Biochemical Pharmacology, 1963, Vol. 12, pp. 489–502. Pergamon Press Ltd., Printed in Great Britain.

BINDING OF γ-AMINOBUTYRIC ACID BY MOUSE BRAIN PREPARATIONS*

KAORU SANO† and EUGENE ROBERTS

Department of Biochemistry, City of Hope Medical Center, Duarte, Calif., U.S.A.

(*Received* 26 *November* 1962; *accepted* 8 *January* 1963)

Abstract—Procedures were described for the study of the binding of γ-aminobutyric acid (γABA)-1-^{14}C, which takes place with various brain preparations but not in similar preparations of several other tissues tested. All the binding activity, which does not require energy and is nonenzymatic, was found in the sedimentable fractions of sucrose homogenates of mouse brain, largely in the "mitochondrial" and "microsomal" fractions. The pH optimum for binding was found to be approximately between 7·3 and 7·5. γABA binding by the preparation was shown to be Na^+-dependent. There is a considerable degree of specificity in the structural requirements for γABA binding, and even minor variations in structure give substances with less affinity than γABA for the binding substance of structures. Experiments with surface-active materials and with sulfhydryl reagents led to the suggestion that the binding capacity is associated with osmotically sensitive macromolecular structural elements, among the components of which may be proteins with reactive sulfhydryl groups. Two antihistamines, two phenothiazine derivatives, and imipramine and desmethylimipramine were found to inhibit γABA binding. Several other pharmacologically active substances which were tested were found to be without effect.

γ-AMINOBUTYRIC acid, a substance that has a unique occurrence in the vertebrate central nervous system (CNS) and which may play an important inhibitory role therein, is formed in the CNS to a large extent, if not entirely, from L-glutamic acid. Various aspects of the distribution of γABA and its metabolism have been studied.[1–3]

Some experimental evidence has appeared recently bearing on the uptake and "binding" of γABA in the CNS. γABA was taken up readily from solution by slices of cerebral cortex, but not by liver or kidney slices or diaphragm sectors from the rat;[4] and by slices of guinea pig cortex, but not by liver and kidney from the latter species.[5] The intracellular concentration of γABA absorbed from the medium in some instances was 30 to 40 times as high as that in the medium itself. There was a rapid oxidative metabolism of the absorbed γABA.[3, 6] Anaerobiosis or the omission of glucose from the medium markedly decreased the ability of the slices to take up or to retain γABA. An interesting finding was that several phenothiazine derivatives, β-dimethylaminoethyl-2-methyl benzhydryl ether, imipramine, and hydroxyzine caused a release of γABA and glutamic acid from slices into the incubation medium.[7] Homogenates prepared in various saline mixtures were shown to contain approximately 60% of the

* This investigation was supported in part by Grants B-1615 and B-2655 from the National Institute of Neurological Diseases and Blindness, and a grant from the National Association for Mental Health.

† Present address: Department of Pharmacology, Medical School, Osaka University, Osaka, Japan.

total γABA in a sedimentable form from which it could be liberated by heat.[8] Recent experiments in our laboratory have shown a binding mechanism for γABA in tissue of the CNS, but not in other tissues.[9] The results showed that the binding of γABA that takes place in brain preparations is nonenzymatic and does not require energy.

The aim of the present work was to delineate further the properties of the binding process of γABA in mouse brain and to attempt to characterize the factor or factors involved. Subsequent communications will deal with other attempts to determine whether or not the specific binding of γABA in the CNS is related to its physiological role.

METHODS

Preparation of binding fraction. Particles generally were prepared from whole brains of mice in the following manner. Immediately after decapitation of the animals and removal of the brains a 10% homogenate in cold 0·25 M sucrose was prepared with a ground-glass homogenizer and was subjected to two successive centrifugations at 1,500 × *g* for 10 min to remove nuclei and cellular debris. All procedures were carried out at 0 to 4°. The supernatant was removed carefully with a syringe with a long needle and was centrifuged at 15,000 × *g* for 15 min. The residue was used in subsequent binding studies as described below.

Assay systems

1. *Direct procedure.* The sediment was rehomogenized lightly after resuspension in a minimal amount of 0·25 M sucrose. An amount of the suspension equivalent to one mouse brain or a given fraction thereof was used in each experimental vessel. The standard incubation mixture contained 0·6 mM NaCl; 0·15 mM Tris buffer, pH 7·5; 3 μg γABA-1-^{14}C (approximately 2 mc/mM; Isotope Specialties Co.); and particle suspension and other additions as required to a total volume of 3·0 ml.

The incubations usually were performed at 0 to 4° with shaking for 60 min. After the incubations, the reaction mixtures were centrifuged at 15,000 × *g* for 15 min in 2-ml centrifuge tubes in the No. 40 head of the Spinco model L centrifuge. To aliquots of the supernatant or the resuspended residue were added 4 volumes of 95% ethanol which liberated the bound γABA immediately. The resulting mixture was centrifuged at low speed and 0·2 ml of the supernatant was used for the measurement of radioactivity. The sample was pipetted into a counting vial and 1 ml of 1 M hydroxide of Hyamine 10-X in methanol was added. Toluene (15 ml), containing 0·3% 2,5-diphenyloxazole and 0·01% 1,4-*bis*-2(5-phenyloxazolyl)-benzene in toluene, was added to each vial, and the vials were counted in a Packard Tri-Carb model 34 liquid scintillation counter for at least five periods of 10 min each.

The volume of the sediment usually was estimated from the residue weight (obtained by subtracting the weight of the empty centrifuge tube from the weight of the tube after pouring off the supernatant) and assuming a density of unity for both the sediment and the suspending medium. Occasionally the volume of the sediment was estimated by measuring accurately the volume of buffer necessary to bring the total volume in the tube back to the original volume after pouring off the supernatant. Conservatively, it was assumed that the average extraparticulate fluid content of the sediment was 90%, and suitable corrections were made in the calculation of the amount of γABA bound.

2. *Equilibrium dialysis.* Many experiments employing equilibrium dialysis were performed. Two-ml aliquots of particle suspension or other tissue preparation in suspending medium were placed in a dialysis sac with a small glass bead and were dialyzed at 0 to 4° for 4 to 6 hr with continuous rocking against 2 ml of the same medium containing 4 μg of γABA-1-^{14}C. Suspending medium without tissue preparation in the sac was employed in control tubes. The radioactivity in the external fluid was estimated as described above. Because the binding was studied under a variety of conditions, some of which interfered with the determinations of protein content, and because the chemical nature of the binding material was not known, the quantitative results on binding were expressed on the basis of the amount of the original particulate suspension employed.

RESULTS

1. *Optimal pH for binding γABA.* The effect of varying the pH of the NaCl–Tris suspending medium was studied by both the direct and dialysis methods. The actual measured pH of the suspension was plotted against the quantity of γABA bound (Fig. 1). Since the pH optimum was found to lie between 7·3 and 7·5 (a value close to

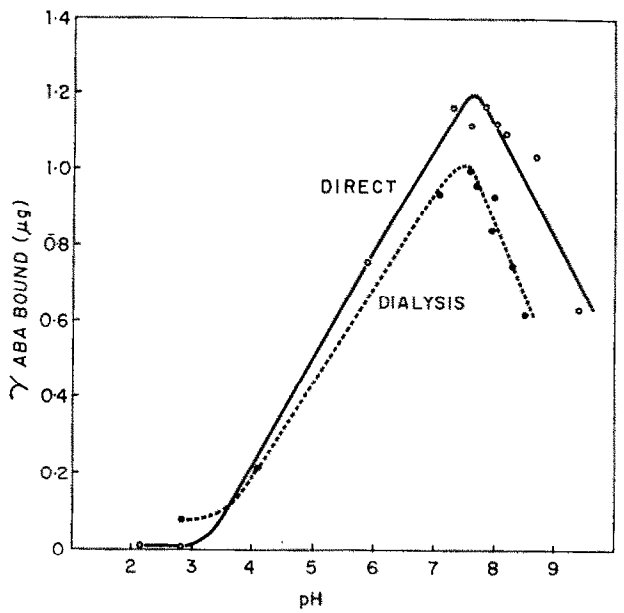


FIG. 1. Influence of pH on binding of γABA by mouse brain homogenates.

the isoelectric point of γABA, 7·3), both amino and carboxyl groups of γABA are approximately maximally charged at the pH that is optimal for binding. The pH optimum did not depend on the nature of the buffer used, since other experiments employing sodium phosphate buffers gave the same value for optimal pH of binding as that found with the suspending medium above.

2. *Rate of binding.* Portions of a particulate preparation of mouse brain were incubated for varying periods of time at 0 to 4° in the medium containing γABA-1-^{14}C prior to centrifugation at 15,000 × *g* for 15 min at the same temperature. An initial quick binding took place after exposure of the particles to the labeled γABA, and

subsequently there was a relatively small increment in uptake for the duration of the experiment, essentially maximal values being attained at 60 min, the period of incubation employed routinely in most of the present experiments (Fig. 2).

3. *Effect of preincubation of brain particles at different temperatures.* After it had been shown that the γABA-binding capacity was stable at low temperatures (0 to 4°)

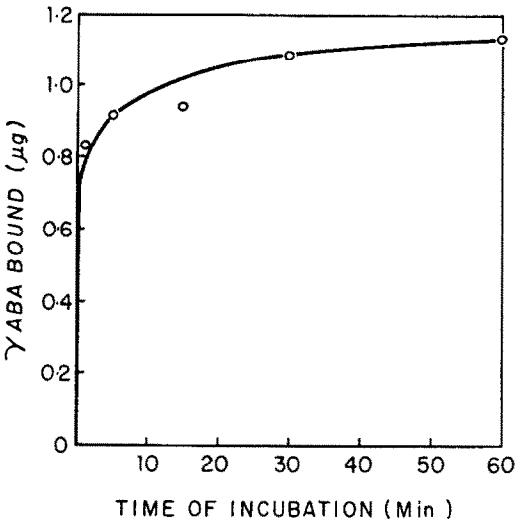


FIG. 2. Extent of binding of γABA as a function of time of incubation.

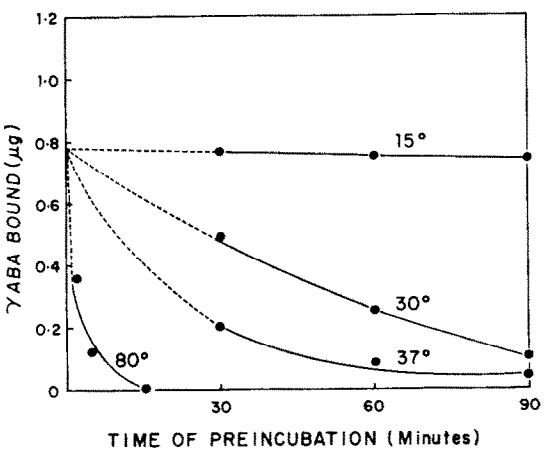


FIG. 3. Influence of time of preincubation at various temperatures on the extent of binding by mouse brain particulates.

for prolonged periods, portions of a resuspended preparation of mouse brain were allowed to stand at 15°, 30°, and 37° for 30, 60, and 90 min, respectively, and at 80° for 2·5, 5, and 15 min, and then were tested for γABA-binding capacity by equilibrium dialysis at 4° for 5·5 hr. When preincubation took place at 15° there was little loss of binding capacity, but at the higher temperatures progressive losses occurred (Fig. 3), suggesting that denaturation or destruction of the cell component responsible for the binding might be taking place.

4. *The Na^+ ion requirement for binding γABA.* A complete Ringer's solution had been used originally for demonstrating the binding of γABA.[9] Further experiments showed NaCl to be the only substance essential for the binding process. The requirement for Na^+ ions can be replaced by Ca^{2+} ions only to a slight extent and not at all by K^+, Li^+, NH_4^+, or Mg^{2+} ions or by sucrose (Table 1). Since binding in the presence of Na_2SO_4 was essentially the same as that observed when NaCl was employed, the anion does not seem to be important. Maximal binding was observed in several experiments when the concentration of Na^+ ion was approximately 0·2 M. There was a decrease in binding capacity, suggesting that the binding material is osmotically sensitive when distilled water instead of 0·25 M sucrose was used as a medium for resuspension of the particulate fraction prior to measurement of binding capacity (experiments 2 and 3, Table 1).

TABLE 1. INFLUENCE OF SEVERAL SALTS AND SUCROSE ON BINDING OF γABA-1-^{14}C BY BRAIN PARTICLES

Experiment	Salt	Concentration (M)	γ-ABA bound (μg)
1	None		0·13
	NaCl	0·2	1·47
	NaCl	0·4	1·27
	Na_2SO_4	0·1	1·44
	Na_2SO_4	0·2	1·38
	Li_2SO_4	0·1	0·10
	Li_2SO_4	0·2	0·16
	K_2SO_4	0·1	0·13
	K_2SO_4	0·2	0·13
	KCl	0·1	0·10
2*	None		0·0
	NaCl	0·2	0·45
	NH_4Cl	0·2	0·0
	NH_4Cl	0·4	0·0
	$MgCl_2$	0·1	0·0
	$MgCl_2$	0·4	0·0
	$CaCl_2$	0·1	0·08
	$CaCl_2$	0·4	0·08
3*	None		0·01
	NaCl	0·2	0·42
	Sucrose	0·25	0·0
	Sucrose	0·50	0·0
	Sucrose	1·00	0·0

* The particulate pellet was resuspended in a small volume of distilled water instead of (the usually employed) 0·25 M sucrose, prior to addition to the incubation mixture.

5. *Osmotic sensitivity of the binding capacity of brain particles.* The effect of tonicity was tested further in the experiments described in Table 2. Brain particles were prepared in the usual fashion and suspended in 0·25 M sucrose. Aliquots of suspension (0·5 ml), containing the particles from one mouse brain, were subjected to different osmotic environments and then tested for binding capacity. The particles in tube 1 were kept in 0·23 M NaCl and 0·055 M sucrose for 1 hr and were found subsequently to have an essentially normal binding capacity. In tubes 2 and 3, particles suspended in a hypotonic medium (0·055 M sucrose) for 30 and 60 min, respectively, were found to

have a greatly reduced binding capacity, the tube in which the exposure to hypotonic medium was longer having the lower activity. On the other hand, although some loss in activity was observed when the particles were contained in a hypertonic solution (tubes 5 and 6, 0·75 M NaCl and 0·516 M sucrose), 74·5% and 71%, respectively, of the activity found in tube 1 were retained in the particles exposed for 30 and 60 min. These results indicate that the binding phenomenon under investigation is indeed associated with the integrity of osmotically sensitive structures which might be broken or distorted by swelling in hypotonic solutions.

TABLE 2. OSMOTIC SENSITIVITY OF BINDING OF γABA

The final incubation mixture contained the following: water, 1·8 ml; 2 M NaCl, 0·3 ml; 0·5 M Tris buffer pH 7·5, 0·3 ml; γABA-1-^{14}C, 0·1 ml; and 0·5 ml of suspension of brain particles in 0·25 ml sucrose. In the present experiment Tris buffer and γABA were added to all tubes at 60 min, and incubation was continued at 0° for an additional 60 min prior to determination of binding by the direct method. In the table are indicated the times at which the water and NaCl solution of the incubation mixture were added to 0·5 ml of particle suspension during the first 60 min.

Tube no.	Time of addition (min)			γABA bound (μg)
	0	30	60	
1	NaCl and H_2O			1·25
2	H_2O	NaCl		0·14
3	H_2O		NaCl	0·05
4	NaCl	H_2O		0·93
5	NaCl		H_2O	0·89

6. *Binding of γABA by different fractions of sucrose homogenates of mouse brain.* On the basis of some preliminary studies of the distribution of the γABA-binding material in whole mouse brain homogenate, the experiments described above were performed on a fraction prepared from whole mouse brain homogenized in 0·25 M sucrose and contained in the pellet obtained by centrifugation at 15,000 × *g*, after discarding the pellet sedimenting at 1,500 × *g*. Results on the distribution of the binding capacity of the various fractions obtained essentially by the procedure of Brody and Bain,[10] as determined by both the direct and equilibrium dialysis methods, are shown in Table 3. Each sediment was resuspended in a volume of NaCl-Tris medium so that the particles were suspended in a volume of fluid equal to that from which they were originally derived. The results obtained by both testing procedures were comparable, with the exception that somewhat higher binding activity was found by the direct method in the two most active fractions. This is not surprising since the dialysis experiment took 6 hr while the incubation in the direct method was only 1 hr.

The "mitochondrial" fraction had the greatest total binding capacity, while the fraction with the highest binding capacity per milligram fresh weight contained the "microsomal" particles. On electron microscopy we have found such fractions prepared from whole-brain tissue to be highly heterogeneous, containing diverse structural elements and fragments in addition to mitochondria and microsomes. Experiments similar to that above employing 0·88 M sucrose or 0·32 M sucrose containing 5×10^{-4} M EDTA as suspending media also showed that the largest amount of

binding material present in the homogenates sedimented in those fractions usually called "mitochondrial" and "microsomal".

From these experiments it can be concluded that the unique ability to bind γABA of tissue of the CNS, among the various tissues studied,[9] probably resides largely within some structural component which, at least in part, survives homogenization in 0·25 M sucrose and which sediments with the smaller particulate fractions.

TABLE 3. BINDING OF γABA BY CENTRIFUGALLY DERIVED FRACTIONS FROM WHOLE MOUSE-BRAIN HOMOGENATE

Fraction		Fresh weight per tube	γABA bound			
			Dialysis method		Direct method	
× g	min	(mg)	(μg)	(μg/100 mg)	(μg)	(μg/100 mg)
800	10	93	0·17	0·18	0·17	0·18
1,500	10	36	0·25	0·70	0·25	0·70
15,000	15	95	0·81	0·85	0·88	0·94
23,000	30	14	0·25	1·78	0·35	2·50
Supernatant		62*	0·13	0·21		

* This weight was obtained by subtracting the total weight of the first four fractions from the original fresh weight of material used in making the homogenate.

7. *Attempts to solubilize and characterize the material that binds γABA.* All the binding activity of the homogenates employed was recoverable in the sedimentable fractions. Many unsuccessful attempts were made to dissolve the γABA-binding material of the sediments in such form that it could still be detected by the equilibrium dialysis procedures. If the material had become solubilized, but was of sufficiently high molecular weight so as not to be dialyzable, it would have been detected. If it were still active, but dialyzable, it would not be detectable by the procedure employed. The binding capacity of all nondialyzable components was destroyed by surface-active agents and detergents (sodium cholate, sodium deoxycholate, Tween-20, Teepol, Triton X-100, Triton X-165), by lyophilization, or by treatment of the particulate fraction with cold acetone, ethanol, butanol, or a 2 : 1 mixture of chloroform and methanol. An acetone powder of mouse brain did not bind γABA.[9] Since a carefully prepared emulsion of total brain lipids or a preparation of purified brain lipoproteins showed no ability to bind γABA, the effect of the organic solvents probably cannot be attributed to the removal of lipids, which themselves might have the capacity to bind γABA, but rather to the destruction of larger complexes or membrane components.

Since the loss of binding activity was relatively small when the particles were incubated for 1 hr at 15° (see Fig. 3), a series of experiments was performed in which the particles were exposed to the action of several enzymes at this temperature prior to equilibrium dialysis. Crystalline or purified enzymes were incubated in 1-mg amounts with 2 ml of particle suspension in NaCl–Tris medium for 1 hr at 15°, and the suspension was then tested for binding capacity by the usual equilibrium dialysis procedure at 0° for 6 hr. The enzymes tested were trypsin, lipase (wheat germ), lysozyme, and papain. None of these enzymes had any effect on the extent of binding of γABA by the preparation, nor did a combination of lipase and trypsin have any effects. However, 1 mg of snake venom (*Crotalus terrificum*) destroyed the binding capacity completely. This suggested the possibility that a surface-active material formed by the

action of the venom on some constituent of the particulate was causing destruction of the binding capacity. Subsequently, a highly purified preparation of lysolecithin (prepared from commercial soybean lecithin and characterized according to Rouser's methods[11–13],) containing unsaturated fatty acids and freed from all detectable protein and free fatty acid, was found to destroy the binding. To aliquots (2·4 ml) of the particulate suspension in the NaCl–Tris medium were added 0·1-ml portions of the medium containing various concentrations of lysolecithin and the mixtures allowed to stand on ice for 1·5 hr at 0°. The binding capacity was almost completely destroyed by final concentrations of 0·125% and above, and to the extent of 80% by the lowest concentration employed (Table 4). The optical density of the 10-fold diluted suspension was decreased by all the concentrations employed.

TABLE 4. DESTRUCTION OF γABA-BINDING CAPACITY BY SONICATION OR BY TREATMENT WITH LYSOLECITHIN

Treatment	Optical density* at 640 mμ	γABA bound
		(μg)
Sonication,† control	0·53	0·80
30 sec	0·46	0·17
1 min	0·40	0·12
2 min	0·29	−0·08
4 min	0·16	−0·01
8 min	0·11	0·0
Lysolecithin, control	0·67	0·99
0·063%	0·50	0·20
0·125%	0·33	0·02
0·25%	0·18	0·04
0·5%	0·13	−0·08

* Measured at 10-fold dilution of the suspension.

† Sonication at 10 kc at 0° in a Raytheon sonic oscillator, model DF 101.

Sonication for short periods in the cold gave results similar to those found with lysolecithin. The results with both sonication and lysolecithin, as well as those with the detergents mentioned above, show that a relatively small decrease in optical density is associated with a relatively large decrease in binding capacity. These findings, also, are consistent with the view that some complex structural component may be involved in the binding of γABA.

8. *Effect of sulfhydryl reagents, heavy metals, and other substances on binding of γABA.* Experiments were performed in which the brain particulate fraction was preincubated for 60 min at 20° in a final concentration of 5×10^{-4} M of reagent to be tested prior to the addition of the labeled γABA and incubation at 0° for 60 min (direct method). *p*-Hydroxymercuribenzoate and n-ethylmaleimide were most effective of the organic reagents tested in decreasing binding (Table 5). Both the latter substances also decreased the optical density of the suspension.

In experiments similar to those above, 10^{-3} M concentrations of L-cysteine, reduced glutathione, mercaptoethanol, EDTA, cyanide, arsenate, arsenite, dinitrophenol, adenosine triphosphate, pyridoxal phosphate, hydroxylamine, serotonin,

oxotremorine, *d*-amphetamine, ephedrine, meprobamate, barbital, diphenylhydantoin, urethane, histamine, acetylcholine, ouabain, picrotoxin, and norepinephrine had no effect whatsoever.

Since n-ethylmaleimide (NEM) has somewhat greater specificity for SH compounds than mercury salts, some further studies were made of the effects of NEM. Inhibition of γABA binding by NEM is a function of the concentration as well as of time of preincubation (Fig. 4). Although the reaction of the reagent with reduced

TABLE 5. INHIBITION OF γABA BINDING BY SULPHYDRYL REAGENTS

Substance tested	Inhibition of binding (%)	Change in optical density at 640 mμ (%)
5×10^{-4} M		
p-Hydroxymercuribenzoate	98	−14
n-Ethylmaleimide	53	−6
1,2-Naphthoquinone-4-sulfonate	12	+4
Iodoacetic acid	11	0
Iodoacetamide	10	+1
1×10^{-3} M		
$HgCl_2$	90	
$CuCl_2$	59	
$ZnCl_2$	32	
$CoCl_2$	21	
$FeCl_3$	18	
$Pb(C_2H_3O_2)_2$	17	

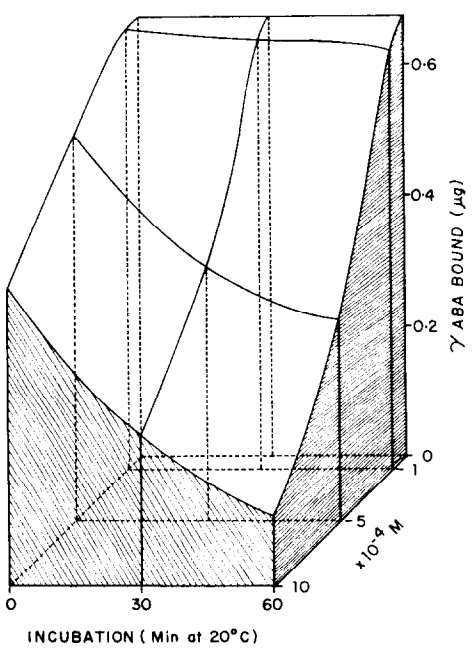
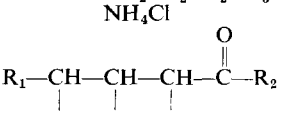
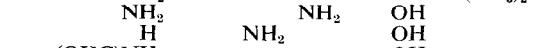
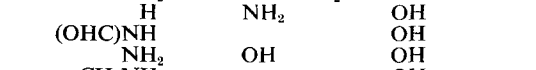
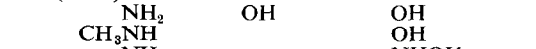


FIG. 4. Influence of concentration and time of preincubation in inhibition of binding by n-ethylmaleimide.

glutathione and cysteine is virtually instantaneous, the reaction with protein SH groups is much slower.[14] The results are therefore compatible with the interpretation that the inhibitory effect of NEM, and probably other SH reagents, is attributable to interaction with protein SH groups in the binding structure. Measurements of optical density before and after the incubation at 20° showed there was no decrease in the control tubes or in those with 1×10^{-4} M NEM even at 60 min. However, in the samples containing 5×10^{-4} M NEM there was a 6% decrease in optical density without preincubation and 9% after 60 min, while at 0 and 60 min the decreases in optical density from the control at 1×10^{-3} M NEM were 14 and 26% respectively. These results suggest that the γABA binding material is associated with a particle containing a protein (or proteins) with reactive SH groups, some of which may be essential for both maintenance of structural integrity and γABA-binding capacity.

TABLE 6. INHIBITION OF BINDING OF γABA BY STRUCTURALLY RELATED COMPOUNDS

Group	No.		Compound	Inhibition (%)
A	1		NH_2CH_2COOH	1
	2		$NH_2(CH_2)_2COOH$	29
	3		$NH_2(CH_2)_4COOH$	42
	4		$NH_2(CH_2)_5COOH$	4
B	5		$CH_3CH_2CH_2COOH$	6
	6		$NH_2CH_2CH_2CH_3$	13
	7		NH_4Cl	12
C				
	8			66
	9	DL		64
	10	DL		61
	11			55
	12	DL		54
	13			52
	14			35
	15			31
	16			9
	17	L		9
	18	L		4
	19			3

9. *Inhibition of binding of γABA by structurally related substances.* The study of specificity of attachment of γABA to the binding material was approached by determining the inhibition of binding of labeled γABA by 100-fold concentrations of substances structurally related to γABA. The labeled γABA (1×10^{-5} M) and test substance (1×10^{-3} M) were added simultaneously, the preparation was incubated at 0° for 60 min, and binding was measured by the direct method. The most effective compounds among the straight-chain ω-amino acids tested (group A, Table 6) were

β-alanine and δ-aminovaleric acid, the two ω-amino acids closest in chain length to γABA. Glycine and ε-aminocaproic acid were ineffective. Both amino and carboxyl groups appear necessary for effective attachment (group B, Table 6), since neither butyric acid nor n-butylamine or ammonium chloride were effective inhibitors.

The requirement of an ω-cationic group for binding was shown by the finding that those substances (Nos. 16–19, group C, Table 6) that do not have such a group are ineffective as inhibitors, while those that do have such a group are inhibitory to approximately the same extent, whether or not the α-carbon (No. 9) or β-carbon (No. 12) contains substituent groups. The carbonyl function is important, but the free carboxylate ion may not be essential for inhibition since the hydroxamic acid derivative of γABA (No. 14) and the isopropyl ester (No. 8) also had inhibitory activity. However, in the case of the latter two compounds great caution must be observed in evaluation of the results since even slight hydrolysis to γABA could give a highly significant dilution of the isotopically-labeled γABA, and an erroneous impression would be given of the extent to which binding of the labeled substance was inhibited. When nonisotopic γABA was used in 1×10^{-3} M concentration, the binding of the ^{14}C-labeled γABA was reduced, as expected, to insignificant levels because of the dilution.

Thus there appears to be a considerable degree of specificity in the structural requirements for the binding of γABA to the brain particles since even minor variations in structure give substances with less affinity than γABA for the binding material.

10. *Inhibition of binding by substances previously found to cause leakage of γABA from brain slices.* It was found in studies with brain slices that several substances caused leakage of γABA and glutamic acid into the medium.[7] This effect appeared to be correlated with an effect on respiration. However, it seemed worth while to study these substances with regard to their ability to prevent the binding of γABA, since it appeared possible that the effect of these substances on both processes might result from alterations in structure of various membrane systems in the cells of the slices (see Spirtes and Guth[15] for some references on effects of chlorpromazine on membranes).

Experiments performed by equilibrium dialysis showed that the antihistamines (Nos. 2 and 3), phenothiazine derivatives (Nos. 1 and 6) and imipramine and a metabolite thereof, desmethylimipramine,[16] (Nos. 4 and 5) all inhibited binding of γABA (Table 7). Studies of the three best inhibitors showed them to have approximately the

TABLE 7. INHIBITION OF γABA BINDING BY SEVERAL PHARMACOLOGICALLY ACTIVE SUBSTANCES

No.	Compound tested	Concentration (M)	Inhibition (%)
1	Chlorpromazine·HCl	10^{-4}	5
		10^{-3}	25
2	Diphenhydramine·HCl (Benadryl)	10^{-4}	12
		10^{-3}	47
3	Orphenadrine·HCl (Disipal)	10^{-4}	15
		10^{-3}	69
4	Imipramine (Tofranil)	10^{-4}	17
		10^{-3}	98
5	Desmethylimipramine	$0{\cdot}9 \times 10^{-4}$	27
		7×10^{-4}	93
6	Randolectil (Riker 595)	$2{\cdot}5 \times 10^{-4}$	47
		10^{-3}	98

same degree of effectiveness (Fig. 5), all giving complete inhibition at 10^{-3} M concentration. At least in the case of imipramine and desmethylimipramine, the inhibition of γABA binding is not caused by direct competition with γABA for a binding site, since the degree of inhibition was not significantly different over an 8-fold difference

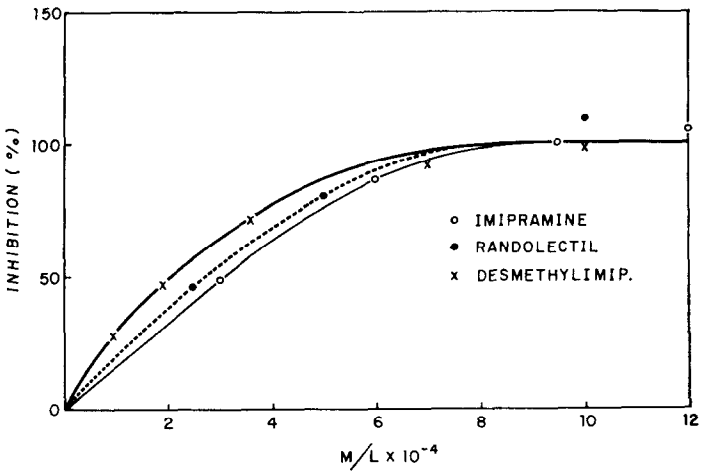


FIG. 5. Inhibition of γABA binding by three psychoactive drugs.

in concentration of γABA (Table 8). However, unlike NEM, sonication, and various surface-active agents, the above substances do not produce any decreases in optical density of the particulate suspensions.

TABLE 8. EFFECT OF CONCENTRATION OF γABA ON INHIBITION BY IMIPRAMINE AND DESMETHYLIMIPRAMINE

γABA concentration (μM)	Inhibition (%) Imipramine (2×10^{-4} M)	Imipramine (3×10^{-4} M)	Desmethylimipramine (2×10^{-4} M)
30	39	79	46
60	49	64	41
120	54	84	57
240		81	63

Quantitative studies with Randolectil showed that, under the usual conditions of the direct experiments, 100, 86, and 91 % of the compound were bound by the particles when $0{\cdot}5 \times 10^{-4}$, 1×10^{-4}, and 2×10^{-4} M concentrations, respectively, were employed. Binding was not reduced by the presence of 10^{-2} M γABA nor, once bound, was the substance released into the aqueous phase by boiling. However, it was extracted quantitatively by water-saturated butanol. These results are in keeping with previous observations which showed that chlorpromazine was bound by both brain and liver particles, and to a greater extent by the brain particles.[17] Inhibition by Randolectil of binding of γABA was found to be dependent on concentration as well as on time of preincubation (Fig. 6).

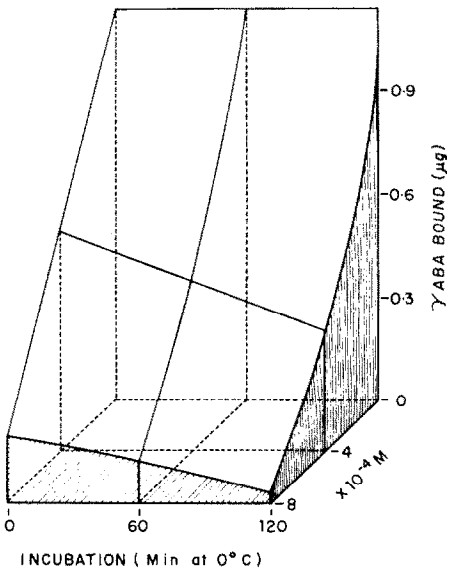


FIG. 6. Influence of concentration and time of preincubation on inhibition of binding by Randolectil.

DISCUSSION

The experiments reported in this paper show that exogenously added γABA can be bound in a nonenzymatic fashion by material found in a centrifugal fraction of mouse brain homogenate which contains mitochondria, microsomes, nerve endings, and structures of a vesicular and membranous nature (see Weinstein *et al.*[18]). A considerable portion of the endogenous sedimentable γABA also is associated with the same fraction.[18] It is not yet known whether or not the exogenously added γABA is bound in the same manner as is the γABA originally present. The results reported in this paper show that the binding structure (or structures) is osmotically sensitive and has other properties expected of a lipoprotein macromolecular complex. The data are remindful of some of the findings made previously on a number of substances active in the CNS, such as acetylcholine, the catecholamines, 5-hydroxytryptamine, and histamine (see Green[19] for extensive review of the literature). Definitive determination of whether γABA is stored within the granules of the vesicles or is bound to them as some of the biogenic amines are believed to be, awaits structural, chemical, and physical study of more homogeneous preparations of individual types of morphological elements, a goal toward which the efforts of our laboratory are now being directed. Only further work along these lines will reveal whether the binding being studied is germane to the known inhibitory effects of γABA in the central nervous systems.

REFERENCES

1. E. ROBERTS and E. EIDELBERG, in *International Review of Neurobiology*, C. C. PFEIFFER and J. R. SMYTHIES, Eds., vol II, p. 279. Academic Press, New York (1960).
2. H. E. HIRSCH and E. ROBINS, *J. Neurochem.* **9**, 63 (1962).
3. G. M. MCKHANN, R. W. ALBERS, L. SOKOLOFF, O. MICKELSEN and D. B. TOWER, in *Inhibition in*

the Nervous System and γ-Aminobutyric Acid, E. ROBERTS, Ed., p. 169. Pergamon Press, Oxford (1960).
4. K. A. C. ELLIOTT and N. M. VAN GELDER, *J. Neurochem.* **3**, 28 (1958).
5. Y. TSUKADA, S. HIRANO, Y. NAGATA and T. MATSUTANI, in *Inhibition in the Nervous System and γ-Aminobutyric Acid*, E. ROBERTS, Ed., p. 163. Pergamon Press, Oxford (1960).
6. K. A. C. ELLIOTT and H. H. JASPER, *Physiol. Rev.* **39**, 383 (1959).
7. M. J. E. ERNSTING, W. F. KAFOE, W. TH. NAUTA, H. K. OOSTERHUIS and C. DE WAART, *J. Neurochem.* **5**, 121 (1960).
8. K. A. C. ELLIOTT and N. M. VAN GELDER, *J. Physiol., Lond.* **153**, 423 (1960).
9. K. SANO and E. ROBERTS, *Biochem. biophys. Res. Commun.* **4**, 358 (1961).
10. T. M. BRODY and J. A. BAIN, *J. biol. Chem.* **195**, 685 (1952).
11. G. ROUSER, A. J. BAUMAN, N. NICOLAIDES and D. HELLER, *J. Amer. Oil Chemists Soc.* **38**, 565 (1961).
12. G. ROUSER, J. O'BRIEN and D. HELLER, *J. Amer. Oil Chemists Soc.* **38**, 14 (1961).
13. G. ROUSER, A. J. BAUMAN, G. KRITCHEVSKY, D. HELLER and J. S. O'BRIEN, *J. Amer. Oil Chemists Soc.* **38**, 544 (1961).
14. E. ROBERTS and G. ROUSER, *Analyt. Chem.* **30**, 1291 (1958).
15. M. A. SPIRTES and P. S. GUTH, *Nature, Lond.* **190**, 274 (1961).
16. B. HERRMANN and R. PULVER, *Arch. int. Pharmacodyn.* **126**, 454 (1960).
17. M. BERGER, *J. Neurochem.* **2**, 30 (1957).
18. H. WEINSTEIN, K. KAKEFUDA and E. ROBERTS, *Biochem. Pharmacol.* **21**, 503 (1963).
19. J. P. GREEN, *Advanc. Pharmacol.* **1**, 349 (1962).