Amino Acid Metabolism in Various Fractions of Rat-Brain Homogenates with Special Reference to the Effect of Ethanol

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1. The increase in brain γ -aminobutyrate, glutamate and aspartate and the decrease in brain glutamine that occur when ethanol is administered to rats *in vivo* could be reproduced by incubating brain homogenates from rats pretreated with ethanol. 2. For the demonstration of the effects of pretreatment with ethanol on the metabolism of γ -aminobutyrate and glutamine, the whole homogenate could be replaced by various supernatant preparations, and even by the soluble protein fraction, which was less active, however. The 'postmitochondrial' sediment could likewise mediate the effects of pretreatment with ethanol. 3. When the brain homogenates from control and ethanol-treated rats were allowed to 'age' at 2° for more than 7 days, the metabolic difference at incubation could no longer be demonstrated. The capacities of the homogenate from the control rats had changed to resemble those of ethanol-treated rats. 4. Data are given on the effects of the incubation time and of the concentration of homogenate.

In a previous paper Häkkinen, Kulonen & Wallgren (1963) showed that the changes in amino acid concentrations (increase of γ -aminobutyric acid, glutamic acid and aspartic acid and decrease of glutamine) observed earlier in the brain of the ethanol-pretreated rat (Häkkinen & Kulonen, 1961) could be reproduced with brain slices *in vitro*, but only when electrical stimulation was applied. We now report an extension of the previous experiments to rat-brain homogenates and their fractions. Preliminary reports of this work have appeared (Kulonen & Häkkinen, 1963; Häkkinen & Kulonen, 1965).

EXPERIMENTAL

Treatment of test animals and preparation of brain homogenates. Rats of the Wistar strain, weighing 202–496 g., were starved for 24–40 hr. before the experiments. As pretreatment *in vivo*, 550 mg. of ethanol/100 g. body wt. was given as an aq. 33% (v/v) solution by stomach tube. The control animals received the same volume of water. The rats were killed by decapitation after 1 hr. The brains were dissected immediately at room temperature, weighed on a torsion balance and homogenized.

The homogenization medium was a fourfold volume (v/w) of a cold iso-osmotic salt solution (0.13 M-KCl–0.01 M-KH₂PO₄–K₂HPO₄ buffer, pH 7.4; DiPietro & Weinhouse, 1959). To test the possible effect of different homogenization media, 0.25 M-sucrose solution was used as an additional medium in one series of experiments (data not presented). One half of each brain was then homogenized in sucrose solution and the other half in the usual salt solution. The

effect of ethanol could be demonstrated to the same extent in both media. The pellet of mitochondria and nuclei was more compact in the salt solution, which was therefore preferred and used throughout this work.

Throughout the work the same Potter-Elvehjem-type homogenizer (size 'B' from A. H. Thomas, Philadelphia, Pa., U.S.A., reported clearance 0.005–0.007 in., pestle diameter 19.0 mm.) was used in conjunction with a 0.02 h.p. motor of 2000 rev./min. nominal speed. We tried to keep the conditions constant during the preparation of brain homogenates, as emphasized by Aldridge, Emery & Street (1960). The Teflon pestle was passed six times to the bottom, which took altogether 45–60 sec. The homogenates were kept cold and either used immediately in the experiments or fractionated further with minimum delay.

The pooled brain homogenates from at least two ethanol-pretreated and two control rats were used in each experiment.

Fractionation with centrifugation. An international model PR-2 refrigerated centrifuge or a Spinco model L preparative ultracentrifuge was used. The average centrifugal force and the time are given in Table 3. In some series of experiments the whole homogenate was first centrifuged at 5000g for 10 min. to remove the nuclei and mitochondria, and the supernatant fractionated further or used instead of the whole homogenate. In the incubation experiments this postmitochondrial supernatant and the whole homogenate gave corresponding results on γ -aminobutyric acid and on glutamine (see Table 3b–c). Further, the supernatant could be more accurately measured with a pipette than the whole homogenate. The volumes of the fractions of the homogenate were adjusted to that of the original homogenate and the mixtures rehomogenized when required.

The morphological features of the microsomal sediment, obtained by centrifuging the postmitochondrial supernatant

for 90 min. at 25 000g, were checked by electron microscopy (Whittaker, 1959) by Professor A. Telkkä, Department of Electron Microscopy, University of Helsinki. The most conspicuous particles were vesicles (about 1000 Å) and clusters of dense particles (200 Å) but there were no mitochondria.

Fractionation with gel filtration. For a part of these experiments, the postmicrosomal supernatant obtained by centrifuging the homogenate at 100 000g for 60 min. was separated into fractions of large and small molecular size, i.e. soluble protein fraction and low-molecular-weight fraction, by gel filtration (Flodin, 1962) with the dextran gel Sephadex G-25 (fine grade) in bead form. A column of 1.8 × 30 cm., with a bed volume of 70 ml., was used. The volume of the sample was either 4, 8 or 12 ml., and 40 mM-sodium phosphate buffer, pH 7.4, was used as eluent. The flow rate was about 0.5 ml./min.

In a typical experiment, 4 ml. (corresponding to 0.8 g. of original brain tissue) of the postmicrosomal supernatant was allowed to drain into the column followed by 10 ml. of the eluent. The column was then eluted under a pressure of about 15 cm. solution. The first colourless effluent fraction of about 12 ml. was discarded and then the orange protein-containing fraction (about 16 ml.) was collected. The next 40 ml. of effluent contained the low-molecular-weight substances. The amount of proteins (soluble protein fraction) and amino acids (low-molecular-weight fraction) in the effluent was determined by the Folin (Lowry, Rosebrough, Farr & Randall, 1951) and ninhydrin (Hawk, Oser & Summerson, 1947) colour reactions (with bovine serum albumin and glycine as respective standards). The separation into these two fractions was good.

Incubations. Incubation of the whole homogenates and their sedimentation fractions was carried out in conventional Warburg flasks attached to constant-volume respiratory manometers. The incubation medium, adopted from DiPietro & Weinhouse (1959), contained the following basal components in the final concentrations designated: 77 mM-KCl; 10 mM-glucose; 8 mM-MgSO₄; 0.1 mM-fumarate; 40 mM-Na₂HPO₄-NaH₂PO₄ buffer, pH 7.4. When stated, this medium was enriched with the following: 1.4 mM-ATP; 2 mM-NAD⁺; 0.06 mM-cytochrome c. To each flask was added 0.5 ml. of homogenate, corresponding to 100 mg. of brain, except in one series of experiments (Fig. 2) where the

amounts of homogenate corresponded to 33, 100 and 200 mg. of brain. The total volume of incubated sample was 2.5 ml. The gas phase was air. The consumption of O₂ was recorded to check the viability of the samples. When ethanol was added *in vitro*, it was placed in the side arm of the flask in 0.2 ml. of the incubation medium and tipped into the flask after equilibration for about 10 min. in the bath. The final concentration of ethanol in the incubation mixture was always 0.4% (w/w).

The fractions of homogenate obtained by gel filtration were incubated in ordinary Erlenmeyer flasks because of the larger volume of the samples. The sample corresponded to either 200 mg. (Tables 3 and 4) or 300 mg. (Fig. 1b) of fresh brain tissue. The volume of incubated sample varied from 4 to 17.5 ml., depending on the fractions of homogenate.

Glutamic acid was added as an exogenous substrate in most of the experiments. In some series of experiments γ -aminobutyric acid or glutamine was added instead of glutamic acid (Table 2).

The incubation temperature was 37.5° and the duration 1 hr., except in the experiments on incubation time (Figs. 1a and 1b). The incubation period was ended by chilling the flasks in crushed ice. All the incubations were carried out in duplicate at least.

Determination of amino acids. For the analysis of amino acids in the incubation mixture the method used in the experiments with brain slices (Häkkinen *et al.* 1963) was followed in the first part of this work (Table 2). In the rest of the experiments the amino acids were analysed by the paper-electrophoretic method described by Häkkinen & Kulonen (1963).

In the present electrophoretic determination, glutamine is not resolved from alanine, glycine, serine and taurine, and its calibration curve is based on the assumption that the other amino acids mentioned are metabolically stable in the samples, which we have found to be true in the brain during ethanol poisoning (Häkkinen & Kulonen, 1961). This is not necessarily so *in vitro*, especially in the subcellular particles. When the incubation mixture did not contain the original amino acids of the brain sample, the standard curve for glutamine was drawn through the origin. Such values of glutamine are given in parentheses (Table 3d).

Reagents. Ethanol was purified 'for spectrometric research' by the Finnish State Alcohol Monopoly. γ -Amino-

Table 1. *Conversion of added amino acids into other amino acids on incubation of whole-brain homogenates in the presence of ethanol added in vitro*

The homogenates were incubated in the basal unenriched medium containing 77 mM-KCl, 10 mM-glucose, 8 mM-MgSO₄, 0.1 mM-fumarate and 40 mM Na₂HPO₄-NaH₂PO₄ buffer, pH 7.4. The concentration of ethanol, when added, was 0.4% (w/w). The preparation of the homogenates, amounts of amino acids added and other conditions of incubation are given in the legend to Table 2. The results are averages of duplicate determinations on two rats.

Additions to incubation medium		Concn. (μ moles/100 g. of original brain)			
Amino acid	Ethanol	γ -Aminobutyric acid	Aspartic acid	Glutamic acid	Glutamine
None	Absent	430	330	940	550
	Present	240	260	850	490
γ -Aminobutyric acid	Absent	—	460	1890	1390
	Present	—	380	1830	1110
Glutamic acid	Absent	990	750	—	1440
	Present	840	900	—	1530

butyric acid and fumaric acid were obtained from Fluka A.-G., Buchs, Switzerland, L(+)-glutamic acid and L(+)-aspartic acid were from E. Merck A.-G., Darmstadt, Germany, and L(+)-glutamine was from F. Hoffmann-La Roche and Co. Ltd., Basle, Switzerland. The disodium salts of ATP and NAD⁺ were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., and cytochrome *c* was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The cation-exchange resin Dowex 50 (mesh 200-400) was manufactured by the Dow Chemical Co., Midland, Mich., U.S.A., and the dextran gel Sephadex G-25 by AB Pharmacia, Uppsala, Sweden.

RESULTS

Experiments with the whole homogenate

Addition of ethanol in vitro. The results (Table 1) of the addition of ethanol *in vitro* closely resemble those obtained analogously with non-stimulated slices (Häkkinen *et al.* 1963). The effects of ethanol observed with intact brain tissue could not be reproduced. For example, instead of an increase in the amount of γ -aminobutyric acid, the addition of

Table 2. *Conversion of added amino acids into other amino acids on incubation of whole-brain homogenates from control and ethanol-pretreated rats*

Iso-osmotic salt solution (0.13M-KCl-0.01M-KH₂PO₄-K₂HPO₄ buffer, pH 7.4) was used as homogenization medium. A 0.5 ml. sample of homogenate, corresponding to 100 mg. of brain, was incubated in the medium containing 77 mM-KCl, 10 mM-glucose, 8 mM-MgSO₄, 0.1 mM-fumarate and 40 mM-Na₂HPO₄-NaH₂PO₄ buffer, pH 7.4, fortified with 1.4 mM-ATP, 2 mM-NAD⁺ and 0.06 mM-cytochrome *c*. The total volume of the incubation mixture was 2.5 ml., the temperature was 37.5° and the duration 1 hr. Where indicated, 12.5 μ moles of γ -aminobutyric acid, 24.0 μ moles of glutamic acid or 10.0 μ moles of glutamine were added to each incubated sample. The results are averages of duplicate determinations with two rats, except for the group marked 'None' where the experiments with enriched medium were performed with four rats each.

Additions to incubation medium		Treatment of rats	Concn. (μ moles/100 g. of original brain)			
			γ -Aminobutyric acid	Aspartic acid	Glutamic acid	Glutamine
None	Absent	Control	350	510	1050	670
	Absent	Ethanol	500	530	1160	530
	Present	Control	510	360	930	520
	Present	Ethanol	590	400	1010	470
γ -Aminobutyric acid	Absent	Control	—	830	1590	560
	Absent	Ethanol	—	740	1460	400
	Present	Control	—	750	1470	850
	Present	Ethanol	—	840	1570	680
Glutamic acid	Absent	Control	1050	810	—	1210
	Absent	Ethanol	1400	970	—	1000
	Present	Control	1700	780	—	1250
	Present	Ethanol	1910	920	—	1140
Glutamine	Absent	Control	450	800	4650	—
	Absent	Ethanol	580	900	5180	—
	Present	Control	940	720	4140	—
	Present	Ethanol	1270	910	4440	—

Statistical evaluation (by the t test) of the effect of the pretreatment with ethanol

The data were calculated in relative units (the values referring to control rats were taken as 100) and treated as non-independent samples arranged in inherent pairs. Those values obtained with four rats (instead of the usual two) were counted twice. N.S., Not significant.

Change tested	Data used for calculations	
	No amino acids added, enrichments present	All values, in relative units
Increase of γ -aminobutyric acid	$P < 0.001$	$P < 0.001$
Increase of aspartic acid	N.S.	$P < 0.02$
Increase of glutamic acid	$P < 0.02$	$P < 0.05$
Decrease of glutamine	$P < 0.025$	$P = 0.001$

ethanol *in vitro* caused an even greater decrease in the whole homogenate than in the non-stimulated slices.

Pretreatment with ethanol *in vivo*. In all the conditions studied (Table 2) the final concentration of γ -aminobutyric acid was increased when the homogenate was prepared from the brains of rats pretreated with ethanol. Further, the net increase of γ -aminobutyric acid after the addition of glutamic acid or glutamine was also augmented as an effect of ethanol (except after addition of glutamine to unenriched medium). The following features observed *in vivo* were also reproduced as an effect of ethanol: a decrease in the final concentration of glutamine and an increase in the glutamic acid and aspartic acid, except when γ -aminobutyric acid was added to unenriched medium. These findings were corroborated when the net conversions of amino acids were calculated.

The enrichments (ATP, NAD⁺, cytochrome c)

caused an increased conversion of added glutamine and glutamic acid into γ -aminobutyric acid and of added γ -aminobutyric acid into glutamine.

Experiments with various fractions of the homogenate

Sedimentation fractions. The final concentrations of γ -aminobutyric acid in the incubated supernatants reached the same levels as in the whole homogenate (Table 3a-c). After the addition of glutamic acid (Table 3b-c), the concentration of γ -aminobutyric acid increased more than threefold. The expected effect of pretreatment with ethanol *in vivo* was evident in all the fractions of homogenate, whether they were incubated with or without added glutamic acid.

After incubations without added glutamic acid (Table 3a) the concentration of glutamine in the supernatants was the same as that in the whole homogenate, and the increase in the concentration

Table 3. Concentrations of amino acids in various incubated fractions of brain homogenates from control and ethanol-pretreated rats

Preparation of the homogenates and the composition of the incubation medium are given in the legend to Table 2. In the series (b) and (c) glutamic acid was added, 24.0 μ moles/incubated 2.5 ml. sample, containing the homogenate from 100 mg. of brain, and in the series (d) 27.6 μ moles/incubated 17.5 ml. sample, corresponding to 200 mg. of brain. Concentrations are calculated/wt. of original brain.

Conditions of centrifugation and fractions of homogenate	γ -Aminobutyric acid		Glutamine		Aspartic acid	
	Control (μ moles/100 g.)	Effect of ethanol (%)	Control (μ moles/100 g.)	Effect of ethanol (%)	Control (μ moles/100 g.)	Effect of ethanol (%)
(a) Centrifuged at 25000g for 10 min.						
Whole homogenate	280	+ 6.0	320	- 16.6	420	+ 5.8
Particles	40	+ 32.5	0	—	140	+ 11.9
Supernatant	280	+ 9.9	360	- 14.3	360	+ 6.9
(b) Centrifuged at 25000g for 10 min.						
Whole homogenate	1040	+ 13.0	1500	- 13.5	2600	+ 3.7
Particles	270	+ 6.7	100	- 15.2	2540	- 5.3
Supernatant	1030	+ 10.0	1340	- 11.5	1110	+ 14.1
(c) Centrifuged at 25000g for 90 min.*						
Postmitochondrial supernatant	1020	+ 9.5	1020	- 12.8	920	+ 22.8
Particles	290	+ 21.8	0	—	940	+ 15.4
Supernatant	1070	+ 12.0	860	- 4.6	310	+ 9.1
(d) Centrifuged at 100000g for 60 min.†						
Particles + soluble protein + low-mol.wt. fraction‡	1010	+ 11.4	590	0	530	+ 14.2
Soluble protein + low-mol.wt. fraction	840	+ 26.0	330	- 17.5	590	- 5.4
Particles + low-mol.wt. fraction	800	- 12.9	0	—	580	+ 11.1
Particles + soluble protein	600	+ 17.3	(720)	- 26.0	130	+ 66.1
Particles	570	- 2.3	(280)	- 26.5	350	- 9.3
Soluble protein	650	+ 27.8	(460)	- 26.2	320	- 3.4

* Instead of the whole homogenate the postmitochondrial supernatant, obtained by centrifuging the whole homogenate at 5000g for 10 min., was used in this series.

† The postmitochondrial supernatant was used for this centrifugation. The 100000g supernatant was then separated by gel filtration into the soluble protein and low-molecular-weight fractions.

‡ The low-molecular-weight fraction also in the control combination originated from ethanol-pretreated rats, exceptionally.

caused by the added glutamic acid was two- to four-fold (Table 3b-c). Very little glutamine was found in the incubated particles. The effect of ethanol pretreatment was about the same in the whole homogenate and in the supernatant, at least after 10 min. centrifugation.

The decrease in the concentration of the endogenous glutamic acid during the incubation of the supernatant (Table 3a) was equal to the combined increases in the concentrations of γ -aminobutyric acid and glutamine.

With aspartic acid the concentrations in the supernatants remained considerably lower than in the whole homogenates, whereas in the particle fractions the values approached those in the whole homogenates (Table 3b-c). The addition of glutamic acid caused remarkably large increases in the concentrations of aspartic acid, especially in the sediment fractions. The effect of ethanol was observed in all the supernatants but most clearly in the postmitochondrial supernatant.

From the data in Table 3(a)-(c) it was concluded that the various supernatants of the homogenates could be used for the demonstration of the effect of ethanol on the amino acid metabolism of the brain. The postmitochondrial supernatant was employed in several subsequent experiments (cf. the Experimental section).

Gel-filtered fractions. The purpose of the experiments shown in Table 3(d) was to learn whether the

effect of ethanol can be demonstrated with the soluble protein fraction of the supernatant, or depends on the metabolic cofactors of low molecular weight.

The expected effect of ethanol on γ -aminobutyric acid, i.e. an increased concentration, appears only when the soluble protein fraction is present in the incubation medium. The findings on glutamine are less clear. As an effect of ethanol the largest absolute decrease was obtained with preparations consisting of both the particle fraction and soluble protein fraction, but the relative change was the same with either of these fractions alone. In the absence of the soluble protein fraction the concentrations of glutamine are low.

It appears that the soluble protein fraction, even alone, can mediate the effect of ethanol on the metabolism of γ -aminobutyric acid and glutamine in the brain, but with regard to aspartic acid the particles in combination with other fractions seem also to be required.

Mutually interchanged fractions. Further experiments were performed with mutually interchanged fractions from control and ethanol-pretreated rats (Table 4). The particle, supernatant and soluble protein fractions were all found to contain the components required for the demonstration of the effect of ethanol, at least on one amino acid. When both fractions originated from ethanol-treated rats, the effects were additive.

Table 4. Incubation experiments with mutually interchanged fractions of brain homogenates from ethanol-pretreated and control rats

Comparisons within the various series are shown on a percentage basis, taking as 100 the values of those preparations in which all the components originated from control rats. In series (a) and (b) the centrifugations, the gel filtration and the conditions during incubation were the same as those referred to in Table 3(d). In series (c) are combined three experiments with particles and supernatant, where the conditions were analogous to those described in Table 3(c), 3(d) and 3(d) without gel filtration respectively. The means and ranges of the values (%) are given.

Composition of brain sample			Final concn.		
Particle fraction	Soluble protein fraction	Low-mol.wt. fraction	γ -Aminobutyric acid	Glutamine	Aspartic acid
(a) —	Control	Control	100	100	100
—	Ethanol	Control	105	88	104
—	Control	Ethanol	101	100	118
—	Ethanol	Ethanol	115	86	121
(b) Control	—	Control	100	100	100
Ethanol	—	Control	103	—	104
Control	—	Ethanol	111	79	107
Ethanol	—	Ethanol	121	68	128
Supernatant					
(c) Control	Control		100	100	100
Ethanol	Control		114 (110-122)	90 (79-98)	103 (80-115)
Control	Ethanol		112 (107-121)	91 (87-96)	102 (100-104)
Ethanol	Ethanol		121 (115-128)	84 (74-99)	120 (114-130)

Fig. 1. Effect of incubation time on the net gains of the amino acids indicated. The enriched medium (see the legend to Table 2) was used. (a) The postmitochondrial supernatant of brain homogenate was incubated with glutamic acid (24.0 μ moles/incubated 2.5 ml. sample, containing the homogenate from 100 mg. of original brain tissue). The values at 60 min. are averages of three different series of experiments. (b) The soluble protein fraction of brain homogenate was incubated with glutamic acid (10.3 μ moles/incubated 4.0 ml. sample, containing the homogenate from 300 mg. of original brain tissue). ●, Preparations from control rats; ○, preparations from rats pretreated with ethanol.

Effects of some experimental variations

Effect of incubation time. The net formation of γ -aminobutyric acid, aspartic acid and glutamine from glutamic acid was followed during the incubation of the postmitochondrial supernatant (Fig. 1a) and of the soluble protein fraction (Fig. 1b). The increases in the amino acids in the postmitochondrial supernatant were rapid during the first 20 min., but then levelled off, and the concentration of aspartic acid even declined after 90 min. When the soluble protein fraction was incubated, the pattern was similar with regard to aspartic acid, whereas γ -aminobutyric acid and glutamine increased almost linearly throughout the experimental period.

Considering the concentrations of the brain preparations in the medium, the soluble protein fraction was less active than the postmitochondrial supernatant in converting glutamic acid into γ -aminobutyric acid and glutamine, and especially into aspartic acid. The amount of γ -aminobutyric acid formed by the soluble protein fraction was about half of the corresponding amount of glutamine, which is confirmed in other experiments (Fig. 2).

The effect of ethanol appeared in all the experiments after about 20 min. incubation and then remained almost constant to the end of the experimental period. Even considering the different scales in Figs. 1(a) and 1(b) the effect of ethanol

with the soluble protein fraction is rather small but still consistent.

Effect of concentration of the homogenate. The net increase of glutamine, and to some extent that

of γ -aminobutyric acid, is directly proportional to the concentration of homogenate (Fig. 2). Also, the net gain of aspartic acid, and likewise the consumption of oxygen, increased with the concentration of homogenate, but not linearly. The effect of ethanol was again observed, especially in the values of γ -aminobutyric acid and aspartic acid, but not in the consumption of oxygen.

Effect of storage on the homogenate. When the postmitochondrial supernatant of the homogenate was kept at 2° for 15 days (Table 5), its ability to form glutamine decreased by 21.7%, but the net gains of γ -aminobutyric acid and aspartic acid increased by 5.1% and 21.2% respectively, i.e. similar changes occurred to those caused by pre-treatment with ethanol *in vivo*. After storage at -18° without thawing these changes were not observed. The changes in the consumption of oxygen did not run parallel with the changes in the metabolism of amino acids.

As shown in Fig. 3, the effect of ethanol could not be reproduced after storage of the preparations at 2° for 1-7 days. After storage at -18° the effect of ethanol still remained, even after 15 days, but occasional thawing to room temperature was detrimental to the demonstration of the effect of ethanol on amino acid metabolism.

DISCUSSION

The present findings prove that the effect of ethanol on the amino acid metabolism of the brain observed earlier *in vivo* (Häkkinen & Kulonen, 1961) and in stimulated slices (Häkkinen *et al.* 1963) can be reproduced by incubating brain homogenates and their fractions, but only if ethanol is given *in vivo* and the animal is killed when poisoned. Ethanol thus acts through a metabolic system that functions in integrated tissue only. Also, Scheig & Isselbacher (1965) state that 'experiments based solely on the action of ethanol *in vitro* must be interpreted with caution'.

Fig. 2. Effect of various concentrations of the postmitochondrial supernatant of brain homogenate on the net gains of the amino acids indicated and on the consumption of O₂. The supernatant was incubated for 1 hr. with glutamic acid (24.0 μ moles/incubated 2.5 ml. sample). The enriched medium (see the legend to Table 2) was used. ●, Preparations from control rats; ○, preparations from rats pretreated with ethanol.

Table 5. *Effect of storage on the amino acid metabolism of incubated brain homogenates*

The homogenate had been centrifuged at 5000*g* for 10 min. and the supernatant was used for the experiment. Glutamic acid was added, 24.0 μ moles/incubated 2.5 ml. sample, containing the homogenate from 100 mg. of original brain tissue. The effect of ethanol is shown in Fig. 3. The conditions of incubation are explained in the legend to Table 2.

Storage	Net gain (μ moles/100 g. of original brain)			Consumption of O ₂ (m-moles/100 g.)
	γ -Aminobutyric acid	Glutamine	Aspartic acid	
None	990	1430	660	1.38
24 hr. at 2°	1070	1400	740	2.45
7 days at 2°	1080	1200	780	0.96
15 days at 2°	1040	1120	800	0.31
15 days at -18°	1000	1500	670	0.88
15 days at -18°, thawed five times	880	1210	640	0.02

Fig. 3. Influence of storage on the effect of ethanol on amino acid metabolism of incubated postmitochondrial supernatant of brain homogenates. The effect of ethanol (administered *in vivo*) on the net changes of the amino acids indicated is calculated as percentages of the respective control values. The experimental details are given in Table 5. The enriched medium (see the legend to Table 2) was used. ●, Homogenates stored at 2°; ○, homogenates stored at -18°; Δ, homogenates stored at -18°, but thawed five times during storage for 15 days.

The main reason for the addition of glutamic acid to the incubation medium was to raise the final concentrations of the other amino acids and thus make the analytical data more reliable. The addition of glutamic acid and glutamine is known to bring about the following changes in brain cortex slices: a decreased content of creatine phosphate, increased inorganic phosphate, increased glycolysis and increased respiration (McIlwain, 1952). We do not know whether similar changes occur in the incubated homogenate, and whether they eventually influence the metabolism of amino acids. However, the effects of ethanol remain qualitatively similar to those observed without added glutamic acid.

Clear-cut evidence of the subcellular target of the action of ethanol was not obtained. The general conclusion drawn from the experiments with the mutually interchanged fractions of homogenates (Table 4c) is that both the particle fraction and the supernatant fraction mediate the effect of ethanol. This is hardly surprising: many enzymes in the brain are present in both the particle and soluble fractions and some enzymes may also be released from the particles into the soluble environment at homogenization (Tower, 1959). The enzymes involved in the metabolism of γ -aminobutyric acid and originally located in the particles (Salganicoff & De Robertis, 1965) are partly passed to the soluble protein fraction, which thus, even alone, is adequate for the studies of the metabolism of γ -aminobutyric acid. This conclusion is supported by the smaller activities of the enzymes of γ -aminobutyric acid pathway in the soluble protein fraction as compared with the postmitochondrial supernatant (Figs. 1a and 1b).

The effect of storage was studied first for practical reasons to ascertain how long a homogenate was usable. Ethanol pretreatment *in vivo* and storage of the preparations from control rats at 2° produce similar changes in the metabolism of amino acids in the incubated homogenate. The effects of ethanol can no longer be demonstrated with 'aged' homogenates from pretreated rats. Thus the influences of ethanol and of 'aging' may be similar in nature.

To determine the site of action of ethanol in the brain, we think it useful to study the metabolism of glutamine and γ -aminobutyric acid and the closely related metabolism of glucose further with suitable brain preparations to recognize the nature and location of the enzyme functions that are affected.

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