Biochemical Pharmacology, 1964, Vol. 13, pp. 269–279. Pergamon Press Ltd., Printed in Great Britain.

EXOGENOUS AND ENDOGENOUS γ-AMINOBUTYRIC ACID OF MOUSE BRAIN PARTICULATES IN A BINDING SYSTEM *IN VITRO**

S. VARON,† H. WEINSTEIN and E. ROBERTS

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

(*Received* 2 *July* 1963; *accepted* 9 *September* 1963)

Abstract—A study was made of the distribution of endogenous γ-aminobutyric acid (γABA) among different centrifugal fractions from sucrose homogenates of mouse brains. "Mitochondrial" and "microsomal" resuspensions in buffered saline were used to bind ^{14}C-labeled γABA. It was shown that *both* the suspending fluid and the particulate matter of the ^{14}C-γABA binding test systems contained a much larger quantity of γABA than that of the radioactive γABA added. The specific activity of the bound γABA at the end of the experiment was considerably smaller than that of the free γABA in the suspending fluid, indicating that only part of the finally bound γABA participated in the binding process. Thus the occurrence of two "pools" of bound γABA in the final particulates was established. The increase in total amount of bound γABA from the onset to the end of the binding procedure was smaller than was calculated from the specific activity of the free γABA and the ^{14}C-γABA content of the particles at the end of the procedure. These results suggest either that some exchange occurs between bound and free γABA or that appreciable amounts of endogenous γABA are lost by the particulate in the course of the procedure.

THE identification of γ-aminobutyric acid (γABA) as a major constituent of the vertebrate brain and its restriction to the central nervous system have resulted in considerable interest in the biochemical and physiological significance of this compound.[1] After the observation that brain homogenates contained some of their total γABA in a sedimentable form,[2] whole mouse brain homogenates and subcellular fractions were incubated in the presence of labeled γABA, and radioactivity was shown to be accumulated by the particulate matter.[3] The phenomenon was limited to brain tissue, required only the presence of sodium ions in the medium, and took place optimally at pH 7·3–7·5 at 0°. In the present study the relationship between this ^{14}C–γABA "binding" and endogenous γABA present in the test system was investigated.

MATERIALS AND METHODS

Homogenization and centrifugation. Whole mouse brains (average wet weight 400 mg each) were homogenized in nine parts (v/w) of 0·25 M sucrose, using an all-glass

* This investigation was supported in part by Research Grants B-1615 and B-2655 from the National Institute of Neurological Diseases and Blindness (NIH), a grant from the National Association for Mental Health, and grant AF-AFOSR-451-63 from the Air Force Office of Scientific Research.

† Assistant in the Psychiatric Institute of the Medical School, University of Milan, Italy.

homogenizer, and submitted to successive centrifugations at 1,500 *g* (10 min), 15,000 *g* (15 min) and 105,000 *g* (30 min). The pellets, which will be referred to as "nuclear", "mitochondrial", and "microsomal", respectively, were shown by electron microscopic examination to be morphologically heterogenous. All pellets were resuspended in distilled water or saline up to the same volume from which they had been derived originally. All the steps of the preparation and the handling of the particles were carried out at 0–4°. The original homogenate, the supernatant material resulting from each centrifugation, and the resuspended pellets were assayed for total protein content and γABA. In order to correct for the γABA and protein present in the supernatant material that was trapped in the various pellets it was assumed that the entire volume of the pellet consisted of contaminating supernatant material.[3] The amount of γABA and protein contained in this volume of supernatant material was subtracted from the total amounts of γABA and protein present in the pellet. This correction yields values for bound γABA that tend to err on the low side.

Terminology. The following terms will be used throughout.

^{14}C-γABA: the labeled (1-^{14}C) γABA added to the system for the binding assay (specific activity 2·71 mc/mmole; radiopurity > 99%).

Specific activity equivalent: the ratio between micrograms of ^{14}C-γABA and micrograms of total γABA in any given fraction. This designation is justified by the fact that such a ratio is proportional to the conventional specific activity.

Free γABA: the γABA present in solution in the suspending fluid. It consists of the added ^{14}C-γABA (exogenous) as well as some of the γABA carried over into the system from the previous sucrose supernatant material (endogenous insofar as the original source, though now extraparticulate). Since after the centrifugation some supernatant material remained with the final particulate as entrained fluid or possibly even as freely diffusible intraparticulate fluid, some of the γABA associated with the final pellet may still be called free γABA. This is the amount of γABA for which maximal correction has been applied (see calculations).

Bound γABA: any *state* of γABA within the particle other than the free form, irrespective of the nature of its association with the particles (adsorption, binding, concentration of soluble γABA inside the particles, etc.).

γABA binding: the actual *process* by which originally free γABA becomes bound, regardless of the mechanism (exchange, uptake, etc.). ^{14}C-γABA binding will apply only to the binding of the exogenous ^{14}C-γABA.

^{14}C-γABA binding procedure. Thoroughly drained "mitochondrial" and "microsomal" pellets were resuspended in a solution of NaCl (0·2 M) and Tris-HCl buffer (0·05 M, pH 7·3). The concentration* of particles as well as reagents was the same as that described previously.[3] Greater amounts of ^{14}C-γABA were bound by particles that were resuspended directly in the incubation medium than by particles that were suspended first in 0·25 M sucrose and then added to the incubation medium as had been done in earlier experiments.[3] After addition of the ^{14}C-γABA (final concentration 0·75 μg/ml), incubation was carried out at 0–4°, usually for 30 min, and aliquots of the final suspension were employed for the determination of radioactivity, protein, and total γABA. A measured volume of the remaining suspension was centrifuged

* The concentrations for NaCl and buffer given here are the correct ones. The figures reported in a previous publication[3] (p. 490) should read 0·6 and 0·15 *mmoles*, instead of mM, respectively for NaCl and Tris buffer contents).

under the same conditions under which the original particles had been obtained which, in the presence of NaCl, results in complete sedimentation. The supernatant fraction was collected as completely as possible and the volume measured. The firmly packed pellet was resuspended in water to the volume from which it had been sedimented. Aliquots of both supernatant fraction and resuspended residue also were assayed for radioactivity, protein, and total γABA. The free state of the ^{14}C-γABA in the supernatant fraction was verified by: (a) dialysing 3 ml of supernatant material against 6 ml of buffered saline; equilibration was attained between the inside and outside of the dialysis tube within 3 hr; and (b) applying 0·4 ml of the supernatant material to a Sephadex G25 column (17 × 0·9 cm) and washing with the buffered saline; the effluent showed a single peak of radioactivity which coincided with that from 0·4 ml of a saline solution of ^{14}C-γABA.

Radioactivity determination. In early experiments the radioactive samples were first extracted with 4 volumes of 95% ethanol, and 0·2-ml aliquots of the ethanolic supernatant materials were transferred to 1 ml of Hyamine base. The transfer of 40-μl aliquots from the radioactive samples directly to the counting vials containing 1 ml Hyamine is more convenient and, provided such aliquots are mixed with the Hyamine without delay, the same counts are obtained as with the more laborious procedure. This direct transfer was therefore adopted. 15 ml of counting fluid (3 g 2,5-diphenyloxazole, plus 100 mg 1,4-*bis*-2(5-phenyloxazolyl)-benzene in 1 l. cold toluene) were then added to each vial and the vials counted five times for 10 min in a Packard Tri-Carb scintillation counter. Blank vials containing 40 μl water instead of sample also were counted with each batch and their average counts subtracted from those of the sample vials. Vials containing 40-μl aliquots of 0·25 M sucrose, buffered saline, or particle suspensions gave substantially the same readings as those with the same volume of water alone.

Protein determination. Protein determination was carried out according to the method of Lowry *et al.*[4] after appropriate dilutions of the samples with distilled water. The sucrose from the homogenization procedure did not affect the reaction, and therefore albumin standards were used in aqueous solution. On the other hand, the buffered saline of the ^{14}C-γABA binding experiments depressed color development unless diluted more than 30 times. As this could not be done with the supernatants from such experiments because of their low protein content, their readings were referred to a standard curve derived from albumin solutions diluted similarly in buffered saline.

Total γABA determination. Suitable volumes of each sample, kept at 0–4° prior to processing, were acidified to pH 4–5 with HCl, heated in a boiling water bath for 5 min, and centrifuged 5 min at 1,000 *g*. The pellet was resuspended in water and recentrifuged and the supernatant added to the previous one. Losses were monitored by a known amount of ^{14}C-γABA added (when none was already present) prior to the addition of HCl and found to be negligible after this one wash. The protein-free extracts (pH 4 to 5) were then passed through a Dowex-2 column (—OH^- form), the columns were washed with water, and the γABA eluted with 1 N acetic acid. The eluates were dried under an infrared lamp in the presence of a good air flow, redissolved in water, redried a number of times to eliminate the acetic acid, and finally taken up in 1 ml 0·1 M Na–pyrophosphate (pH 8·3). Replicate aliquots were assayed enzymatically for γABA.[5] Other aliquots (40 μl) were counted, with the appropriate pyrophosphate

blanks and internal standards, to establish the over-all recovery of the γABA throughout the column procedure. Recoveries varied between 70 and 95 per cent of the ^{14}C-γABA present in the samples prior to the column treatment. The possibility that a significant amount of ^{14}C-γABA was metabolized during the binding procedure was excluded by subjecting aliquots of the protein-free extract to one-dimensional paper chromatography in three different solvent systems (butanol–acetic acid–H_2O, 12:3:5; butanol–pyridine–H_2O, 1:1:1; ethanol–NH_3–H_2O, 18:1:1). The strips were scanned in a gas-flow counter (Nuclear Chicago, Actigraph II). In all the extracts tested only one radioactive peak was found. This corresponded to that obtained when the labeled γABA was added to heat-inactivated particles prior to processing.

Calculation of bound γABA. The volumes of the pellets at the end of the binding assay were found to vary between 2 and 4 per cent of the volumes of suspension from which they had been derived. The dry weight of the pellet was found to be 10–15 per cent of its wet weight, hence the solids of the pellet contributed less than 1 per cent to the suspension volume. Thus the supernatant material can be assumed to occupy all of the pellet volume and therefore to have the same volume as the total suspension, with an error of no more than 2–3 per cent. Protein and γABA measurements were carried out on equal volumes of total suspension, supernatant material, and particles resuspended in water to their original volume. The difference between the figures from the total suspension and those from the supernatant material agreed within the same limit of error of 2–3 per cent with the figures obtained from the resuspended pellet (corrected for the entrained supernatant material), irrespective of the amount of contamination by the supernatant material. In routine experiments, therefore, such calculation could conveniently replace the direct pellet measurements.

The values reported throughout refer to 1 ml of total saline suspension. Percentage of ^{14}C-γABA bound was calculated by the formula:

$$\frac{\text{counts in total suspension} - \text{counts in supernatant}}{\text{counts in total suspension}} \times 100$$

The bound ^{14}C-γABA in micrograms per milliliter of test system was derived from the percentage of ^{14}C-γABA bound and the actual amounts of ^{14}C-γABA that had been added. The nonradioactive γABA content of all samples was calculated by subtracting the ^{14}C-γABA value from the enzymatically measured total γABA.

RESULTS

Centrifugal fractions

The distribution of protein and γABA in various centrifugal fractions was examined in three experiments and found to be fairly consistent, with variations usually within ±5 per cent. The average figures are shown in Table 1 expressed in per cent of the original homogenate. Table 2 shows calculations and actual values for the "mitochondrial" and the "microsomal" fractions. The γABA content of the homogenate, 35 μg γABA/100 mg fresh mouse brain (Table 1), is in agreement with that previously reported.[6] No significant increase in γABA content was found in samples stored for 4 hr in ice prior to inactivation and extraction. The final supernatant material after removal of the microsomal pellet showed a very high content of γABA, most of which probably is in the free form. It was therefore apparent that appreciable amounts of

free γABA would be carried over into the ^{14}C-γABA binding system with the sucrose supernatant material entrained by the pellet.

Bound ^{14}C-γABA as a function of protein content

In early experiments with concentrated particle suspensions it was found that considerable variations in the protein content of the test system were not always

TABLE 1. PROTEIN AND γABA PERCENTAGE DISTRIBUTION IN CENTRIFUGAL FRACTIONS OF MOUSE BRAIN HOMOGENATES*

Procedure 10% homogenate (in 0·25 M sucrose)		Protein 12·5 mg/ml = 100% (%)	γABA 34·4 μg/ml = 100% (%)
(1) 10% Homogenate 10 min at 1,500 *g*	Pellet (nuclei and debris)	23	8
(2) Supernatant fraction from (1) 15 min at 15,000 *g*	Pellet ("mitochondrial")	42	19
(3) Supernatant fraction from (2) 30 min at 105,000 *g*	Pellet ("microsomal")	10	3·7
	Supernatant	23	72
Recovery		98	102·7

* Average of three experiments.

TABLE 2. PROTEIN AND γABA, PER MILLILITER, IN "MITOCHONDRIAL" AND "MICROSOMAL" CENTRIFUGATES*

	Mitochondrial		Microsomal	
	Protein	γABA	Protein	γABA
A. Concentration in supernatant	4·17 mg/ml	26·45 μg/ml	2·93 mg/ml	24·97 μg/ml
B. Volume of pellet	0·13 ml	0·13 ml	0·04 ml	0·04 ml
C. Max. amt. entrained supernatant (A × B)	0·54 mg	3·44 μg	0·12 mg	1·00 μg
Supernatant collected	3·63 mg	23·01 μg	2·81 mg	23·97 μg
Supernatant entrained by pellet (C)	0·54 mg	3·44 μg	0·12 mg	1·00 μg
Total supernatant	4·17 mg	26·45 μg	2·93 mg	24·97 μg
Total pellet	5·72 mg	10·02 μg	1·34 mg	2·28 μg
Supernatant entrained (C)	0·54 mg	3·44 μg	0·12 mg	1·00 μg
Net particles	5·18 mg	6·58 μg	1·22 mg	1·28 μg
Supernatant (total) + particles (net)	9·35 mg	33·03 μg	4·15 mg	26·25 μg
Found before centrifugation	9·54 mg	33·25 μg	4·17 mg	26·45 μg

* Average of three experiments.

paralleled by appreciable changes in the bound ^{14}C-γABA. To investigate the appropriate conditions for quantitative comparative studies, different dilutions of the same particle suspension in buffered saline were assayed for ^{14}C-γABA binding and protein content in such a manner that the final concentrations of NaCl, Tris-HCl buffer, and

^{14}C-γABA were the same for each tube. Figure 1 shows the relationship between bound ^{14}C-γABA and protein content in mitochondrial and microsomal preparations. In both cases, the amounts of ^{14}C-γABA bound were directly proportional to the protein content at the lower protein levels. Sharp breaks occurred in the slopes of the lines at 3·5 and 2·5 mg protein, respectively, for the mitochondrial and microsomal

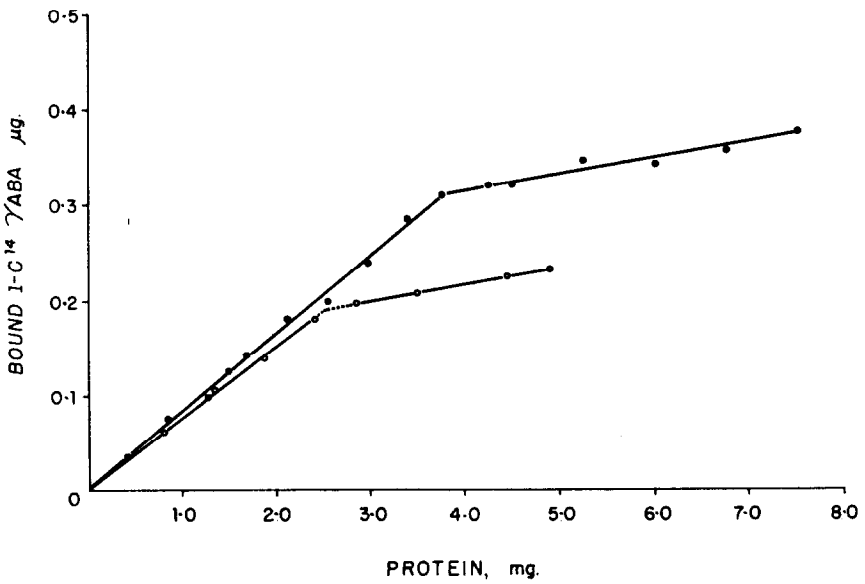

FIG. 1. Bound 1-^{14}C-γABA as a function of increasing concentrations of "mitochondrial" (● —— ●) and "microsomal" (○ —— ○) fractions. The sucrose-derived pellet was suspended and serially diluted in buffered saline. The same amount of 1-^{14}C-γABA was then added to equal volumes of the diluted suspensions and incubated for 30 min at 0–4°. The values, derived from three experiments, are expressed on a per milliliter basis.

materials, corresponding to 0·42 and 0·25 μg of bound ^{14}C-γABA. Beyond those points the linear relationship was resumed at a decreased ratio of bound ^{14}C-γABA to protein content. The slopes were strikingly similar in the mitochondrial and microsomal plots. This close coincidence suggests that the major protein constituents of both particulate fractions may be related to the binding of ^{14}C-γABA. The reasons for the occurrence of the sharp breaks are not apparent and are under further investigation. Because of their occurrence, however, only suspensions containing less than 3·5 and 2·5 mg protein, respectively, for mitochondrial and microsomal materials were deemed appropriate for quantitative experimental work.

The protein contents of total suspension, supernatant material, and particles in the assay system are shown in Table 3. The increase in nonparticulate protein as a result of the experimental procedure was greater for the microsomal than for the mitochondrial fraction. Corresponding decrements were seen in the particulate proteins. Thus it appears that components of the microsomal pellet lose protein to the medium to a greater extent than do those of the mitochondrial fraction.

γABA distribution in the ^{14}C-γABA binding system

Table 3 shows the nonradioactive, radioactive, and total γABA contents and distributions in 1 ml of test system for both types of particulates, at the end of 30min incubation at 0°. In addition, values "at the time of resuspension" were recalculated from Table 2 for the amounts of γABA bound to the particles and of γABA carried over to the test system as entrained sucrose supernatant material. The latter is shown in Table 3 under the heading "suspending medium".

TABLE 3. PROTEIN AND γABA DISTRIBUTION IN "MITOCHONDRIAL" AND "MICROSOMAL" TEST SYSTEMS FOR ^{14}C-γABA BINDING*

	Mitochondrial			Microsomal		
	Total suspension	Particulate	Suspending medium	Total suspension	Particulate	Suspending medium
Protein (mg/ml suspension)						
at time of resuspension*†	3·59	3·24	0·35	2·23	2·03	0·20
after 30-min incubation (0–4°)	3·64	3·02	0·53	2·14	1·52	0·59
Nonradioactive γABA (μg/ml suspension)						
at time of resuspension*†	6·26	4·10	2·16	3·80	2·13	1·67
after 30-min incubation (0–4°)	6·28	4·47	1·88	3·68	2·02	1·76
(μg/mg protein)	(1·72)			(1·72)		
^{14}C-γABA (μg/ml suspension)						
at time of resuspension (added)	0·75		0·75	0·75		0·75
after 30-min incubation	0·75	0·34	0·41	0·75	0·19	0·56
Total γABA (μg/ml of suspension)						
at time of resuspension (nonradioactive + ^{14}C-γABA)	7·01	4·10	2·81	4·55	2·13	2·42
after 30-min incubation	7·10	4·81	2·29	4·43	2·21	2·32
Specific activity equivalent = SAE = $\frac{\mu g^{14}\text{ C–γABA}}{\mu g\text{ total γABA}}$	0·106	0·070	0·179	0·170	0·087	0·241
"Traceable" bound γABA = $\frac{\mu g/ml\ ^{14}\text{C-γABA}}{\text{SAE of supernatant}}$		1·90			0·79	
"Nontraceable" γABA = (total γABA − traceable γABA) μg/ml		2·91			1·42	
same, in μg/mg protein		(0·96)			(0·93)	

* Average of three experiments.

† The mitochondrial pellet from 1 ml of 1,500 *g* supernatant (Table 1) was resuspended in saline to a final volume of 1·6 ml. The microsomal pellet from 1 ml of 15,000 *g* supernatant (Table 1) was resuspended to a final volume of 0·6 ml. It is assumed that the resuspension resulted in the uniform distribution in the resuspending medium of the supernatants entrained by the pellets (calculated in Table 2). The distributions of protein and γABA, at the time of resuspension, were calculated on the basis of Table 2 figures and the final volumes of the saline suspensions.

Three major points may be derived from these data. Since they apply equally to either particulate, only the figures for the "mitochondrial" one will be discussed below.

(a) The added ^{14}C-γABA accounted for only 8 per cent of the total γABA of the mitochondrial system. About one-third of the remaining, nonradioactive γABA was found in the free form. Therefore, the added ^{14}C-γABA must have been diluted by nonradioactive γABA in the suspending medium. Consequently the final bound radioactivity must represent a larger amount of bound γABA than would have been calculated from the specific activity of the added ^{14}C-γABA. This quantity may be

designated as "traceable" bound γABA. At the end of the incubation the suspension medium contained 0·41 μg ^{14}C-γABA and 2·29 μg total γABA. Thus, its "specific activity equivalent" was 0·41/2·29 = 0·179 and the ^{14}C-γABA found in the particles at the end of the incubation, i.e. 0·34 μg, would represent 0·34/0·179 = 1·90 μg. This, therefore, is the maximal amount of the traceable bound γABA.

(b) Comparison of this amount with that of the γABA observed in the final particulate (4·81 μg) showed that the traceable bound γABA does not make up all of the final bound γABA. The excess (2·91 μg) will be henceforth designated as "nontraceable". The same conclusion could have been reached by noting the higher specific activity equivalent of the supernatant material (0·179) as compared with that of the pellet (0·070). The occurrence of a nontraceable pool of bound γABA could have been a reflection of the presence in the supernatant material of radioactive compounds other than ^{14}C-γABA or else of a lack of completion of the binding

TABLE 4. PROTEIN AND γABA DISTRIBUTION IN 1 ML OF A "MITOCHONDRIAL" ^{14}C-γABA-BINDING SYSTEM AFTER DIFFERENT TIMES OF INCUBATION AT 0° TO 4°

	Time of incubation, min	Particles	Supernatant	Particles + supernatant
Proteins (mg)	5	2·97	0·57	3·54
	30	3·12	0·59	3·71
	120	3·12	0·64	3·76
Nonradioactive γABA (μg)	5	3·88	1·68	5·56
	30	3·80	1·75	5·55
	120	3·39	2·16	5·55
^{14}C-λABA (μg)	5	0·22	0·52	0·74
	30	0·30	0·45	0·75
	120	0·31	0·44	0·75
Total γABA (μg)	5	4·10	2·20	6·30
	30	4·10	2·20	6·30
	120	3·70	2·62	6·32
Specific activity equivalent = $\frac{^{14}\text{C-}\gamma\text{ABA}}{\text{Total }\gamma\text{ABA}}$	5	0·054	0·236	0·118
	30	0·073	0·205	0·119
	120	0·084	0·168	0·119
Traceable γABA (μg) $\frac{(^{14}\text{C-}\gamma\text{ABA})}{(\text{SAE supernat})^*}$.	5	0·85		
	30	1·46		
	120	1·85		
Nontraceable γABA (μg) (total − traceable)*	5	3·25		
	30	2·64		
	120	1·85		

* See text.

process. The first possibility was ruled out by paper-chromatography tests (see Methods) and the second by running an experiment in which the mitochondrial suspension was incubated for 5, 30 and 120 min under test conditions (Table 4), which showed that the accumulation of radioactivity occurred entirely within the first 30 min.

(c) The final amount of bound γABA (4·81 μg) was significantly lower than the sum (6·00 μg) of the originally bound γABA (4·10 μg) and the traceable bound γABA (1·90). This indicated that during the test procedure some of the nonradioactive γABA of the particles must have been released to the medium.

DISCUSSION

The two most immediate interpretations of the accumulation *in vitro* of ^{14}C-γABA by brain particulates in a saline resuspension are that of a unidirectional *uptake*, leading to a static situation, and that of an *exchange*, leading to a dynamic equilibrium. In the following discussion, the experimental data reported here and the operational concept of two pools of bound γABA will be used to evaluate the plausibility and merits of either hypothesis.

(a) In an uptake process, free γABA of the medium would associate with the particles and remain bound to specific sites or possibly retained in intraparticulate spaces by an outward barrier. Two γABA pools would be found in the final particulate. One, exclusively nonradioactive, would correspond to the γABA present in the particles prior to their resuspension in saline and, in the procedure used, might also include some γABA bound after the resuspension but before the ^{14}C-γABA addition. The other would consist exclusively of newly bound γABA containing ^{14}C-γABA with the same specific activity (or specific activity equivalent) as that of the free γABA. The experimental distinction of the traceable and nontraceable pools, although consistent with such a hypothesis, does not adduce evidence for or against the existence of two or even more distinct types of binding mechanisms or binding sites, since it could merely be the reflection of the presence or absence of labeled γABA at different stages of the experimental procedure.

Two obvious consequences of such a hypothesis are that the specific activity of the free γABA should remain constant throughout and that the amount of bound γABA should increase from beginning to end by at least the amount of the traceable pool. The time experiment of Table 4 contradicts the former and the figures of Table 3 the latter point. Both groups of data, however, become consistent with the hypothesis if the additional assumption is made that nonradioactive γABA is concomitantly lost to the medium by the nontraceable pool. Thus its progressive decrease (Table 4) will partially offset the uptake of traceable γABA and result in a lower net gain of bound γABA (Table 3). The specific activity equivalent of the free γABA also would drop progressively in the medium (Table 4), thereby making the calculation of a traceable pool by use of the specific activity equivalent of the final supernatant material no longer accurate. By using the data of Table 3 for a "mitochondrial" preparation, the two extreme values of specific activity equivalent for the free γABA (that is before and after the 30-min incubation) can be calculated as 0·267 and 0·179, with corresponding sizes for the traceable pool of 1·28 and 1·90 μg/ml respectively. Since the over-all increase of bound γABA was (4·81 − 4·10 =) 0·71 μg/ml, the required losses of nontraceable bound γABA would range between (1·28 − 0·71 =) 0·57 and (1·90 − 0·71 =) 1·19 μg/ml, or 14 to 29 per cent of the originally bound γABA. A similar calculation for the "microsomal" preparation (Table 3) would lead to specific activity equivalents of 0·310 and 0·241, to traceable pool sizes of 0·61 to 0·79 μg/ml and to required losses of 0·53 to 0·71 μg/ml or 25 to 34 per cent of the originally bound γABA. The higher loss of nontraceable γABA from microsomal

components is in keeping with their already discussed higher loss of protein. The situation may be further complicated in the standard procedure by the possibility that some of the nonradioactive free γABA of the saline suspension already becomes bound during the short interval before the ^{14}C-γABA addition to the system. Such γABA will represent a newly-added nontraceable compartment and will raise correspondingly the size of the required loss from the nontraceable pool.

(b) In the exchange hypothesis, the γABA binding *in vitro* is conceived as a reversible process involving both free and bound molecules. If this hypothesis should be correct, the finding of a nontraceable pool would mean that under the experimental procedure used, most, if not all, of the γABA originally bound to the mitochondrial or microsomal particles tested was incapable of exchanging with the free γABA of the system. The experimental data of Tables 3 and 4 still require, as in the uptake hypothesis, a release into the medium of part of the original bound, nonradioactive γABA. However, the dilution by it of the free ^{14}C-γABA will be paralleled in an equilibrating system by a similar dilution of the traceable pool, and the specific activity equivalent of the final supernatant material, being the same at equilibrium as that of the traceable γABA, will allow for a correct calculation of the size of the two pools as well as the size of the required release of original bound γABA (1·19 and 0·71 μg/ml, respectively, for the mitochondrial and the microsomal preparations). Moreover, there is no longer any need to consider separately the free γABA that could become bound in the saline suspensions prior to the ^{14}C-γABA addition, since such a newly bound γABA will continue to exchange and thus participate to the final equilibration. If none of the original bound γABA were exchangeable, all of the required release has to be conceived, as in the uptake hypothesis, as an irreversible loss from an original nontraceable pool. The final traceable pool would then involve an entirely different set of binding sites or a new binding mechanism. An alternative interpretation offered by the exchange hypothesis is, however, that part of the original bound γABA was "potentially" exchangeable. The final nontraceable pool will in such case represent that part only of the original bound γABA that was incapable of exchanging, whereas the final traceable pool will constitute the other part of it as well as any net increase of the bound γABA. The sum of the final free and traceable bound γABA will in both cases represent all the γABA ultimately involved in the binding *in vitro* as an equilibrating system.

In conclusion, the uptake hypothesis requires that about one-third or more of the original bound γABA be lost to the medium during the procedure. It implies that a delayed addition of ^{14}C-γABA to the saline suspension will to some extent be reflected in a reduced ^{14}C-γABA binding, although not necessarily in a reduced level of newly bound γABA. It also implies that the traceable pool be irreversibly bound to the particulate, at least under conditions that protect the *in vitro*-bound γABA. It does not necessarily implicate different properties of the nontraceable and traceable pools. Conversely, the exchange hypothesis requires a similar loss of original bound γABA only as an alternative to the assumption that some of it is potentially exchangeable. Provided sufficient time is allowed for an equilibrium to be attained, the ^{14}C-γABA binding should reach the same level regardless of the order or the delay of the ^{14}C-γABA addition. The traceable pool being in a dynamic equilibrium, ^{14}C-γABA should be removable or even replaceable by nonradioactive γABA under appropriate conditions without any drastic change in the binding ability of the particles. Finally,

traceable and nontraceable pools would differ in at least their ability to exchange with the free γABA and therefore be likely to represent distinct binding sites or mechanisms. The data reported thus far are consistent with either hypothesis. However, since the differences outlined above can be approached experimentally, it is hoped that further investigations will allow discrimination between the two interpretations or possibly suggest additional ones. A significant aspect of this problem may lie in the similarities observed between "mitochondrial" and "microsomal" particulates in respect of the binding *in vitro* as well as original content of γABA (Table 3 and Fig. 1).

A better understanding of the process or processes involved in the formation of the two pools, as well as of their properties, is of course required before the question can even be asked whether they reflect a similar situation *in vivo* or are to be viewed as mere *in-vitro* artifacts. The sodium-dependent binding may even reflect a potential, rather than actual, property of brain tissue, for example if the procedure *in vitro* caused the exposure to γABA of structures which *in vivo* never come into contact with it or do so only in some special functional stages (e.g. receptor sites).

Elliott and Van Gelder[2] suggested that the "free" and "occult" Factor I found in saline homogenates may be representative respectively of a tightly and a loosely bound Factor I *in vivo* and considered the lower sucrose homogenate levels as the result of a partial "loss" of original occult Factor I. Our results offer an alternative interpretation of the relationship between the occult γABA found in saline and sucrose homogenates, by suggesting that the additional occult γABA found in the former might be the result of the same sodium-dependent binding *in vitro* as that studied here, rather than a measure of particle impairment by sucrose.

REFERENCES

1. E. Roberts, Ed., *Inhibition in the Nervous System and γ-Aminobutyric Acid.* Pergamon Press, Oxford (1960).
2. K. A. C. Elliott and N. M. Van Gelder, *J. Physiol. (Lond.)* **153**, 423 (1960).
3. K. Sano and E. Roberts, *Biochem. Pharmacol.* **12**, 489 (1963).
4. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
5. C. F. Baxter, *Meth. med. Res.* **9**, 192 (1961).
6. E. Roberts, P. J. Harman and S. Frankel, *Proc. Soc. exp. Biol. (N.Y.)* **78**, 799 (1950).