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EFFECTS *IN VIVO* AND *IN VITRO* OF L-GLUTAMIC ACID-γ-HYDRAZIDE ON METABOLISM OF SOME FREE AMINO ACIDS IN BRAIN AND LIVER*

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Abstract—Further research on the effect of L-glutamic acid-γ-hydrazide (GAH) on free amino acids metabolism in brain is reported. Similar studies of free amino acid metabolism in liver are also presented.

The addition of GAH to mice brain homogenates inhibited the activities of γ-aminobutyrate aminotransferase (ABAT), and of glutamate decarboxylase (GAD). The activities of alanine aminotransferase (AAA) and of aspartate aminotransferase (ASA) were not affected by GAH in these conditions.

Brain homogenates from GAH-treated mice showed lower GAD, ABAT, AAA, and β-alanine-α-ketoglutaric aminotransferase (βAA) activities than brain homogenates from control mice. GAD activity was inhibited by GAH to a considerably lesser extent *in vivo* than *in vitro*.

In liver, GAH produced a striking increase in the concentrations of almost all the free amino acids, especially of alanine and β-alanine.

The addition of GAH to mice liver homogenates inhibited the βAA activity, but neither AAA nor ASA activity was affected by the same concentration of GAH. Liver homogenates from GAH-treated mice showed diminished βAA and AAA activity in comparison with liver homogenates from control mice.

Some differences in the conversion of uniformly labeled ^{14}C- glucose to liver and brain free amino acids were found between control and GAH-treated mice. In the brain of the latter animals, aspartic and glutamic acids and glutamine exhibited initially a higher specific activity than in control mice. In the case of γ-aminobutyric acid and alanine, opposite results were obtained. In the GAH-treated animals these two amino acids showed no significant decay of the acquired radioactivity.

In liver, the decay of the acquired radioactivity of alanine tended to be slower in GAH-treated animals than in control animals.

IT HAS been reported that the administration of GAH† modified the free amino acid levels in mouse brain.[1] In this paper it is shown that the levels of several amino acids are also increased in liver by GAH treatment; other experiments suggest that in the case of alanine and β-alanine this effect is related to inhibitory changes in AAA and βAA activities in brain as well as in liver. The effect of GAH on the mentioned

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† The abbreviations used in this work are: GAH, L-glutamic acid-γ-hydrazide; GABA, γ-aminobutyric acid; ABAT, aminobutyrate aminotransferase (EC 2.6.1.19); AAA, alanine aminotransferase (EC 2.6.1.2); ASA, aspartate aminotransferase (EC 2.6.1.1); GAD, glutamate decarboxylase (EC 4.1.1.15); βAA, β-alanine-α-ketoglutaric aminotransferase (not reported in the Enzyme Commision nomenclature); U-^{14}C-glucose, uniformly labeled ^{14}C-glucose; PALP, pyridoxal phosphate.

enzymatic activities and on ASA *in vitro* was studied in brain and in liver homogenates. GAD and ABAT activities were also studied in brain homogenates in the absence and presence of GAH and in brain homogenates from GAH-treated mice.

Since glucose is rapidly converted *in vivo* to amino acids, especially in brain,[2, 3] the conversion of U-^{14}C-glucose to several amino acids in brain and liver of control and GAH-treated mice was studied in order to obtain additional information on the mode of action of GAH.

MATERIALS AND METHODS

In all experiments adult mice (local strain), weighing 25–30 g and fed with a commercial diet (Purina laboratory chow) were used. Different experimental conditions of doses and times of treatment, described below, were employed.

Measurement of free amino acids in liver. Different doses (40 to 1440 mg/kg) of GAH (hemihydrate, A grade, from Calbiochem) were injected intraperitoneally, and the mice were killed by decapitation 6·5 hr later. In other experiments the dose of GAH was 160 mg/kg and the mice were killed 3·5 hr after treatment. The livers were frozen in liquid air and the method of Awapara[4] was used for obtaining protein and lipid-free extracts containing the amino acids. Previously described procedures for the bidimensional paper chromatographic separation of amino acids[1] were followed (80% phenol and butanol:acetic acid:water, 4:1:1). The location of GAH in the chromatograms was studied by usual recovery tests. The quantitative estimation of individual amino acids was carried out according to the colorimetric method of Naftalin.[5] For the readings (570 mμ) a Beckman B spectrophotometer was employed. The recoveries obtained by this procedure for the amino acids studied are at least 85 per cent; for glutamic and aspartic acids it is about 95 per cent.[6]

Measurement of effects on enzyme activities in vivo. The effects *in vivo* of GAH on enzyme activities were estimated by measuring the activities in brain or liver homogenates of mice injected i.p. with 160 mg GAH/kg; the animals were sacrificed 3·5, 6·5, or 24 hr after the injection. Control mice were injected with saline solution.

GABA production in a 2-hr incubation period according to the methods of Roberts and Frankel[7] and Rindi *et al.*,[8] with slight modifications, was taken as a measure of GAD activity. In some experiments the conventional manometric technique for measuring the CO_2 production in a Warburg apparatus was used; the results obtained by this technique were similar to those reported here. AAA, ASA, and βAA activities were estimated according to the procedure of Awapara and Seale[9] with slight modifications. The same technique was used for ABAT determination, but with the incubating conditions established by Bessman *et al.*[10] and Baxter and Roberts.[11] In all cases the value of a blank tube incubated without substrate was subtracted from those of the experimental tubes. Recovery tests for the glutamic acid measured in these determinations gave an error not greater than 10 per cent.

Measurement of effects on enzyme activities in vitro. The above-mentioned techniques for the determination of the enzyme activities were used to assay the effect of GAH *in vitro*. In this case GAH was added to the incubation systems from the beginning of the incubation period at a $3{\cdot}2 \times 10^{-3}$ M final concentration. In some experiments PALP was also added to the incubation mixture (see Results). In the chromatographic technique used, excellent separation between GAH and glutamic acid and GABA was obtained.

Measurement of the conversion of U-^{14}C-*glucose to amino acids.* After the injection (6·5 hr) of saline solution (control mice) or GAH (160 mg/kg), 5μc U-^{14}C-glucose (Calbiochem) in 0·1 ml with 2 mg carrier glucose was injected i.p. to each animal (weighing 25 g). The mice were sacrificed at 0·5, 1·0, or 1·5 hr after the administration of radioactive glucose; control animals were sacrificed at corresponding times. This type of experiment was repeated four times. In other types of experiments (repeated three times), saline solution or GAH (160 mg/kg) was injected 24 hr before the administration of U-^{14}C-glucose, and the mice were killed at 0·5, 1·0, or 1·5 hr after glucose administration.

The brain and liver of each animal were frozen in liquid air and the routine procedure for the separation of amino acids[1] was used. The chromatograms were run in triplicate and, after treating them with 0·02% ninhydrin for the localization of amino acids, one of them was used for the colorimetric quantitative determination of amino acids according to Naftalin.[5] In the other two chromatograms the spots corresponding to the individually measured amino acids [aspartic acid, glutamic acid, glutamine, alanine, and GABA (brain)] were pooled and eluted with 90% acidulated acetone and washed three times with the same solvent. In order to be sure that all the amino acid had been extracted from the paper, each washing was carried out at 37° for 0·5 hr. The eluate containing the amino acids was evaporated to dryness, and the residue was redissolved in acetone (0·2 ml), stirred, and placed in planchets. The residue was rinsed three times with 0·1 ml acetone; these volumes were added to the planchet and evaporated under an i.r. lamp. The rest of the ninhydrin-positive spots in the chromatograms were pooled and their radioactivity measured as described above. The possibility of a contamination of the radioactive amino acid spots with some intermediaries of the Krebs cycle was studied by chromatographic samples of lactic, pyruvic, α-ketoglutaric, and succinic acids in the same conditions employed for the separation of the amino acids, and revealing the chromatograms with 0·1% bromphenol blue. An excellent separation between the acids and the amino acids measured was obtained.

The radioactivity was counted three times in a Nuclear Chicago (model C110B) automatic counter. The background activity was subtracted from all radioactivity values.

RESULTS

Effect of GAH *on free amino acid levels in liver*

Striking changes in the concentrations of all the amino acids, except aspartic acid, were found 3·5 to 6·5 hr after the administration of different doses of GAH (Tables 1 and 2). An almost direct relation was found between the dose of GAH and the level of alanine. Serine + glycine and cystine concentrations were decreased by low doses of GAH, whereas doses higher than 360 mg/kg increased them. Glutamic acid and β-alanine were also increased, especially the latter (Table 2). More intense spots of lysine, tyrosine, leucine + isoleucine, and valine were observed in the chromatograms of GAH-treated mice relative to control mice. Some other unidentified spots appeared when the chromatograms were revealed with ninhydrin, one of them of considerable intensity near the glutamine spot. For this reason the values in the tables of glutamine in GAH-treated animals should be considered approximate

TABLE 1. FREE AMINO ACIDS IN LIVER OF CONTROL MICE AND OF MICE TREATED WITH GAH (160 mg/kg) AND SACRIFICED 3·5 hr. AFTER TREATMENT*

Group	Aspartic acid	Glutamic acid	Glutamine	Alanine	Serine + glycine	Threonine	Cystine
Control (4)	13·6 ± 1·47	30·9 ± 3·13	46·3 ± 4·57	8·3 ± 1·23	17·6 ± 2·21	2·07 ± 0·32	56·7 ± 6·80
Treated (4)	15·5 ± 2·38	53·9 ± 5·19	60·4 ± 5·67	101·4 ± 6·40	17·9 ± 3·00	4·27 ± 1·07	50·7 ± 3·60

* The figures are mg/100 g; mean ± S.E.M. Number of animals in parentheses.

Statistical significance of results according to *t* test

Glutamic acid 30·9–53·9	P <0·01	Alanine 8·3–101·4	P <0·001
Glutamine 46·3–60·4	<0·05	Threonine 2·07–4·27	<0·05

TABLE 2. FREE AMINO ACIDS IN LIVER OF CONTROL MICE AND OF MICE TREATED WITH DIFFERENT DOSES OF GAH, SACRIFICED 6·5 HR AFTER TREATMENT*

Dose of GAH (mg/kg)	Aspartic acid	Glutamic acid	Glutamine	Alanine	Serine + glycine	Cystine	Threonine	β-Alanine
Control (27)	13·8 ± 0·49	31·8 ± 0·98	46·7 ± 1·62	8·8 ± 0·44	20·6 ± 0·88	49·4 ± 2·82	2·4 ± 0·67	trace
40 (8)	11·5 ± 0·92	31·1 ± 1·53	42·1 ± 4·22	24·3 ± 0·39	14·8 ± 0·65	29·1 ± 1·21	4·0 ± 0·94	
80 (6)	9·1 ± 1·39	35·6 ± 3·61	50·3 ± 3·85	37·6 ± 2·32	13·9 ± 0·87	40·1 ± 2·95	4·3 ± 0·61	
160 (6)	10·7 ± 0·55	50·5 ± 4·25	94·0 ± 12·1	98·0 ± 13·9	21·5 ± 2·81	31·6 ± 1·45	8·5 ± 0·78	42·8 ± 3·12
360 (4)	14·1 ± 2·87	43·0 ± 5·46	113·0 ± 15·5	118·7 ± 31·8	23·1 ± 4·60	26·7 ± 3·08	4·7 ± 0·12	
720 (4)	9·7 ± 0·84	60·3 ± 10·7	105·7 ± 15·0	190·0 ± 31·4	49·7 ± 9·02	54·6 ± 2·73	12·4 ± 2·30	
1440 (4)	13·2 ± 1·19	50·0 ± 3·08	114·7 ± 2·24	162·7 ± 15·8	51·8 ± 6·30	53·0 ± 5·42	16·2 ± 3·70	

* The values are mg/100 g; mean ± S.E.M. Number of animals in parentheses.

Statistical significance of results according to *t* test

Glutamic acid	*P*	Glutamine	*P*	Alanine	*P*	Serine + glycine	*P*	Cystine	*P*	Threonine	*P*
31.8 vs 50·5	<0·001	46·7 vs 50·3	<0·5	8·8 vs 24·3	<0·001	20·6 vs 14·8	<0·001	49·4 vs 29·1	<0·001	2·4 vs 4·0	<0·01
31.8 vs 43·0	<0·01	46·7 vs 94·0	<0·001	8·8 vs 37·6		20·6 vs 13·9	<0·001	49·4 vs 40·1	<0·5	2·4 vs 4·3	<0·01
31.8 vs 60·3	<0·001	46·7 vs 113·0	<0·001	8·8 vs 98·0		20·6 vs 23·1	<0·5	49·4 vs 31·6	<0·01	2·4 vs 8·5	<0·001
31.8 vs 50·0	<0·001	46·7 vs 105·7	<0·001	8·8 vs 118·7		20·6 vs 49·7	<0·001	49·4 vs 26·7	<0·01	2·4 vs 4·7	<0·01
		46·7 vs 114·7	<0·001	8·8 vs 190·0		20·6 vs 51·8	<0·001	49·4 vs 54·6	<0·5	2·4 vs 12·4	<0·001
				8·8 vs 162·7	<0·001			49·4 vs 53·0	<0·5	2·4 vs 16·2	<0·001

values. The spot corresponding to GAH almost overlapped the cystine site, and therefore the values of this amino acid are also only approximations.

Effects of GAH *on enzyme activities* in vivo

Brain. GAH administered at a dose of 160 mg/kg decreased ABAT activity within 2 to 24 hr after treatment, with a maximal inhibition at 24 hr (43·5 per cent). At 36 hr after GAH injection the activity was again normal. GAD activity was inhibited 30 per cent 6·5 hr after GAH administration. PALP completely reversed GAD inhibition when added to the incubation mixture containing the brain homogenate of animals sacrificed 3·5 hr after GAH treatment. The cofactor did not affect ABAT inhibition (Table 3).

βAA activity was inhibited 26 per cent 6·5 hr after treatment. AAA activity was diminished 52 per cent (Table 3).

TABLE 3. EFFECT OF GAH ON MICE BRAIN ENZYMATIC ACTIVITIES *in vivo**

Enzyme	Time after treatment (hr)	Control mice	Mice treated with GAH (160 mg/kg)	Inhibition (%)
GAD†	2	2·04 ± 0·25 (4)	2·05 ± 0·32 (4)	0
	3·5	1·75 ± 0·22 (5)	1·62 ± 0·21 (5)	7·5
	3·5 (PALP added to the incubation mixture)	3·19 ± 0·16 (4)	3·88 ± 0·19 (4)	0
	6·5	1·62 ± 0·22 (9)	1·13 ± 0·24 (9)	30
	24	1·87 ± 0·31 (5)	1·63 ± 0·24 (5)	13
ABAT‡	2	2·50 ± 0·13 (6)	2·15 ± 0·12 (6)	14
	3·5	2·52 ± 0·11 (8)	1·67 ± 0·17 (8)	34
	3·5 (PALP added to the incubation mixture)	2·30 ± 0·19 (3)	1·72 ± 0·15 (3)	25
	6·5	2·35 ± 0·07 (8)	1·58 ± 0·09 (8)	33
	24	2·60 ± 0·22 (6)	1·47 ± 0·07 (6)	43·5
AAA‡	6·5	3·27 ± 0·10 (12)	1·57 ± 0·12 (12)	52
βAA‡	6·5	1·54 ± 0·08 (12)	1·14 ± 0·08 (12)	26

* The figures are means ± S.E.M.; number of determinations in parentheses.

† Micromoles GABA produced by 100 mg wet tissue in 2-hr incubation at pH 6·3.

‡ Micromoles glutamic acid produced by 100 mg wet tissue in 1-hr incubation at pH 8·2 (ABAT) or 7·6 (AAA and βAA).

Statistical significance of results according to *t* test

GAD	*P*	ABAT	*P*	AAA	*P*	βAA	*P*
1·62–1·13	< 0·001	2·50–2·15	< 0·1	3·27–1·57	< 0·001	1·54–1·14	< 0·01
1·87–1·63	< 0·5	2·52–1·67	< 0·001				
		2·30–1·72	< 0·1				
		2·35–1·58	< 0·001				
		2·60–1·47	< 0·001				

TABLE 4. EFFECT OF GAH ON MICE BRAIN ENZYMATIC ACTIVITIES *in vitro*, IN ABSENCE AND IN PRESENCE OF PALP*

Enzyme	Control	GAH ($3{\cdot}2 \times 10^{-3}$ M)	Inhibition (%)
GAD without PALP	$1{\cdot}70 \pm 0{\cdot}013$ (8)	$0{\cdot}34 \pm 0{\cdot}013$ (8)	80
With PALP	$4{\cdot}45 \pm 0{\cdot}81$ (3)	$2{\cdot}70 \pm 0{\cdot}76$ (3)	39·5
ABAT			
Without PALP	$2{\cdot}67 \pm 0{\cdot}14$ (10)	$1{\cdot}44 \pm 0{\cdot}09$ (10)	46
With PALP	$2{\cdot}45 \pm 0{\cdot}16$ (3)	$1{\cdot}74 \pm 0{\cdot}19$ (3)	29
AAA			
Without PALP	$3{\cdot}30 \pm 0{\cdot}16$ (9)	$3{\cdot}30 \pm 0{\cdot}20$ (9)	0
With PALP	$3{\cdot}80 \pm 0{\cdot}33$ (3)	$4{\cdot}50 \pm 0{\cdot}35$ (3)	0
ASA			
Without PALP	$21{\cdot}9 \pm 1{\cdot}13$ (9)	$24{\cdot}8 \pm 0{\cdot}45$ (9)	0
With PALP	$20{\cdot}7 \pm 2{\cdot}93$ (3)	$21{\cdot}9 \pm 3{\cdot}19$ (3)	0
βAA			
Without PALP	$1{\cdot}42 \pm 0{\cdot}08$ (12)	$1{\cdot}18 \pm 0{\cdot}08$ (12)	17

* Legends as in Table 3. PALP final concentration: 10^{-4} M.

Statistical significance of results according to *t* test

GAD	*P*	ABAT	*P*	βAA	*P*
1·70–0·34	< 0·001	2·67–1·44	< 0·001	1·42–1·18	< 0·05

Liver. Only AAA and βAA activities were measured in the liver of mice treated with GAH (160 mg/kg). Both enzymes were inhibited (67·4 and 60 per cent respectively) 6·5 hr after GAH treatment (Table 5).

Effects of GAH *on enzyme activities* in vitro

Brain. As can be seen in Table 4, $3{\cdot}2 \times 10^{-3}$ M GAH inhibited GAD activity of brain homogenates to 20 per cent of control value; addition of PALP to the incubation mixture reversed the inhibition (39·5 per cent inhibition in presence of PALP). ABAT activity was inhibited 46 per cent by GAH in the absence of PALP and only 29 per cent in the presence of the cofactor. It is to be noted that in the control tubes PALP increased GAD activity and slightly decreased ABAT activity. βAA was inhibited 17 per cent by GAH, but neither AAA nor ASA activity was affected by the same concentration of GAH in the absence or presence of PALP (Table 4).

Liver. Unexpectedly, AAA activity was not affected by $3{\cdot}2 \times 10^{-3}$ M GAH even when the drug was preincubated without substrate for 15 or 30 min. ASA activity was also normal in the presence of GAH, but βAA was notably inhibited (60·6 per cent) (Table 5).

TABLE 5. EFFECT OF GAH ON MICE LIVER ENZYMATIC ACTIVITIES *in vitro* AND *in vivo**

Enzyme	*In vitro*			*In vivo* (6·5 hr after treatment)		
	Control	GAH ($3{\cdot}2 \times 10^{-3}$ M)	Inhibition (%)	Control	GAH (160 mg/kg)	Inhibition (%)
AAA	$22{\cdot}7 \pm 0{\cdot}85$ (12)	$25{\cdot}1 \pm 0{\cdot}96$ (12)	0	$21{\cdot}3 \pm 0{\cdot}29$ (5)	$6{\cdot}95 \pm 0{\cdot}32$ (6)	67·4
Preincubated 15 min with GAH	$17{\cdot}6 \pm 0{\cdot}38$ (6)	$19{\cdot}0 \pm 0{\cdot}62$ (6)	0			
Preincubated 30 min with GAH	$17{\cdot}7 \pm 0{\cdot}81$ (6)	$19{\cdot}0 \pm 0{\cdot}49$ (6)	0			
βAA	$2{\cdot}25 \pm 0{\cdot}28$ (15)	$0{\cdot}89 \pm 0{\cdot}04$ (15)	60·6	$1{\cdot}94 \pm 0{\cdot}14$ (12)	$0{\cdot}78 \pm 0{\cdot}05$ (11)	60·0
ASA	$20{\cdot}2 \pm 0{\cdot}78$ (11)	$23{\cdot}8 \pm 1{\cdot}12$ (11)	0			

* The values are micromoles of glutamic acid produced in 1-hr incubation at pH 7·6; mean ± S.E.M. Number of determinations in parentheses.

Statistical significance according to *t* test

AAA	*P*	βAA	*P*
21·3 − 6·95	<0·001	2·25 − 0·89	<0·001
		1·94 − 0·78	<0·001

Experiments with U-^{14}C-*glucose*

Brain. In brain, 92 per cent of the total radioactivity found in free amino acids was detected in aspartic acid, glutamic acid, glutamine, and GABA, both in treated and in control mice. In the brain of animals treated with a single dose of GAH 6·5 hr before the administration of labeled glucose, the specific activity (counts/min/μmole amino acid/100 mg wet tissue) of glutamic acid, aspartic acid, and glutamine reached an apparent maximum 30 min after the injection of U-^{14}C-glucose; in control animals the maximum was reached 60 min after the injection. Conversely, GABA and alanine incorporated initially less radioactivity in GAH-treated than in control mice. In the case of the decay of the acquired radioactivity, GABA and alanine specific activity was practically the same in the 1·5-hr experimental period in GAH-treated animals, whereas that of the other three amino acids diminished. In control animals the rate of decay of specific activity was almost equal in aspartic and glutamic acids, glutamine, and GABA; in alanine, which showed initially a very high specific activity, an extremely rapid rate of decay was observed (Fig. 1).

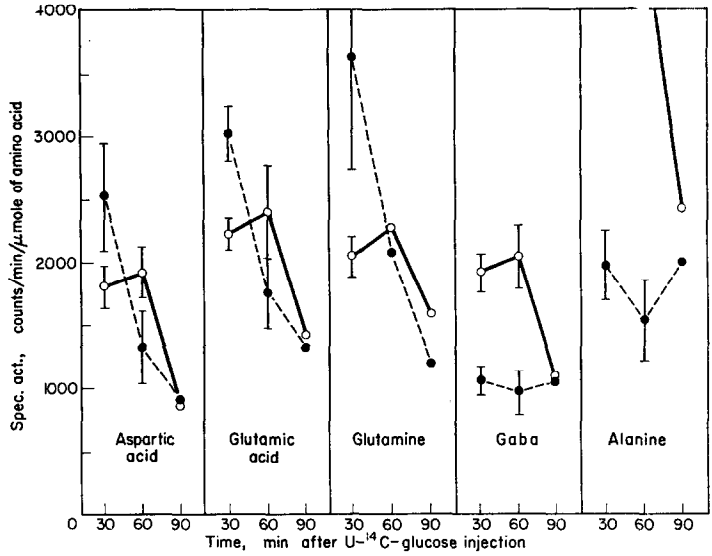

FIG. 1. Incorporation of radioactivity into amino acids 30, 60, and 90 min after the injection of U-^{14}C-glucose in brain of control mice and of mice previously treated with GAH. Each point represents mean ± S.E.M.; the standard error is not indicated when that corresponding to control mice overlapped that of GAH-treated mice.

○—○, Control mice (alanine values at 30 and 60 min were 6090 ± 887 and 4740 ± 950 respectively);

●—●, mice treated with GAH (160 mg/kg) 6·5 hr before the administration of radioactive glucose.

The percentage of acquired radioactivity in GABA and alanine was higher, and that of glutamine lower after U-^{14}C-glucose injection, in the GAH-treated than in control animals at the 30-, 60-, and 90-min periods. This percentage in control mice was constant in glutamic acid, GABA, and alanine throughout the experimental period, whereas in GAH-treated mice that in glutamic acid decreased and that in GABA and alanine increased; the radioactivity percentage in aspartic acid and glutamine showed a constant level throughout the experimental period both in control

TABLE 6. PERCENTAGE OF ACQUIRED RADIOACTIVITY OF AMINO ACIDS AFTER U-^{14}C-GLUCOSE ADMINISTRATION IN BRAIN OF CONTROL MICE AND OF MICE TREATED WITH GAH (160 mg/kg)*

Group	Time after glucose injection (min)	Aspartic acid	Glutamic acid	Glutamine	Alanine	GABA
Control	30	14·1 ± 1·06 (5)	52·5 ± 1·83 (5)	17·2 ± 0·65 (4)	1·84 ± 0·27 (5)	8·72 ± 0·29 (5)
	60	12·8 ± 0·66 (4)	53·4 ± 1·80 (4)	17·3 ± 0·73 (4)	1·55 ± 0·41 (4)	8·74 ± 1·37 (4)
	90	10·6 ± 1·43 (4)	53·6 ± 2·25 (4)	20·4 ± 1·83 (4)	1·48 ± 0·22 (4)	7·94 ± 0·91 (4)
Treated	30	13·8 ± 0·79 (3)	56·8 ± 1·20 (3)	10·4 ± 1·04 (3)	3·18 ± 0·85 (3)	14·4 ± 1·48 (3)
	60	10·7 ± 0·88 (4)	42·7 ± 2·42 (4)	10·5 ± 1·29 (4)	4·07 ± 0·39 (4)	21·5 ± 1·89 (4)
	90	11·3 ± 1·14 (4)	38·0 ± 4·82 (4)	9·17 ± 2·68 (3)	5·47 ± 1·17 (4)	25·5 ± 0·71 (3)

* Percentages refer to total radioactivity in free amino acids. Mean ± S.E.M. Number of animals in parentheses. GAH was injected 6·5 hr before the administration of radioactive glucose.

TABLE 7. PERCENTAGE OF ACQUIRED RADIOACTIVITY OF AMINO ACIDS AFTER U-^{14}C-GLUCOSE ADMINISTRATION IN BRAIN OF CONTROL MICE AND OF MICE TREATED WITH GAH (160 mg/kg)*

Group	Time after glucose injection (min)	Aspartic acid	Glutamic acid	Glutamine	Alanine	GABA
Control	30	11·3 ± 0·53 (3)	54·3 ± 0·64 (3)	19·2 ± 1·58 (3)	1·84 ± 0·30 (3)	8·28 ± 0·55 (3)
	60	10·4† 14·0	52·0 ± 1·58 (3)	22·2† 21·0	0·93 ± 0·08 (3)	8·21 ± 0·22 (3)
	90	11·1 ± 0·89 (3)	45·8 ± 2·56 (3)	23·5 ± 1·54 (3)	1·25 ± 0·28 (3)	8·88 ± 0·86 (3)
Treated	30	13·1 ± 2·59 (3)	49·7 ± 3·35 (3)	14·8 ± 1·15 (3)	3·57 ± 0·35 (3)	13·6 ± 1·40 (3)
	60	11·7 ± 1·42 (3)	42·9† 41·5	20·6† 22·8	3·00† 3·81	18·4 ± 1·61 (3)
	90	7·80 ± 0·57 (3)	42·6 ± 0·99 (3)	24·3 ± 1·30 (3)	2·47 ± 0·18 (3)	17·8 ± 1·28 (3)

* Percentages refer to total radioactivity in free amino acids. Mean ± S.E.M. Number of animals in parentheses. GAH was injected 24 hr before the administration of radioactive glucose.
† Values of the only two data obtained.

and treated animals; glutamine percentage, however, was lower in treated than in control mice (Table 6).

When GAH was injected 24 hr before U-^{14}C-glucose, essentially the same percentage differences in acquired radioacitvity described for the 6·5-hr trial was found between treated and control animals with regard to GABA. The differences in radioactivity percentage between control and treated animals in glutamic acid, glutamine, and alanine were less than those found in the 6·5-hr experiments. Aspartic acid percentage showed a diminished value in the treated mice relative to control mice at the 60-min period, which was not observed in the 6·5-hr experiments (Table 7).

Liver. In liver, the specific activity (counts/min/μmole amino acid/100 mg wet tissue) of glutamic acid, aspartic acid, glutamine, and alanine tended to be higher in control than in treated mice (GAH injected 6·5 hr before U-^{14}C-glucose) in the first 30 min. The decay of radioactivity in the first three amino acids was approximately the same in control and treated animals, whereas in alanine a slower decay was observed in GAH-treated mice in the last 30 min (Fig. 2).

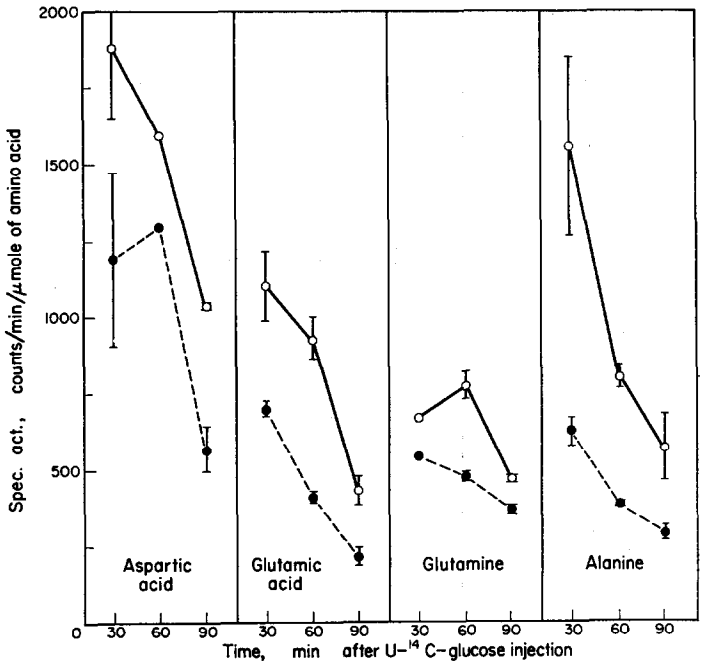

FIG. 2. Incorporation of radioactivity into amino acids 30, 60, and 90 min after the injection of U-^{14}C-glucose in liver of control mice and of mice previously treated with GAH. Each point represents mean ± S.E.M.; the standard error is not indicated when that corresponding to control mice overlapped that of GAH-treated mice.

○—○, Control mice; ●—●, mice treated with GAH (160 mg/kg) 6·5 hr before the administration of radioactive glucose.

DISCUSSION

Of the changes observed *in vivo* in the livers of GAH-treated mice only the increases of alanine and β-alanine could be explained as a consequence of the blockade of their respective aminotransferases. In this regard it is of interest that hydroxylamine, an inhibitor of some PALP-dependent enzymes, also induces an increase in

liver β-alanine concentration.[12] However, an interesting problem arises on the correlation of effects *in vivo* and *in vitro*, since GAH did not affect AAA activity *in vitro* whereas *in vivo* an important inhibition was observed.

It is difficult to explain the increases of the other amino acids induced by GAH in liver. Similar increases in glutamine[13] and glutamic and aspartic acids[14] have been found in animals fed with a vitamin B_6-deficient diet. Thus it is possible that GAH *in vivo* induces a general blockade of the catabolic pathways of almost all the free amino acids, which can be attributed to a quantitatively important inhibition of several PALP-dependent enzymes.

In brain, the inhibition *in vivo* of the aminotransferases of GABA, alanine, and β-alanine induced by GAH could explain the increases of the first two amino acids observed previously in GAH-treated mice.[1] As in the case of liver, AAA activity was not inhibited *in vitro*, whereas ABAT and βAA activities were inhibited almost to the same extent *in vivo* and *in vitro*.

It is of interest that the ASA activity was not affected by GAH in either of the experimental conditions used in this work, in spite of its being a PALP-dependent enzyme. This fact can be explained by postulating that PALP is bound to the ASA protein more firmly than to the aminotransferases inhibited by GAH, such as ABAT or βAA. However, data in this regard are still lacking.

The discrepancy between the effects *in vivo* and *in vitro* of GAH on AAA activity deserves further comment. Assumed that GAH is equally distributed in all organs, its maximal theoretical concentration in brain and liver after a dose of 160 mg/kg would be approximately 200-fold less than the concentration used *in vitro*. This fact seems to indicate that the difference of action *in vivo* and *in vitro* on such aminotransferase was not due to differences in concentration of the inhibitor. Whatever the explanation may be, since GAH inhibited βAA activity to the same extent *in vivo* and *in vitro* in the same experimental conditions, it is possible to suggest that there are some factors involved in the inhibition of some enzymes *in vivo* which are not apparent *in vitro*, and that such factors apparently do not affect equally all the enzymes of a given tissue. It is noteworthy that a situation similar to that described for AAA has been observed by Cedrangolo,[15] who used isonicotinoylhydrazide and cycloserine as inhibitors of AAA and ASA activities.

Another interesting discrepancy is the fact that GAD activity in brain was considerably inhibited by GAH *in vitro*, while it was much less affected *in vivo*. On the other hand, ABAT activity was equally inhibited in both conditions. According to certain reports,[12] hydroxylamine and aminooxyacetic acid are similar to GAH with respect to their effects on GAD and ABAT activities. The results obtained with the former two drugs are compatible with the hypothesis of a compartmentalization of these enzymes in brain tissue,[16] which would permit these drugs to reach ABAT, but not GAD, *in vivo*. The inhibitory effect of GAH plus PALP treatment on GAD activity *in vivo*[17] may be accounted for by the assumption of a participation of PALP in such a way that GAH reaches the site of GAD. This interpretation agrees with the compartmentalization hypothesis. However, recent work in our laboratory suggests that GAH combines with PALP to form the corresponding PALP-hydrazone *in vivo*, which apparently is indirectly responsible for the GAD inhibition.* Thus, the mechanism of the inhibition of GAD activity by GAH *in vivo* is apparently different from

* Unpublished results.

that *in vitro*. In the latter conditions GAH seems to act simply by trapping PALP and thus diminishing the coenzyme available to the apoenzyme, whereas *in vivo* the formation of PALP would be inhibited by the PALP-hydrazone in a form similar to the inhibition produced by some pyridoxal hydrazones.[18, 19]

Experiments with U-^{14}C-*glucose*

Both in liver and brain, the results obtained in the experiments on the incorporation of ^{14}C from labeled glucose into the amino acids in control mice in general agree with recent data obtained in similar experiments; if the data by Gaitonde *et al.* in brain[3] are recalculated with the radioactivity in free amino acids considered as 100 per cent (as is done in the present work), the values of each amino acid in percentage of acquired radioactivity at equivalent times are in excellent agreement in both papers.

Our experiments on the conversion of isotopic glucose into amino acids in GAH-treated mice lend support to the conclusions reached with the reported experiments on enzymatic activities. In brain, the inhibition of ABAT and AAA activities *in vivo* agrees with the results obtained in these experiments, which indicate a slower decay in the specific activity of GABA and alanine in GAH-treated animals relative to control animals. The percentage of acquired radioactivity in GABA and alanine also show the blockade of their metabolism, since in GAH-treated mice it increased from 30 to 90 min, whereas that in glutamic acid decreased. On the other hand, in control animals GABA, alanine, and glutamic and aspartic acid percentages were constant throughout the experimental period.

An interesting feature was that the initial specific activity (after 30 min) of aspartic acid, glutamic acid, and glutamine was higher in treated than in control mice, while that of GABA and alanine was higher in control than in GAH-treated animals. Apparently, the conversion of isotopic glucose to the first three amino acids was stimulated, and in the last two amino acids it was inhibited, by GAH. The inhibition *in vivo* of AAA activity could explain the great difference in the specific activity of alanine between treated and control mice at 30 and 60 min. In the case of GABA, since the only known route of synthesis is the decarboxylation of glutamic acid, a correlation between the differences in specific activities at 30 and 60 min is probably related to the inhibition of GAD activity 6·5 hr after GAH treatment.

In liver, the tendency to a slower decay of specific radioactivity of alanine in GAH-treated mice, in comparison to control animals, agrees with the inhibition of AAA activity *in vivo*. Further experiments along this line could throw some light on the mechanism of the effect of GAH on the levels of other hepatic free amino acids.

In these radioactivity experiments, the possibility exists that some of the radioactive compounds derived from U-^{14}C-glucose contained in the protein and lipid-free extracts used for the paper chromatographic analysis could contaminate the amino acids studied in the present work. However, according to the chromatographic experiments already mentioned, the contamination with lactate, succinate, pyruvate and, α-ketoglutarate did not occur. Furthermore, data on the chromatographic behavior of other intermediates of the Krebs cycle[20] indicate that these compounds do not overlap with the amino acids studied in this work. However, we consider that our data are tentative and that they must be confirmed by more accurate techniques.

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