produced lactate at increased rates. These effects of ethanol were even larger when glucose-6-phosphate was used as substrate, but smaller with glucose-1-phosphate. Measurement of the utilization of U-[^{14}C]glucose in a system designed for the assay of hexokinase showed that the formation of acidic metabolites was also augmented as a result of ethanol treatment. By contrast, when ethanol was added *in vitro* in corresponding concentrations, the catabolism of glucose decreased. Data are presented on the effects of varied conditions during incubation, of the use of homogenates from other tissues, of the interval from administration of ethanol to killing, and of the amount of ethanol administered. The concentration of γ-aminobutyric acid during incubation with glutamic acid increased with increasing concentration of glucose in the medium, while the concentrations of glutamine and aspartic acid decreased. The concentrations of γ-aminobutyric acid and glutamine increased continuously as functions of time, but the concentration of aspartic acid was maximal at an early phase of incubation. It is concluded that under the influence of ethanol the brain utilizes increased amounts of glucose, while the catabolism of γ-aminobutyric acid and glutamic acid as well as the formation of glutamine decrease.

REPORTS on the way in which ethanol affects glucose consumption in the intact brain are conflicting.[1] Sutherland *et al.*[2] found no difference in the consumption of glucose in the brains of normal and alcoholic subjects. In slices of rat brain the uptake of glucose increased but no change was observed in the formation of lactate.[3] Stimulation of the phosphogluconate pathway in brain mince has been reported.[4] Ammon, Estler and Heim[5] concluded that during ethanol intoxication the breakdown of glycogen increased in the whole brain, and that glycolysis and the utilization of pyruvate in the tricarboxylic acid cycle were inhibited, but that direct oxidation through the pentose phosphate cycle was increased. It is suggested that ethanol causes transient activation of phosphorylase in the brain by increasing permeability to adrenaline.[6] In the presence of oxygen, glycolysis in brain tissue was greatly increased by low concentrations of several narcotics.[7]

The purpose of this work was to investigate the utilization of glucose by the same brain preparations which in our earlier studies have been used to demonstrate the following effects of ethanol on amino acid metabolism: increases in the levels of γ-aminobutyric acid, glutamic acid and aspartic acid and a decrease in glutamine.[8] The experiments were extended to the interactions of carbohydrate and amino acid metabolism, with special reference to ethanol. Part of this work has been presented as an abstract.[9]

EXPERIMENTAL

Treatment of rats and preparation of brain fractions. Rats of the Wistar strain, weighing 219–413 g but matched in each series, had fasted for 24–40 hr. As a pretreatment *in vivo* they received 550 mg of ethanol/100 g body wt. as a 33% (v/v) aqueous solution by stomach tube. For the rat this dose is adequate: the animals become strongly but not lethally intoxicated. The control animals were given an equal volume of water. In three series of experiments the effect of the concentration of ethanol, given *in vivo*, was tested over the concentration range from 5·5 to 55 per cent.

The rats were killed by decapitation, usually after 1 hr, and in special experiments from 2 to 5 hr after administration of ethanol. The brains were dissected immediately at room temperature, blotted, weighed and homogenized with a Potter–Elvehjem apparatus (cooled in crushed ice) in a 4-fold volume (v/w) of cold isotonic KCl–K_2HPO_4 solution, pH 7·4.[8,10]

The postnuclear, postmitochondrial and postmicrosomal supernatants of the brain homogenate were obtained by differential centrifugation at 0°, for 10 min at 700 *g*, for 10 min at 5000 *g*, and for 60 min at 100,000 *g*, respectively. The soluble protein fraction was prepared from the postmicrosomal supernatant by gel filtration through a Sephadex G-25 column[8,11] and thus did not contain any small-molecular metabolites from the original tissue. The preparations were stored frozen until used.

Incubations. The standard incubation medium was somewhat modified from that of DiPietro and Weinhouse,[10] and had the following composition: 6·0 mM ATP, 1·2 mM ADP (in studies on amino acids 1·4 mM ATP only), 2·0 mM NAD^+, 0·06 mM cytochrome *c*, 10 mM glucose, 1·72 mM glutamic acid (in studies on amino acids 2·58 mM), 8·0 mM $MgSO_4$, 0·2 mM fumaric acid, 77 mM KCl, and 40 mM Na_2HPO_4–NaH_2PO_4 buffer, pH 7·4. The brain preparations were diluted, with a few exceptions, so that 1 ml of the incubation mixture corresponded to 50 or 75 mg of original brain tissue. The incubations were carried out in duplicate in a Gallenkamp shaking incubator (Cat No. 1 H 350). The incubation temperature was 37° and the time usually 30 or 60 min. After incubation the vessels were immediately chilled in crushed ice.

In several experiments the concentration of glucose was varied from zero to 200 mM, or glucose was replaced by hexose phosphates. Glucose-1-phosphate as disodium-tetrahydrate was prepared by the California Corporation for Biochemical Research (Calbiochem), Los Angeles 63, California, USA, and glucose-6-phosphate as the disodium salt by C. F. Boehringer & Soehne G.m.b.H., Mannheim, Germany. Additionally, a large number of variations in the incubation medium were tested systematically. Those with marked effects are presented in Table 4.

In the experiments with U-[^{14}C]D-glucose (Radiochemical Centre, Amersham, England) as a substrate for hexokinase, the incubations were performed either as explained above or mainly in a medium containing 5 mM U-[^{14}C]glucose (specific activity 1 μc/ml incubation mixture), 10 mM $MgSO_4$, 10 mM ATP, 20 mM mercaptoethanol, 1 mM EDTA and 50 mM tris-buffer, pH 7·5.[12] The amount of the postmitochondrial fraction of the brain homogenate in the sample corresponded to 33 mg of original tissue per ml of incubation mixture. In some experiments inorganic phosphate (Na_2HPO_4) or glucose-6-phosphate was added. The incubation time was either 30 or 60 min and the temperature 28°.

Analyses. For the determination of glucose the incubated samples were deproteinized with 0·16% (w/v) uranyl acetate, and for the determination of lactic acid with 6% (w/v)

perchloric acid. The amounts of glucose[13] and lactic acid[14] were then determined enzymatically, using the reagent kit (TC-M and TC-B) of C. F. Boehringer & Soehne G.m.b.H., Mannheim, Germany.

α-Oxoglutaric acid and pyruvic acid were isolated from the incubated samples as their 2,4-dinitrophenylhydrazones,[15] separated by thin-layer chromatography[16,17] and measured spectrophotometrically.[18]

For the estimation of amino acids the samples were precipitated immediately after incubation by addition of a 3-fold volume of cold absolute ethanol, kept overnight at 4° and then centrifuged at 24,000 *g* for 1 hr at 0°. The precipitate was resuspended in 5 ml of cold 75 per cent ethanol and recentrifuged for 30 min. The combined supernatants were evaporated in an air stream at room temperature. The dry residues were dissolved in water. From these solutions the amino acids were determined by a paper-electrophoretic method.[19]

To estimate the hexokinase activity, the radioactivity of the catabolic products of [^{14}C]glucose was determined according to the method of Newsholme *et al.*[12] Samples (20 μl) of the incubation mixture were pipetted on the discs of DEAE-cellulose paper (Reeve Angel & Co., Ltd., London) and the remaining glucose was removed by washing the discs (the radioactive disc and two unused ones underneath) with 100 ml of water. The radioactivity retained by the paper discs was measured in a liquid-scintillation counter (M 3101–4, Ekco Electronics Ltd., Southend-on-Sea, England). It was found most practical to place the discs flat on the bottom of the counting vials.

Comments on the methods. The salt solution was preferred to the sucrose solution as homogenization medium because fine fractionation was not required during the differential centrifugation, and, moreover, a more compact pellet was sedimented in this medium. The soluble protein fraction of brain homogenate was selected to get a wholly soluble and least complicated incubation mixture.

The electrophoretic method used does not separate glutamine from alanine, glycine, serine or taurine, which occur in unfractionated brain tissue. When the soluble protein fraction was used, however, the present incubation mixture did not contain any free amino acids from the original brain. The values of glutamine may contain a contribution from alanine, formed during the incubation from glucose through pyruvate. This error cannot have been very marked, since the values of glutamine were lower at higher concentrations of glucose (Fig. 7).

It might be suspected that treatment with ethanol would change the water content of the brain, which would invalidate the use of the weight of the fresh tissue as a basis for calculation of the concentrations of the metabolites. However, when the protein concentration in the soluble protein fraction and in the 100,000 *g* supernatant was estimated by Folin's reagent,[20] the decrease due to ethanol was only 2·6 and 1·4 per cent, respectively.

RESULTS

Carbohydrate metabolism

Preliminary experiments. The first estimations of the glucose consumption by brain homogenates from ethanol-treated rats were carried out in connection with our studies on amino acid metabolism.[8] The initial concentration of glucose in the incubation medium was 10 mM, i.e. 20 m-moles/100 g of fresh brain. After an incubation period

of 30 min, the consumption of glucose by the control preparation (the soluble protein fraction) was 13·98 m-moles/100 g of fresh brain and by the preparation from ethanol-treated rats 14·74 m-moles/100 g. The respective amounts of lactate formed were 7·05 and 8·63 m-moles/100 g. The differences caused by ethanol were both statistically significant ($P < 0{\cdot}01$, $n = 4$). After 60-min incubation almost all of the glucose added had been consumed and ethanol no longer had any effect on the rate of glucose utilization. Ethanol had no effect on the formation of α-oxoglutarate, but the formation of pyruvate was enhanced, although the increase was significant only in the presence of glutamic acid (from 1000 to 1220 μmoles/100 g of brain, $P < 0{\cdot}02$, $n = 4$).

For the ratio of ATP and ADP, 5:1 was found to be optimal for the catabolism of glucose and for the effect of ethanol. The maximal rates of glucose catabolism and lactate formation were reached with a 7 mM concentration of the combined adenosine phosphates. Omission of ATP or NAD^+ or both reduced utilization of glucose and formation of lactate by about a half.

Time course and glucose concentration. Figure 1 shows the time course of glucose consumption. The effect of ethanol could be recognized after 20 min already. The dependence of the consumption of glucose on the concentration of glucose in the medium is seen in Fig. 2. There is no plateau, but above 5 mM glucose the curve rises more slowly. The curve for lactate production (Fig. 5) is almost level above 4 mM glucose. The effect of ethanol is statistically significant, as specified in the legends to

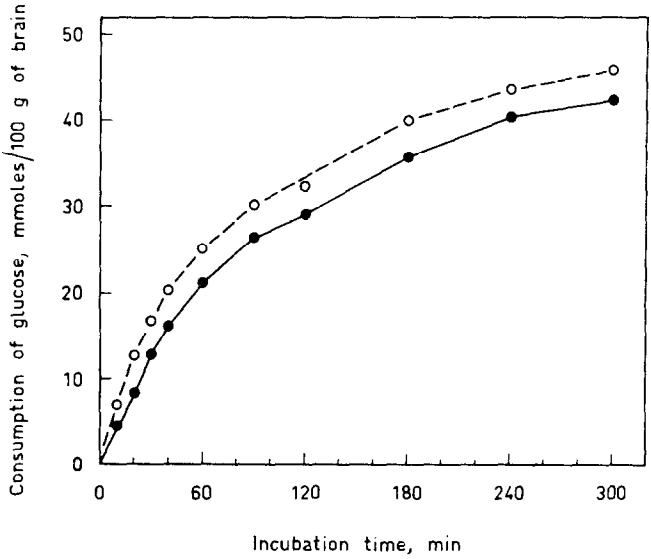

FIG. 1. Effect of the incubation time on the consumption of glucose by the incubated soluble protein fraction of brain. The composition of the incubation medium was 6·0 mM ATP, 1·2 mM ADP, 2·0 mM NAD^+, 0·06 mM cytochrome *c*, 50 mM glucose, 1·72 mM glutamic acid, 8·0 mM $MgSO_4$, 0·2 mM fumaric acid, 77 mM KCl and 40 mM Na_2HPO_4–NaH_2PO_4 buffer, pH 7·4. The results are averages of four different series of experiments, each consisting of the brains of three to five rats, either pooled or separate. Control rats —●—●—, ethanol-treated rats – ○ – ○ –. The effect of ethanol, expressed in per cent, was in average +21·0% $P < 0{\cdot}005$ ($t = 3{\cdot}99$, $N = 12$). Calculated from the absolute numerical values, the effect of ethanol was significant at the level $P < 0{\cdot}001$ ($t = 13{\cdot}07$, $N = 12$).

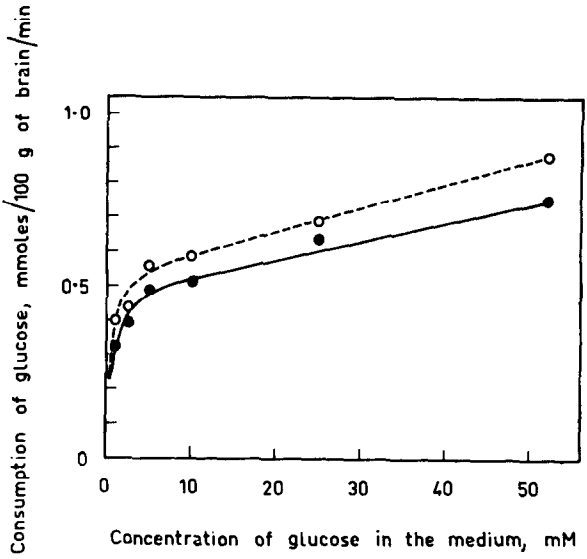

FIG. 2. Effect of the concentration of glucose in the medium on the consumption of glucose by the incubated soluble protein fraction of brain. The composition of the incubation medium is given in the legend to Fig. 1. The actual incubation time varied from 5 to 60 min. The results are averages of three different series of experiments, each consisting of the pooled brains of three rats. Control rats —●—●—, ethanol-treated rats – ○ – ○ –.

The effect of ethanol, expressed in per cent, averaged +14·8%, $P < 0{\cdot}001$ ($t = 6{\cdot}92$, $N = 6$), and calculated from the absolute numerical values $P < 0{\cdot}005$ ($t = 6{\cdot}04$, $N = 6$).

the figures. In another series of eleven samples incubated for 30 min the consumption of glucose in control preparations was 13·25 ± 1·43 m-moles/100 g of brain and in preparations of ethanol-treated rats 16·66 ± 0·77 m-moles/100 g ($P < 0{\cdot}001$). The corresponding figures for the production of lactate were 10·78 ± 0·75 and 12·96 ± 0·48 m-moles/100 g, respectively ($P < 0{\cdot}001$).

Effect of ethanol added in vitro. When ethanol was added to the incubation mixture, 0·5 or 1·0% (v/v), the effect on the catabolism of glucose was the opposite of that reported above, i.e. ethanol decreased the consumption of glucose by 21·7 and 54·7 per cent, respectively. The formation of lactate was decreased correspondingly. Ethanol thus has different effects on the integrated brain tissue *in vivo* and on the homogenate. A similar conclusion has already been reached in studies on amino acid metabolism.[8]

Intoxication time. The effect of ethanol on the consumption of glucose and on the formation of lactate lasts about 4 hr (Fig. 3) and is maximal 1–2 hr after oral administration of ethanol. This period is somewhat longer than that observed in studies on amino acid metabolism[21] but corresponds approximately to the duration of the ethanol intoxication.

Concentration of ethanol. The dose of ethanol was varied in the range of 92–920 mg/100 g of body wt. by changing the concentration of ethanol from 5·5 to 55% (v/v). There was some variation in the level of consumption of glucose between the three different series of experiments but the maximal effect of ethanol, both in the consump-

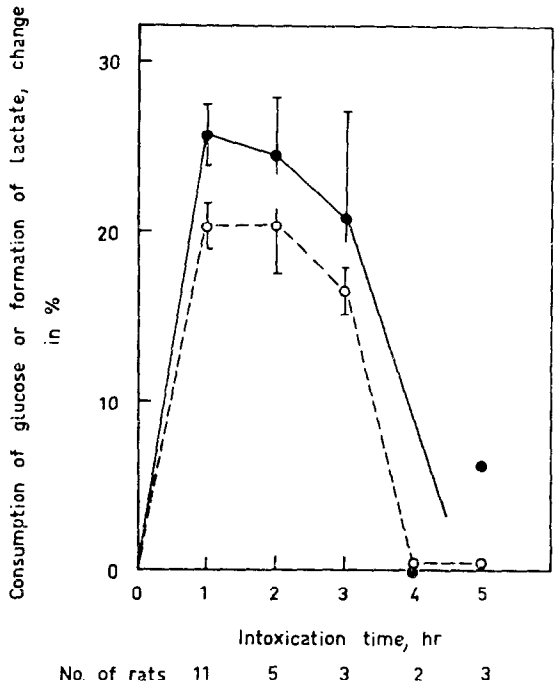

FIG. 3. Effect of the intoxication time on the changes in the consumption of glucose and formation of lactate by the incubated soluble protein fraction of brain. The change caused by administration of ethanol is calculated in per cent of the respective control value. The experimental conditions were the same as described in the legend to Fig. 1, except that the concentration of glucose in the medium was 10 mM. The number of experiments and the standard errors of the means are indicated. Consumption of glucose —●—●—, formation of lactate – ○ – ○ –.

tion of glucose and in the formation of lactate, was reached with the 22% (v/v) ethanol (370 mg/100 g of body wt.).

Utilization of other substrates instead of glucose. The formation of lactate from glucose, fructose and mannose was 10·78, 9·26 and 7·59 m-moles/100 g of brain, respectively, and the effect of ethanol was +20·2, +14·2 and +18·2 per cent. Hardly any lactate was formed from galactose, and from glycogen 2·44 m-moles/100 g of brain only. The formation of lactate from glucose-1-phosphate and glucose-6-phosphate is shown in Fig. 4 as a function of incubation time and in Fig. 5 as a function of substrate concentration. In comparison to glucose, the glucose phosphates are utilized better, although saturation of enzymes with substrates is reached at a lower concentration with glucose (Fig. 5). The effect of ethanol decreased in the order glucose-6-phosphate < glucose < glucose-1-phosphate ($P < 0.005$ and < 0.001, respectively).

Hexokinase reaction. For the direct assay of hexokinase activity, experiments were made with U-[^{14}C]glucose according to Newsholme *et al.*[12] (Table 1). In these conditions no detectable lactate was formed. Ethanol increased the uptake of glucose by 13·1 per cent at 30 min incubation. After 60 min incubation, the effect of ethanol was only +4·8 per cent. In the presence of 2 mM inorganic phosphate the effect of ethanol remained unchanged.

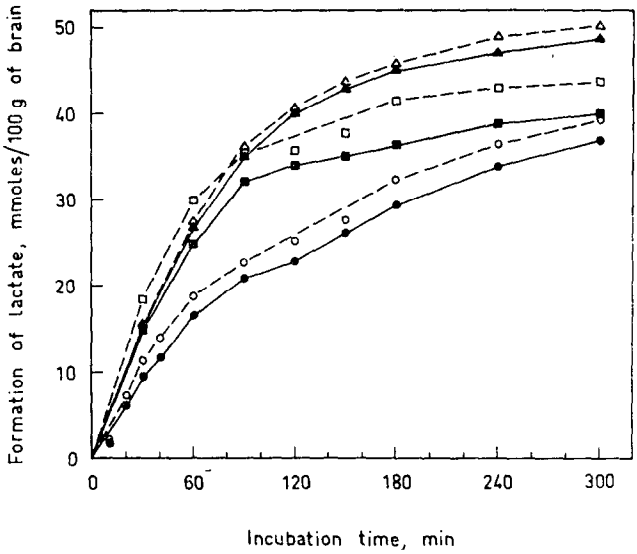

FIG. 4. Effect of the incubation time on the formation of lactate from glucose, glucose-6-phosphate and glucose-1-phosphate during incubation of the soluble protein fraction of brain. The composition of the incubation medium is given in the legend to Fig. 1. The concentration of each substrate in the medium was 50 mM. The results with glucose as substrate (control rats —●—●—, ethanol-treated rats – ○ – ○ –) are averages of four different series of experiments and those with glucose-6-phosphate (—■—■—, – □ – □ –) and glucose-1-phosphate (—▲—▲—, – △ – △ –) of two series, each with brains from three to five rats.

The effect of ethanol, expressed in per cent and with the data treated as non-independent pairs, was significant for glucose at the level $P < 0{\cdot}001$ ($t = 7{\cdot}01$, $N = 12$), for glucose-6-phosphate $P < 0{\cdot}001$ ($t = 5{\cdot}40$, $N = 8$), and for glucose-1-phosphate $P < 0{\cdot}005$ ($t = 5{\cdot}26$, $N = 8$). For the effects calculated in absolute values, P was $<0{\cdot}001$, $<0{\cdot}01$ and $<0{\cdot}005$, respectively.

Corresponding experiments were also made using the medium of DiPietro and Weinhouse,[10] designed for production of lactate from glucose. Less radioactivity was now retained, but the effect of ethanol averaged +17 per cent.

Table 2 shows the significance of incubation time. In the first 3 min no clear effect of ethanol could be observed. Glucose-6-phosphate inhibits the hexokinase reaction, more strongly in the beginning of the incubation. Ethanol does not affect feed-back inhibition by glucose-6-phosphate.

It is claimed that certain surface-active substances increase the hexokinase activity in brain preparations.[22] When Triton X-100 was added to the incubation medium (0·5–1·0 per cent), the activity in the post-mitochondrial fraction increased by 5–8 per cent (cf. Discussion and references 30–32), but the proportional effect of ethanol remained the same.

Other brain fractions and other tissues as sources of the enzyme preparation. Because the effect of ethanol pretreatment appears differently in the various fractions of brain homogenate,[8] the postnuclear, postmitochondrial and postmicrosomal supernatants and the soluble protein fraction were compared in regard to the metabolism of glucose. The consumption of glucose (17·5, 16·7, 13·7 and 10·7 m-moles/30 min/100 g, respectively) and the formation of lactate (16·9, 16·4, 13·0 and 8·9 m-moles/30 min/100 g,

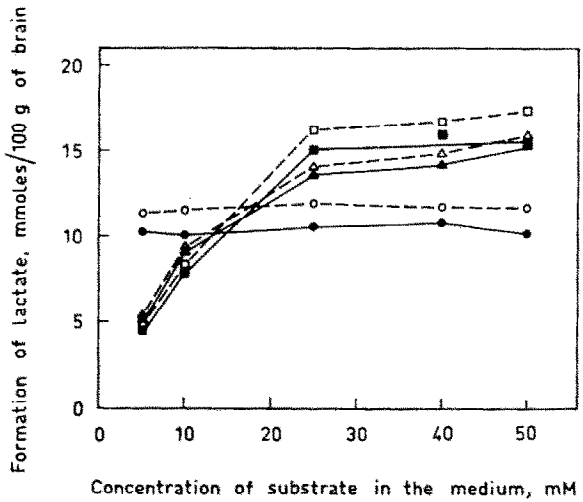

FIG. 5. Formation of lactate during incubation of the soluble protein fraction of brain with various concentrations of glucose, glucose-6-phosphate and glucose-1-phosphate. The composition of the incubation medium is explained in the legend to Fig. 1. The incubation time was 30 min. The results are averages of two series (at the 50 mM point four series) of experiments with pooled brains from three to five rats. The symbols are the same as in Fig. 4.

The effect of ethanol, expressed in per cent and with the data treated as non-independent pairs, was significant for glucose at the level $P < 0{\cdot}001$ ($t = 10{\cdot}85$, $N = 5$), for glucose-6-phosphate $P < 0{\cdot}02$ ($t = 3{\cdot}86$, $N = 5$), and for glucose-1-phosphate $P < 0{\cdot}005$ ($t = 8{\cdot}34$, $N = 5$). For the effects calculated in absolute values, P was $<0{\cdot}001$, $<0{\cdot}05$ (N.S.) and $<0{\cdot}02$, respectively.

respectively) decreased in the order mentioned. The effect of ethanol was only observed on incubation of the postmicrosomal supernatant and soluble protein fractions.

Brain was also compared with muscle, liver and kidney as the source of the postmicrosomal supernatant (Table 3). Brain and muscle, on the one hand, and liver and kidney, on the other, resembled each other in regard to the extent of both the formation of lactate and the effect of ethanol.

TABLE 1. EFFECT OF ETHANOL ON THE UTILIZATION OF U-[^{14}C]GLUCOSE

Conditions for incubation	Additions	Radioactivity (counts/min)		
		Control rats	Ethanol-treated rats	P*
Designed for hexokinase determination[12]	none	6870 ± 161 (10)	7770 ± 155 (10)	< 0·001
	2 mM P_i	7250 ± 171 (10)	7940 ± 140 (10)	< 0·01
Designed for glycolysis to lactate[10]	none exp. 1	3270 ± 108 (5)	3790 ± 114 (5)	< 0·01†
	exp. 2	4220 ± 196 (5)	4980 ± 100 (5)	< 0·01†

The post-mitochondrial fraction of rat brain homogenate was incubated for 30 min with U-[^{14}C]-glucose (radioactivity 12,800 counts/min/sample) in various conditions. The radioactivity of the catabolic products retained by the DEAE paper was determined. The number of rats is given in parentheses.

* By the *t*-test.

† Exp. 1 and 2 combined, $P < 0{\cdot}0005$, as tested by variance analysis.

TABLE 2. EFFECT OF GLUCOSE-6-PHOSPHATE ON THE BRAIN HEXOKINASE REACTION

Incubation time (min)	Concentration of added glucose-6-phosphate (mM)	Radioactivity, counts/min		Effect of ethanol (%)
		Control rats	Ethanol-treated rats	
3	zero	1230 ± 79 (5)	1250 ± 39 (5)	+ 1·6
	1	450 ± 67 (5)	570 ± 15 (5)	+26·7
	10	110 ± 63 (5)	100 ± 52 (5)	− 9·1
30	zero	7790 ± 288 (5)	8820 ± 420 (5)	+13·2†
	0·05*	7070 ± 259 (5)	7700 ± 177 (5)	+ 8·9
	1	7010 ± 517 (5)	8250 ± 923 (5)	+17·7
	10	1230 ± 304 (5)	1320 ± 297 (5)	+ 7·3

The post mitochondrial fraction of rat brain homogenate was incubated with U-[^{14}C]glucose (radioactivity 12,800 counts/min/sample) in the medium designed for hexokinase determination.[12] The radioactivity of the catabolic products retained by the DEAE paper was determined. The number of rats is given in parentheses.

* Taken from a separate series of experiments.

† For the difference between the experimental and control samples $P < 0{\cdot}05$.

TABLE 3. FORMATION OF LACTATE AT THE INCUBATION OF THE POST MICROSOMAL SUPERNATANT OF HOMOGENATES FROM VARIOUS TISSUES

Tissue	Substrate					
	Glucose		Glucose-1-phosphate		Glucose-6-phosphate	
	Control rats (m-moles/ 100 g)	Effect of ethanol (%)	Control rats (m-moles/ 100 g)	Effect of ethanol (%)	Control rats (m-moles/ 100 g)	Effect of ethanol (%)
Brain	11·15	+ 8·3	8·45	+10·8	11·60	+ 3·7
Muscle	7·28	+19·4	12·76	+22·4	18·43	−14·2
Liver	1·28	−11·4	5·46	−50·4	4·91	−56·8
Kidney	2·79	−39·4	7·08	−17·5	8·49	− 5·1

The composition of the incubation medium was the same as explained in the legend to Fig. 1, except that the concentration of each substrate, glucose, glucose-1-phosphate and glucose-6-phosphate, was 10 mM. The incubation time was 30 min. The samples were pooled from two rats.

Interaction of glucose and amino acids

Changes in γ-aminobutyric acid. The higher the concentration of glucose in the medium, the higher was the concentration of γ-aminobutyric acid after incubation with glutamic acid, although the relationship was not linear (Fig. 6). The expected effect of ethanol, i.e. increased concentration of γ-aminobutyric acid, is of the same magnitude throughout the whole range of glucose concentration in the medium. There was a continuous rise in the level of γ-aminobutyric acid as a function of incubation time (Fig. 9).

When the supply of NAD^+ was increased, the concentration of γ-aminobutyric acid

TABLE 4. EFFECTS OF ATP, NAD^+ AND PYRUVATE ON THE FORMATION OF γ-AMINOBUTYRIC ACID, GLUTAMINE AND ASPARTIC ACID AT THE INCUBATION OF THE SOLUBLE PROTEIN FRACTION OF BRAIN WITH GLUTAMIC ACID

Incubation medium	No. of Exp.	γ-Aminobutyric acid (μmoles/100 g of brain)		Glutamine (μmoles/100 g of brain)		Aspartic acid (μmoles/100 g of brain)	
		Control	Ethanol	Control	Ethanol	Control	Ethanol
Standard medium	9	231 ± 12	286 ± 20	545 ± 34	489 ± 40	31 ± 9	42 ± 10
ATP omitted	1	227	253	53	0	65	76
ATP increased	2	257	286	741	648	4	7
NAD^+ omitted	1	207	291	258	217	0	0
NAD^+ increased	2	374	473	415	358	27	33
Pyruvate	3	205	238	910	828	794	841

The composition of the standard incubation medium was 1·4 mM ATP, 2·0 mM NAD^+, 0·06 mM cytochrome *c*, 10 mM glucose, 2·58 mM glutamic acid, 8·0 mM $MgSO_4$, 0·2 mM fumaric acid, 77 mM KCl and 40 mM Na_2HPO_4–NaH_2PO_4 buffer, pH 7·4. The incubation time was 2 hr. The concentration of ATP and NAD^+ was increased by adding the standard amount seven times at 15-min intervals. Pyruvate (10 mM) was added instead of glucose. Each experiment was performed with pooled brain samples from two to four control or ethanol-treated rats. The standard error of the mean is indicated.

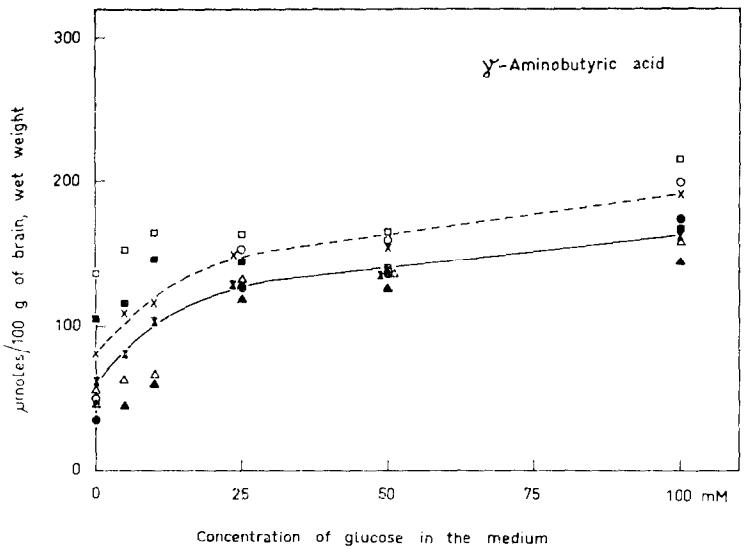

FIG. 6. Concentration of γ-aminobutyric acid after incubation of the soluble protein fraction of brain with glutamic acid at various concentrations of glucose. The composition of the standard incubation medium is explained in the legend to Table 4. The incubation time was 1 hr. The closed symbols ● ■ ▲ (in Figs. 7 and 8 also ▼) and their average ⧗, indicate the mean values of duplicate determinations in three (or four) different experimental series, each with pooled brain samples from five control rats, and the open symbols ○ □ △ (in Figs. 7 and 8 also ▽) and their average ×, the respective experiments with samples from five ethanol-treated rats. The lines, fitted visually, indicate the averages for control and ethanol-treated rats. When the data were treated statistically as non-independent pairs, the effect of ethanol, expressed in per cent for each pair, averaged +20·7%, $P < 0.001$ ($t = 8.30$, $N = 16$).

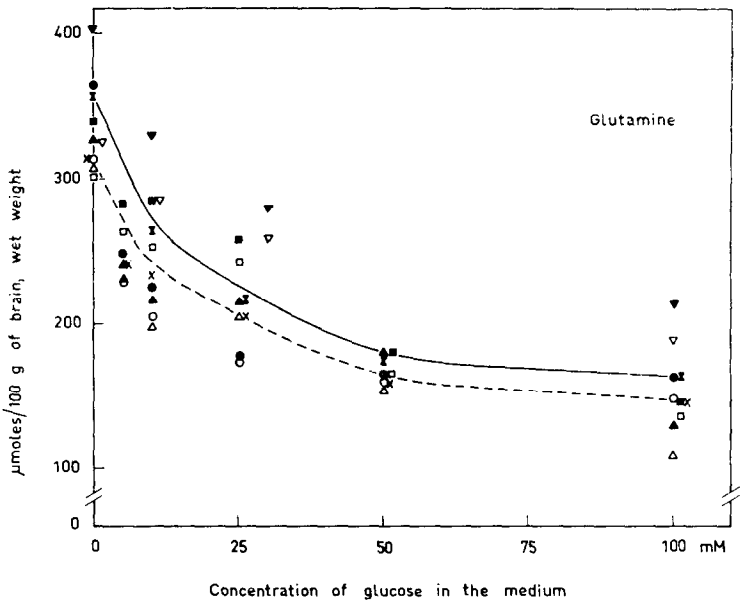

FIG. 7. Concentration of glutamine after incubation of the soluble protein fraction of brain with glutamic acid at various concentrations of glucose. The composition of the standard incubation medium is given in the legend to Table 4. The incubation time was 1 hr. The meaning of symbols and lines is explained in the legend to Fig. 6. When the data were treated statistically as non-independent pairs, the effect of ethanol, expressed in per cent for each pair, averaged −9·3%, $P < 0.001$ ($t = 10.28$, $N = 22$).

was also increased (Table 4). The omission of ATP or NAD^+ as well as the increase of ATP had little effect on the concentration of γ-aminobutyric acid in these conditions.

Changes in glutamine. The changes in glutamine were quite opposite to the changes in γ-aminobutyric acid (Fig. 7). The concentration of glutamine decreased with increasing concentration of glucose in the medium. Pretreatment with ethanol caused an additional decrease in the concentration of glutamine at all glucose concentrations, as could be expected from our earlier work. The addition of ATP was necessary for the synthesis of glutamine (Table 4). When NAD^+ was omitted, the level of glutamine was low. The increase of glutamine noticed on addition of pyruvate instead of glucose may be partly due to the formation of alanine (cf. comments on the methods). The formation of glutamine increased with incubation time, but not quite linearly (Fig. 9).

Changes in aspartic acid. The concentration of aspartic acid decreased with increasing concentration of glucose (Fig. 8). The expected effect of ethanol, i.e. an increase, was observed at all concentrations of glucose. The presence of pyruvic acid instead of glucose greatly enhanced the production of aspartic acid (Table 4). Both repeated additions of ATP and omission of NAD^+ decreased the formation of aspartic acid. As shown in Fig. 9, the concentration of aspartic acid reached a maximum in the beginning of the incubation period, and then decreased.

Ratios of the concentrations of amino acids. The ratio of the concentration of γ-aminobutyric acid to that of glutamine increased with increasing concentration of glucose in the medium. At 10 mM concentration of glucose this ratio was 0·42 for the

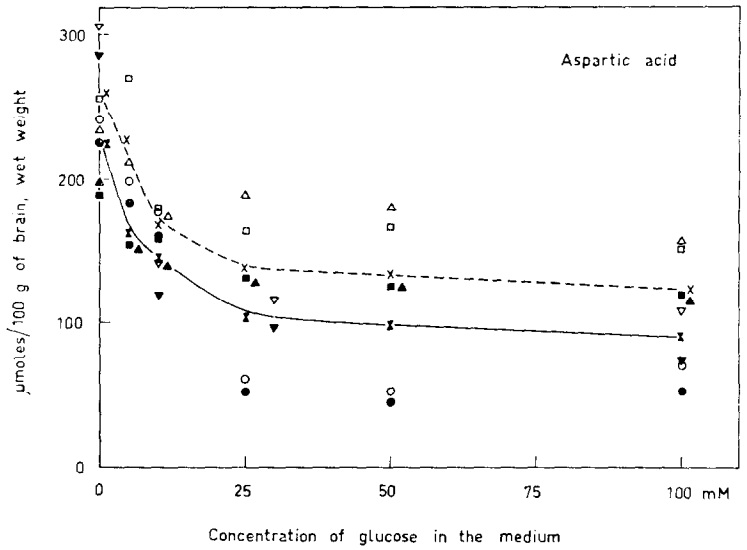

FIG. 8. Concentration of aspartic acid after incubation of the soluble protein fraction of brain with glutamic acid at various concentrations of glucose. The composition of the standard incubation medium is explained in the legend to Table 4. The incubation time was 1 hr. The meaning of symbols and lines is explained in the legend to Fig. 6. When the data were treated statistically as non-independent pairs, the effect of ethanol, expressed in per cent for each pair, averaged +28·1 %, $P < 0{\cdot}001$ ($t = 7{\cdot}63$, $N = 21$).

samples from control rats and for the ethanol-treated rats 0·58 ($P < 0{\cdot}001$, $N = 8$). The sums of the concentrations of these amino acids after incubation were almost the same in samples from control and ethanol-treated rats (802 and 796 μmoles/100 g of brain, respectively).

The ratio of the concentrations of glutamine and aspartic acid decreased linearly with the concentration of glucose, and the effect of ethanol on this ratio remained constant.

DISCUSSION

Glycolysis

The rate of glycolysis in brain preparations can be varied experimentally over a wide range. The increased production of lactate can be caused by high intracellular inorganic phosphate[22] or by anoxia.[24] In the present conditions the supply of inorganic phosphate and oxygen was the same at the incubation of the homogenate fractions from both ethanol-treated and control rats.

Activation of glucose metabolism by ethanol has been observed in the erythrocytes by Redetzki and Redetzki[25] but they could not locate the effect metabolically. Rose and O'Connell[26] suggested that the rate of glucose utilization in the erythrocytes is controlled by the factors which determine the removal of glucose-6-phosphate.

In the brain tissue hexokinase and phosphofructokinase are the key enzymes controlling glycolysis.[27,28] The effect of ethanol in increasing the catabolism of glucose is not limited to the production of lactate, because the ratio of lactate formed to glucose

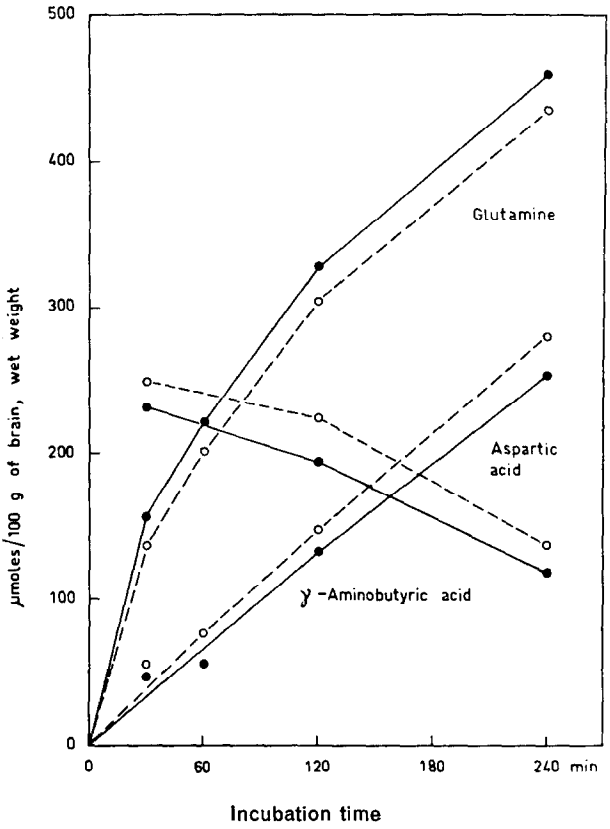

FIG. 9. Formation of γ-aminobutyric acid, aspartic acid and glutamine during incubation of the soluble protein fraction of brain with glutamic acid and glucose. The composition of the standard incubation medium is explained in the legend to Table 4. The points indicate the means of two series of experiments with pooled brain samples from five and six control rats (—●—●—) and from five and six ethanol-treated rats (– ○ – ○ –).

consumed remained unchanged (40·7 per cent in controls and 38·9 per cent in samples from ethanol-treated rats). The experiments with [^{14}C]glucose confirm that one of the affected steps in the utilization of glucose is the hexokinase reaction. Sols and Crane[29] concluded that brain hexokinases possess a third binding site for glucose-6-phosphate, which inhibits the hexokinase reaction noncompetitively. In the experiments shown in Table 2, the inhibition by glucose-6-phosphate did not explain the effect of ethanol.

The catabolism of glucose is obviously affected by ethanol at the phosphorylation of glucose, and even more at the utilization of glucose-6-phosphate (Figs. 4 and 5). The effect of ethanol on the hexokinase activity is uncertain during the first 3 min of the incubation (Table 2). This short lag phase indicates that some metabolite related to glucose catabolism has to be formed before the effect of ethanol becomes manifest.

Brain hexokinase is in part bound to the various particles in brain homogenate.[30–32] On the basis of our experiments with the sedimentation fractions, it would be plausible to suggest that ethanol causes a leakage or detachment of bound hexokinase from the particles to increase its activity in the postmitochondrial supernatant and in the soluble protein fraction. However, there was no significant difference between the

experimental and control samples in the hexokinase activity of the postmitochondrial supernatant released by Triton X-100. A direct assay of the hexokinase activity of the sedimented mitochondrial and microsomal particles was not successful, because glucose was not utilized in these incubation conditions, perhaps due to a shortage of cytoplasmic cofactors. It remains unsettled whether ethanol influences the proportion of bound hexokinase.

The more active catabolism of glucose in the postnuclear and postmitochondrial supernatants of the homogenates may have masked the effects of ethanol, which were observed with the postmicrosomal supernatant and with the soluble protein fraction.

Amino acids

The concentrations of γ-aminobutyric acid, glutamine and aspartic acid are dependent on the concentration of glucose in the medium (Figs. 6–8) and this interdependence has a bearing on the effects of ethanol on the brain.

Elliott and van Gelder[33] found that after incubation of brain slices in aerobic conditions the concentration of γ-aminobutyric acid was higher in the presence of glucose than in its absence. In insulin-induced hypoglycemia, decreased γ-aminobutyric acid concentrations were observed in rat brain.[34,35] The levels on γ-aminobutyric acid in the brain tissue increased during shortage of oxygen.[35,36] These findings support our suggestion that the γ-aminobutyric acid pathway may be involved in those functional changes in brain which are produced by disturbances in carbohydrate metabolism.

Increased consumption of glucose was observed both after pretreatment with ethanol and as a result of increasing the concentration of glucose in the medium. An increase in γ-aminobutyric acid and a decrease in glutamine were observed as results of both increased concentration of glucose and pretreatment with ethanol, but conflicting results were obtained in regard to aspartic acid. Addition of glucose to the medium has been reported to decrease the removal of glutamic acid during incubation of brain slices.[37,38] We have observed decreased consumption of both glutamic acid and γ-aminobutyric acid as an effect of ethanol.[39]

The increase of γ-aminobutyric acid in brain has been reported to be reflected in the levels of other compounds with amino groups like serotonin but not in the level of norepinephrine.[40] Pharmacological evidence is presented to show that γ-aminobutyric acid is likely to be a transmitter at certain inhibitory synapses.[41,42]

An increase in the concentration of aspartic acid in the nervous tissue with shortage of glucose was reported already by Dawson.[34] We observed the maximal level of aspartate at an early phase of incubation and in the absence of glucose (Fig. 8). Haslam and Krebs[38] found that in brain homogenate glutamic acid was converted to aspartic acid, and Häkkinen and Kulonen[43] that the aspartic acid formed in the soluble protein fraction of brain on incubation originated from glutamic acid. Addition of glucose inhibited the formation of aspartic acid from glutamic acid.[38] These findings are in agreement with the observed decline in the level of aspartic acid at higher concentrations of glucose but the effect of ethanol remains unexplained. Balazs *et al.*[44] found that in brain slices the specific radioactivity of γ-aminobutyric acid is first transferred to aspartic acid. The early maximum observed in the concentration of aspartic acid (Fig. 9) is in agreement with their results. Thus there may be a relationship between the increases in the levels of γ-aminobutyric acid and

aspartic acid as a result of ethanol. Yoshino and Elliott[45] studied the conversion of labeled glucose to the free amino acids in brains of rats *in vivo* and found that during pentobarbital narcosis the formation of glutamic acid, aspartic acid and γ-aminobutyric acid from glucose was decreased, but that of alanine was increased. The concentration of aspartic acid was increased, while the concentration of γ-aminobutyric acid remained unchanged. Thus the effects of ethanol do not necessarily parallel those of other depressants.

CONCLUDING REMARKS

The ultimate purpose of this work was to locate a single primary target of ethanol action on the metabolic map, starting from the effects of ethanol on amino acid metabolism in brain. This has not been possible, although the experimental conditions and the effects of the cofactors have been systematically tested. Several effects of ethanol have been demonstrated, but the changes are of the order of 10–30 per cent only, albeit statistically significant. However, even these small changes may be relevant, because ethanol causes a relatively mild and reversible intoxication, and only single doses have been administered.

Other authors have similarly been unable to ascertain the primary effect of ethanol on brain except that it may be in some way related to energy consumption.[1,46] Also it may be said that there is a tendency towards an increase in the reduced members of certain redox pairs, such as glutamic acid, γ-aminobutyric acid and lactic acid. This is also observed *in vitro* without the contribution of any reducing components released into the circulating blood by the liver. An allosteric effect also seems possible.

We believe that we have to consider the possibility that ethanol affects the architecture of the macromolecules in the brain generally and non-specifically. Various alcohols, including ethanol, affect the aggregation of tropocollagens at concentrations in the range of 50–250 mM,[47] a finding which may be generalized to the associations of all macromolecules. Ethanol affects the solubilities of the neuraminic acid-containing components of the brain,[48] presumably by disturbing the association of the molecules, and possibly by competing with water molecules.

It also remains to be investigated whether the effect of ethanol on the nervous tissue is mediated through a hormonal system, either in the hypothalamic region or in the peripheral endocrine glands. Indeed, fed rats which have been pretreated with an artificial glucocorticoid triamcinolone (9α-fluoro-16α-hydroxyprednisolone), become significantly ($P < 0.001$) more intoxicated than controls[49] as measured by the tilted plane test.[50] This difference is not observed with fasted rats. A detailed study of the connection between ethanol inebriation and the enzymes and intermediates involved in the carbohydrate metabolism of the brain is required to assess this effect.

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