Some Metabolic Studies of γ-Aminobutyric Acid.* (23883)

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Relatively large quantities of γ-aminobutyric acid (GABA) have been detected by us in an easily extractable form in the central nervous system of representative members of fish, reptilian, avian, marsupial, and mammalian species, but not in other tissues. GABA was the only extractable ninhydrin-reactive material which showed progressive increases at successive developmental stages in extracts of brains of chick embryo, mouse, salamander, and frog. Both GABA and L-glutamic decarboxylase, the enzyme which catalyzes formation of GABA from L-glutamic acid, were present in uniquely high concentrations in gray matter of the central nervous system. GABA transaminase, an enzyme found in brain as well as other tissues, catalyzes transamination of GABA with α-ketoglutarate (αKG). There exists the possibility that in brain a shunt operates around the αKG oxidase system of tricarboxylic acid cycle by which αKG could be withdrawn through formation of glutamic acid by transamination with GABA, and the carbon chain of GABA could enter the cycle at the succinate level (see reference 1 for pertinent literature). The results of present experiments indicate that the above formulation, based on enzymatic studies, is actually consistent with reactions which occur *in vivo*.

Methods. Fresh specimens were fixed in 75% alcohol immediately after dissection and rapid weighing on torsion balance. Extracts were prepared by homogenizing the tissue in alcohol with ground-glass, motor driven homogenizer, clarifying by centrifugation, and evaporating supernatant fluid in 20 ml beakers under infra-red lamp in air stream of a fan. Each residue was taken up in saturated aqueous solution of picric acid (100 μl/100 mg of original fresh weight of tissue) and the suspension centrifuged. Usually a total of 75 μl of supernatant was placed in 3 applications of 25 μl each onto 18″ x 22″ sheets of Whatman No. 1 filter paper, although occasionally 20 μl of supernatant was placed on 9″ x 11″ sheets. Descending 2-dimensional chromatography was performed in water-saturated phenol followed by water-saturated lutidine. The picric acid migrated with solvent front in lutidine and did not interfere with subsequent detection of ninhydrin-reactive constituents, visualized by spraying with 0.1% ninhydrin in butanol. In some instances quantitative estimations of amounts of amino acids were made by eluting the spots with water from chromatograms and immediately reading the colors in Beckman DU spectrophotometer at 570 mμ. Standard curves were prepared from suitable mixtures of amino acids chromatographed in the same manner and at same time. Radioautographs of some of chromatograms were prepared by placing unsprayed sheets in contact with Type K Industrial X-ray film for suitable time. Quantitative measurements of radioactivity of eluates of areas corresponding to some amino acids were made by standard technics in a gas-flow counter. Other procedures will be described in sections dealing specifically with their use.

Results. Conversion of carbon chain of L-glutamic acid to GABA in brain of intact mouse; inhibition by thiosemicarbazide. Previous work has shown that the carbon chain of uniformly C^{14}-labeled glutamic acid can be converted to carbon chain of GABA *in vitro* by mouse brain preparation and studies were made of properties of L-glutamic acid decarboxylase, the enzyme which forms GABA from glutamic acid(1). The following experiments show that glutamic acid is, indeed, a major precursor of GABA in the intact brain of living animal.

Approximately 35 γ of L-glutamic acid-U-C^{14} (11.3 mc/mM; Nuclear Inst. and Chem. Corp.) contained in 0.03 ml of water was in-

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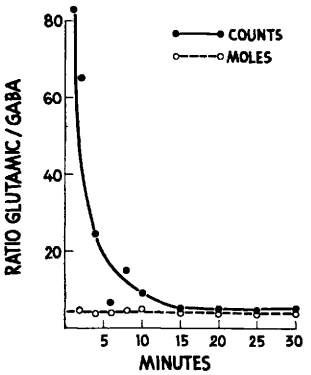

FIG. 1. Glutamic acid/GABA ratios in extracts of brains of mice at various times after intracer. inj. of L-glutamic acid-U-C^{14}. The 30 min. value is the avg for 5 animals, all other points are for individual animals.

jected intracerebrally into each of 9 mice of Webster-Swiss strain weighing 15 to 17.5 g. The mice were sacrificed by cervical dislocation at 1, 2, 4, 6, 8, 10, 15, 20, and 25 minutes after injection and the brains analyzed in usual manner. The results were expressed in ratios of radioactivity found in glutamic acid to that observed in GABA. The ratios of radioactivities decreased from initial value higher than 80 to that of approximately 4 within 15 minutes after injection (Fig. 1). The molar ratios of glutamic acid to GABA also were approximately 4. Therefore, specific activities (counts/min/μM) of GABA and glutamic acid were essentially the same after 15 minutes. These results show that the carbon chain of GABA can be formed from the carbon chain of L-glutamic acid in brain *in vivo*.

Chromatograms and radioautographs of extracts of the 4 and 25 minute samples are shown in Fig. 2 and 3, 4 and 5, respectively. All compounds detected at later times on these radioautographs were radioactive even in the one minute sample, indicating an extremely rapid entry of glutamic acid carbon into metabolic pathways. A very high level of radioactivity was found in glutamine at all times. Increase in radioactivity of GABA with time is seen by inspection of photographs of the radioautographs. An experiment was then performed in which 5 mice received 0.4 mg of thiosemicarbazide intraperitoneally 15 minutes prior to intracerebral administration of 0.03 ml of C^{14}-labeled glutamic acid as described previously. The animals were killed and the brains removed for analysis 30 minutes after glutamic acid was given. No convulsions occurred during the study. A group of control mice received only glutamic acid 30 minutes prior to removal of brains. The results in Table I show that the amount of

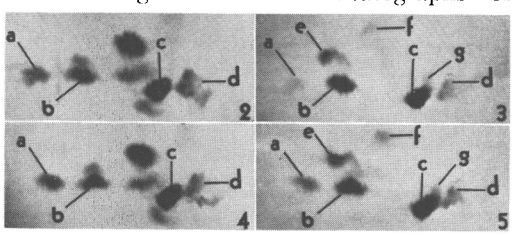

FIG. 2-5. Chromatographs and radioautographs of extracts of mouse brain after intracer. inj. of L-glutamic-U-C^{14}. Fig. 2 and 4 are the chromatograms prepared at 4 and 25 min., respectively, and Fig. 3 and 5 are the corresponding radioautographs. a, GABA; b, glutamine; c, glutamic acid; d, aspartic acid; e, probably pyrrolidonecarboxylic acid (formed from glutamine); f, unknown; g, succinic acid.

TABLE I. Glutamic Acid/GABA Ratios in Extracts of Mouse Brains 30 Minutes after Intracerebral Injection of L-glutamic Acid-U-C^{14}.

Treatment	Glutamic acid/GABA	
	Molar	Radioactivity
Controls	3.9	4.7
	4.6	4.0
	3.0	3.4
		4.2
	2.5	4.0
Avg	3.5	4.1
Thiosemicarbazide*	4.0	7.1
	3.4	15.9
	3.5	7.6
	4.2	11.2
	3.7	13.1
Avg	3.8	11.0

* 0.4 mg/mouse given intraper. 15 min. prior to intracer. inj. of glutamic acid.

glutamic acid relative to GABA was not increased significantly by thiosemicarbazide. However, a highly significant increase in ratios of radioactivities of these 2 amino acids was noted, showing decreased rate of flow of isotope from glutamic acid into GABA. These results confirmed, by measurements in intact animal, the results obtained by Killam(2,3) who showed that convulsant hydrazides inhibited glutamic acid decarboxylase activity in brain, and inferred that the convulsant ac-

TABLE II. Radioactivity of Succinic and α-Ketoglutaric Acids in Urine of Rats after Administration of γ-Aminobutyric Acid-1-C^{14}.

Exp. No.	Radioactive compound administered	Dose*	Amt of catabolite administered (mg)	Catabolite isolated	Specific activity of isolated catabolite†
1	Acetate-1-C^{14}	1.3×10^7	400	α-Ketoglutarate‡	180
			"	Succinate§	46
2	γ-Aminobutyric acid-1-C^{14}	9.8×10^6	"	α-Ketoglutarate	53
			"	Succinate	240

* Disintegrations/min. † Disintegrations/min./mMole $\times 10^{-3}$. ‡ Isolated as the 2,4-dinitrophenylhydrazone. § Isolated as succinic acid.

tion of these drugs was related to this effect.

Conversion of the carbon chain of GABA to succinic acid in the intact rat. One and two-tenths mg of GABA-1-C^{14} (Tracerlab) and 400 mg of succinate (neutralized with NaOH) in 5 ml of H_2O were injected intraperitoneally into a female Sprague-Dawley rat (250 g) fasted previously for 24 hours. The urine was collected 18 hours, made alkaline, and extracted continuously for 10 hours with ether. The aqueous solution was acidified and then extracted for another 12 hours with fresh ether. The extract was dried over sodium sulfate, filtered, and evaporated to dryness. The residue was recrystallized from ether-benzene mixture, dissolved in water, treated with Norit, filtered, evaporated, and dried. In this manner 73 mg of white crystals were obtained and sublimed at 100° at 1 mm pressure to obtain 69 mg of white solid succinic acid. To this was then added 69 mg of inert succinic acid and the mixture recrystallized 3 times from ether-benzene mixture to constant specific activity. In a similar experiment the same amount of GABA-1-C^{14} was injected simultaneously with 400 mg of αKG into another rat. Urines were collected, treated with excess of 2-4 • dinitrophenylhydrazine in 2N HCl, the precipitate collected and recrystallized repeatedly from 10% ethanol and ethyl acetate-chloroform mixture to constant specific activity. Similar experiments were performed in other rats using acetate-1-C^{14} and cold succinate or αKG.

The results are shown in Table II. Injection of labeled acetate together with either succinate or αKG gave a 4-fold higher specific activity in αKG than in the succinate isolated. This result is in keeping with the known facts of tricarboxylic acid cycle, the carbon from acetate reaching succinate only after passing through αKG. On the other hand, when GABA-1-C^{14} was administered with succinate or αKG the specific activity of the isolated succinate was approximately 4.5 times greater than that of the αKG. The latter result indicates that the carbon of GABA probably enters the tricarboxylic acid cycle at the succinate level. That the conversion to succinate represents a pathway of metabolism of GABA in the intact animal is indicated by the fact that as much as 2% of the radioactivity of GABA administered was recovered in the succinate isolated. The latter results are consistent with the findings on metabolism of the closely related substances, β-alanine (4) and δ-aminovaleric acid(5), and suggest that transamination and subsequent oxidation of the carbon chain are major metabolic reactions of GABA *in vivo*. The isotope-trapping technic described above has been used previously for study of lysine metabolism(6).

Metabolism of γ-aminobutyric acid-1-C^{14} after intracerebral injection in mice. Seven male Swiss mice weighing approximately 25 g were injected intracerebrally with 0.03 ml aqueous solution containing approximately 560,000 counts of GABA-1-C^{14} (250 γ). The mice were killed by cervical dislocation at different intervals after injection, the brains removed immediately, and prepared for analysis. Total activity recovered in alcoholic extracts is shown in Table III. The highest recovery of radioactivity, 44% of that administered, was achieved in the mouse killed at 11.5 minutes after injection. The proportion of radioactivity recovered decreased at

TABLE III. Radioactivity in Extracts of Mouse Brain and in GABA at Various Times after Intracerebral Administration of GABA-1-C^{14}.*

Min. after inj.	Total counts in extract (counts/min.)	Counts in GABA (counts/min.)	% of total counts of extract in GABA
	× 1000	× 1000	
2	188.8	183.0	97
5	146.4	117.0	80
11.5	244.0	211.0	87
27	80.4	33.0	41
61	18.4	6.0	32
120	10.8		
240	3.5		

* Each mouse received an intracer. inj. of 0.03 ml of solution containing 560,000 counts/min. of GABA-1-C^{14}. Radioactivity was measured in a gas-flow counter.

rapid rate, only 3% of injected amount being detected after 1 hour. After preparation of 2-dimensional chromatograms and development of radioautographs of these chromatograms, the area containing GABA was cut out, eluted, and a suitable aliquot counted. The percentage of total activity found in GABA decreased after 11.5 minutes. Even 2 minutes after injection, radioactivity was detectable in aspartic and succinic acid areas and in glutamine. At 27 minutes the glutamine showed greatest radioactivity of constituents other than GABA. Radioactivity of all observable constituents decreased in brains obtained one and 2 hours after injection, glutamine and GABA remaining the most highly radioactive materials in the extracts.

Metabolism of γ-aminobutyric acid-1-C^{14} during incubation with brain minces. Confirmation of above results was obtained in experiments with brain minces. Two whole mouse brains were minced into 3 ml of glucose-containing bicarbonate-saline(7) in each of four 20 ml beakers. To each beaker was added 0.02 ml of GABA-1-C^{14} (390,000 counts), and incubated at 30° in 95% oxygen and 5% carbon dioxide. At 15, 30, 60, and 90 minutes, respectively, the contents of individual flasks were poured into 90 ml of boiling 95% ethyl alcohol contained in ground glass homogenizer and the samples homogenized and worked up for analysis in usual manner. After chromatography and radioautography the spots corresponding to aspartic, glutamic, and succinic acids and GABA and glutamine were eluted and counted. The results are shown in Table IV. The major radioactive areas on the radioautographs were attributable to GABA, aspartate, glutamate and glutamine at all time intervals. With the exception of GABA, the largest amount of radioactivity was found in the aspartate. Finding of the label in succinate and amino acids which can arise from the keto-acids of tricarboxylic acid cycle is consistent with experiments on the intact animal. Since completion of above experiments, similar findings have been made with guinea pig brain slices and rat brain mitochondria(8).

Correlation of biochemical and physiological findings. Although the chief pathways of formation of GABA in nervous tissue and its utilization were known(1), there were no clues from the biochemical information regarding the possible function and importance in the central nervous system of the uniquely high concentrations of GABA and of the enzyme which forms it from glutamic acid. An extensive series of studies by Florey (see 9 for references) on a factor isolated from beef brain which has potent physiological activity

TABLE IV. Distribution of Radioactivity at Various Times after Incubation of Mouse Brain Minces with GABA-1-C^{14}.

Min. of incubation	Total counts in alcoholic extract (counts/min.)	Total counts in constituents of picric acid filtrates* (counts/min.)				
		GABA	Aspartic acid	Glutamic acid	Glutamine	Succinic acid
15	389,600	374,000	5,770	1,425	483	544
30	376,600	311,000	7,840	1,140	149	1,605
60	289,600	229,500	7,550	2,595	4,170	1,180
90	243,800	197,500	10,470	1,960	6,080	763

* The substances were visualized by radioautography of 2 dimensional chromatograms of the picric acid filtrates, eluted, and suitable aliquots were counted. Amount of radioactivity in the picric acid precipitate was not determined.

in a number of test systems has culminated recently in the identification of this factor as GABA(10), and a number of lines of evidence have been adduced in favor of assigning to it the role of a transmitter substance of inhibitory neurons in decapod crustaceans (9). GABA appears to act by increasing the permeability of the synaptic membrane of the crayfish stretch receptor to chloride and potassium, but not to sodium, a mode of action also ascribable to the inhibitory axon which forms synapses with the dendrites of the receptor(11,12). It is not known whether the pharmacological effects of GABA are attributable to the interaction of GABA itself with receptor sites or whether some metabolite which is formed rapidly from GABA is involved. It was reported recently that γ-guanidobutyric acid is found in mammalian brain(13) and that it can be synthesized in kidney from GABA and an amidine donor (14), but GABA has proven to be much more active in the crustacean test system than the guanido derivative(11,12). Although γ-butyrobetaine could conceivably be formed from GABA, neither betaine(15) nor γ-butyrobetaine (Florey, personal communication) affected the stretch receptor preparation, nor was it possible to show the presence of γ-butyrobetaine or its esters in brain extracts (unpublished, this laboratory) by a combination of isolation and paper chromatographic procedures(16). Succinic semialdehyde, formed on transamination of GABA with αKG(17), is oxidized readily by brain preparations(8), but its effectiveness in pharmacological test systems has not been reported.

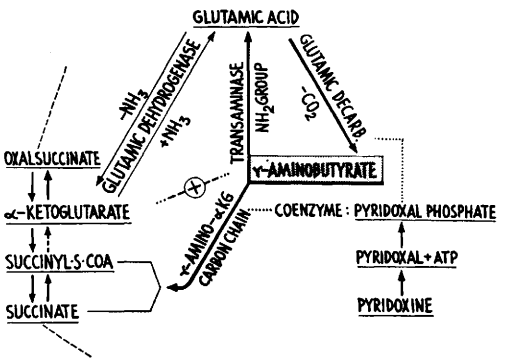

FIG. 6. Known metabolic relationships of GABA.

Since GABA may play an important role in regulating activity in the central nervous system, it is important to consider the various factors which might be involved in maintenance of given, relatively constant amounts of this substance. Widely differing levels of GABA have been found in various areas of the brain. The coordination of the metabolic sequences shown in Fig. 6 requires the proper functioning of several enzyme systems which could be affected by genetic or environmental factors.

Both glutamic acid decarboxylase(1) and GABA transaminase(18) require for activity pyridoxal phosphate, which can be synthesized in brain from ATP and pyridoxal(1). Work to date indicates that the apoenzyme of glutamic decarboxylase has a lower affinity for the coenzyme than that of GABA transaminase(18). Observations in many laboratories have established a relationship of convulsive seizures to pyridoxine deficiency in experimental animals and in human beings. Dietary pyridoxine deficiency of insufficient severity to produce seizures in rats resulted in a marked but readily reversible decrease in the activity of glutamic decarboxylase(1). Convulsant hydrazides, compounds which react with the coenzyme, have been shown to produce decreases in the activity of glutamic decarboxylase and the content of GABA in the brains of rats(2) and in the present study to decrease the rate of formation of GABA from glutamic acid *in vivo* in mice. A good correlation was obtained between electrically detected seizure activity and decreases in glutamic decarboxylase activity in affected areas in brains of cats(3). Topical application of GABA to the cortex has reversed seizures in cats induced by parenteral administration of hydrazides,‡ while the placing of solutions of GABA on the cortex of normal animals has been shown to have a depressant action(19-21). Convulsions in mice produced by the injection of methoxypyridoxine could be prevented completely by the injection of small amounts of pyridoxine or large amounts

‡ Dasgupta, S. R., Killam, E. K., Killam, K. F., presented before the Am. Soc. for Pharm. and Exp. Therap., Baltimore, Sept. 6, 1957.

of GABA (Gumnit and Gammon, personal communication). All of the above results fit the tentative hypothesis that seizure activity in pyridoxine deficiency and hydrazide poisoning results from the decrease of activity of the glutamic acid decarboxylase with a consequent decrease in ability to produce GABA. Studies have not yet been made of the relative degree of inhibition of glutamic decarboxylase and GABA transaminase by pyridoxine deficiency or by the convulsant hydrazides. However, the presently available results would suggest that glutamic decarboxylase is preferentially inhibited whenever pyridoxal phosphate becomes a limiting factor.

Picrotoxin was found to antagonize the inhibitory action of GABA on both the crayfish stretch receptor and heart(9). It is interesting that smaller amounts of both semicarbazide(22) and picrotoxin(23) were required to produce seizures in monkeys with experimentally induced epilepsy than in controls. GABA and acetylcholine have been found to be antagonistic to each other in a number of test systems(9). It appears possible that the glutamic decarboxylase-GABA-GABA transaminase system might be a companion system to the choline acetylase-acetylcholine-cholinesterase system in regulation of activity in the central nervous system (see 24 for discussion of role of acetylcholine in seizures). The balance between these systems, rather than the absolute amount of any single substance or enzymatic activity, might be important in the regulation of activity in a given region of the central nervous system and disturbances in this balance might be involved in at least some cases of clinical epilepsy. It is possible to begin the analysis of the action of a number of pharmacologically active materials in terms of imbalances produced by their action on specific enzymes in the GABA and acetylcholine sequences.

The pH activity relationships of cerebral glutamic decarboxylase(1) and GABA transaminase§ are shown in Fig. 7. The results for each of the enzymes are plotted as % of maximal activity under optimal conditions of assay. Glutamic decarboxylase possesses maximal activity at pH 6.5, GABA transaminase at 8.2. The two curves intersect (possess same percentage of maximal potential activity) at pH 7.5, which might be assumed to be close to intracellular pH of brain. It is apparent that small shifts in pH in the physiological range could result in large changes in relative activity of the 2 enzymes and so might alter the content of GABA at the loci where its physiological effects are exerted. *Intracellular* acidosis would tend to increase the content of GABA and alkalosis to decrease it. *Metabolic* acidosis has been generally found to decrease the incidence of epileptic seizures and some types of experimental seizures and alkalosis to increase them (see 25 for pertinent references).

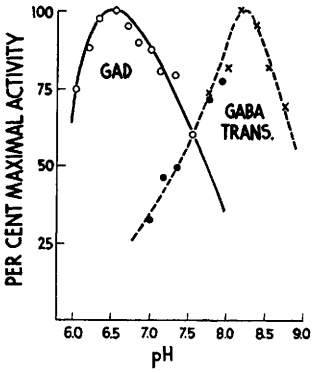

FIG. 7. pH activity relationships of cerebral glutamic decarboxylase and GABA transaminase. ○——○ and ●- - -●, phosphate buffer; ×--×, borate buffer.

Summary. In agreement with previously reported enzymatic studies, experiments with intracerebrally administered L-glutamic-U-C^{14} in intact mice showed that carbon chain of L-glutamic acid is a source of the carbon chain of GABA formed in brain and that thiosemicarbazide decreases the rate of conversion of cerebral glutamic acid to GABA. It was shown that carbon of GABA can enter the tricarboxylic acid cycle at the succinate level in intact rats in experiments with intraperitoneally administered GABA-1-C^{14} employing the isotope trapping technic. Experiments in which GABA-1-C^{14} was injected intracerebrally to mice, or incubated with minces of mouse brain, indicated that utiliza-

§ The details of properties of GABA transaminase are being reported elsewhere.

tion of GABA in brain probably takes place by the same mechanism as in the whole animal. The results of present and past biochemical studies can be correlated with relevant physiological data.

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Hemadsorption (Adsorption-Hemagglutination) Test for Viral Agents in Tissue Culture with Special Reference to Influenza. (23884)

ALEXIS SHELOKOV,† JOHN E. VOGEL† AND LOTTA CHI (Introduced by D. J. Davis)
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In a recent publication(1) we described "adsorption-hemagglutination" which occurs when appropriate erythrocytes are added directly to monkey kidney cell cultures infected with influenza A virus. The present report indicates some practical applications of this reaction, now designated as *hemadsorption,* in the diagnosis of influenza, mumps, and CA (croup-associated) virus infections. In addition, its usefulness with other viruses was investigated using prototype strains in tissue culture systems.

Materials. Several types of trypsinized monolayer cell cultures propagated in usual growth media were used. Most experiments employed monkey kidney (MK) cells prepared by modification of standard methods (2); chick embryo and monkey testicular cells were similarly processed; amnion cultures were made according to procedure developed in this laboratory(3); HeLa and bovine kidney cells were purchased from Microbiological Associates. Tissue culture (TC) tubes

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