Biochemical Pharmacology, 1965, Vol. 14, pp. 1213–1224. Pergamon Press Ltd., Printed in Great Britain.

SODIUM-DEPENDENT BINDING OF γ-AMINOBUTYRIC ACID BY MORPHOLOGICALLY CHARACTERIZED SUBCELLULAR BRAIN PARTICLES*

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

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

(*Received* 29 *January* 1965; *accepted* 10 *March* 1965)

Abstract—The association of ^{14}C-γ-aminobutyric acid (^{14}C-γABA) with a mixture of brain subcellular particles has been ascribed to a Na^+-dependent binding of the ^{14}C-γABA to carrier sites on the membrane of spheroidal particles and to the carrier-mediated appearance of the ^{14}C-γABA in the internal fluid volume of the particles.

It is now shown by subfractionation of mitochondrial and microsomal preparations on a density gradient and by electron microscopic identification that there are at least three types of membrane-bounded particles (mitochondria, nerve-ending fragments, and microsomes) which must be considered in the localization of the endogenous γABA, in the incorporation of isotopically labeled γABA, and in the Na^+-dependent binding of γABA. The results establish that the three phenomena are associated with microsomes and are most probably associated with the nerve-ending fragments. The mitochondria contain endogenous γABA, but the results do not permit a definite conclusion in regard to the existence of Na^+-dependent binding sites nor to the entry of isotopically labeled γABA into the particle.

INTRODUCTION

IN VERTEBRATES γ-aminobutyric acid (γABA) appears to occur almost entirely in the central nervous system. Extensive studies pertaining to the role of γABA in the metabolism and function of the central nervous system have been carried out.[1–3] The observation that γABA can be sedimented in association with particulate fractions of brain homogenates[4] has led to attempts to define the subcellular components with which γABA may be associated.[5–8] Particles recovered from sucrose homogenates were shown to accumulate ^{14}C-γABA *in vitro* at 0–4° provided Na^+ ions were present.[9] Subsequent studies demonstrated the Na^+-dependent binding of ^{14}C-γABA and suggested that more than one pool of γABA was associated with crude mitochondrial and microsomal particles after incubation with Na^+. An extension of these studies[10] carried out on crude mitochondrial preparations indicated that the ^{14}C-γABA of the particles is in two pools. One pool, the rapidly equilibrating pool (REP), appears to be established by the activation by Na^+ ions of binding sites on which γABA remains in rapid equilibrium with γABA in the saline medium. This pool can be rapidly and selectively abolished by removing the Na^+ from the medium. A slowly equilibrating

* This work was supported in part by Grants NB-2655 and NB-1615 from the National Institute of Neurological Diseases and Blindness, National Institutes of Health; by grants from the National Association for Mental Health and the United States Air Force; and by the Hector Escobosa Fund.

† Present address: Department of Genetics, Stanford University, Palo Alto, Calif.

‡ Reprint requests and enquiries should be sent to the Dept. of Biochemistry, City of Hope Medical Center, Duarte, Calif.

pool (SEP) is required to account for the radioactivity that the above treatment fails to remove from the particles. It was suggested that the SEP may include all or part of the endogenous γABA that is sedimented with the particles.

The purpose of the present experiments was to subfractionate the mitochondrial and microsomal fractions which had been used previously for studies of the binding phenomenon,[8, 10] and to determine whether or not the phenomena related to γABA binding could be associated with particular, identifiable morphological components.

METHODS

The procedures for the preparation of "crude mitochondrial" and "microsomal" pellets and their binding of ^{14}C-γABA in a saline medium have been described in detail in a previous paper.[8] All experimental manipulations were conducted at 0–4°. Whole mouse brains were homogenized in 9 parts (v/w) of 0·25 M sucrose in an all-glass homogenizer. Three pellets were obtained by successive differential centrifugations of the suspension. They were a 1,500-*g* (10-min) pellet, a 15,000-*g* (15-min) pellet, and a 105,000-*g* (30-min) pellet. The latter two pellets will be referred to as "crude mitochondrial" and "microsomal" pellets respectively; they were resuspended in final volumes of buffered saline plus ^{14}C-γABA which were respectively 1·6 and 0·6 times the volume of the sucrose suspension from which the pellets were derived. These saline resuspensions of the two fractions were at a final concentration of 0·2 M NaCl, 0·05 M Tris-HCl (pH 7·3), and 0·75 μg ^{14}C-γABA/ml (specific activity 2·71 mc/mmole: radiopurity > 99%). After 30 min in the presence of the isotope, 33-ml aliquots of the crude mitochondrial and microsomal suspensions were sedimented, respectively, at 15,000 *g* (15 min) and 105,000 *g* (30 min). The supernatants were thoroughly decanted. Two molar sucrose (approximately 4 ml) was added to the radioactive pellets. The pellets were gently rehomogenized by hand, and each fraction was brought up to a final volume of 5 ml. The latter suspension was centrifuged for 5 min at 5,000 rpm in the Spinco centrifuge (SW39 rotor). This procedure resulted in the rapid rise of clumped aggregates to the surface. Approximately 3 ml of the contents of the lower half of the tube was collected through a small hole punched in the bottom of the tube. This contained finely dispersed particles. Assays for protein, radioactivity, and γABA content[8] showed that the concentrations were similar in the discarded upper fraction and lower one. Aliquots (0·75 ml) of the latter material (finely dispersed particles) were placed at the bottom of three centrifuge tubes and 0·5 ml-aliquots of sucrose solutions at 1·4, 1·3, 1·2, 1·1, 1·0, 0·9, 0·8, and 0·7 M concentrations were layered in that order into the tubes. A final 0·25-ml layer of 0·35 M sucrose was placed on top of the tube, and the tubes were centrifuged for 120 min at 35,000 rpm with the SW39 rotor. After centrifugation, holes were punched into the bottom of the tubes and successive fractions of 0·5 ml were collected. Corresponding fractions from the three gradient tubes were pooled and protein, radioactivity, and total γABA measured in each pooled fraction. For electron microscopy, aliquots of each fraction were fixed for 30 min at 4° with equal volumes of 1% osmic acid in veronal acetate buffer, pH 7·4, containing 0·4 M sucrose, and centrifuged in a Beckman-Spinco microfuge for 15 min at 15,000 *g*. The pellets were dehydrated in acetone, embedded in epoxy resin in the microfuge tubes, sectioned, and double-stained with uranyl acetate and lead hydroxide in a nitrogen atmosphere. The electron micrographs were obtained with a Hitachi HU-10 electron microscope.

RESULTS

Morphological observations

Electron micrographs of subfractions prepared by density gradient procedures from crude mitochondrial and microsomal particulates are presented in Figs. 1–7.

The unfractionated mitochondrial preparation was highly heterogeneous. In the subfractioned material of the gradient no particles could be obtained from fraction 1. Fractions 2 and 3 (Fig. 1) consisted largely of free mitochondria. In the subsequent fractions the number of mitochondria decreased markedly, relative to other types of particles. In fraction 4 a number of mitochondria was enclosed within larger, irregularly shaped particles which were rich in vesicular material. These have been described as pinched-off presynaptic nerve endings.[11, 12] Nerve endings were even more conspicuous in fraction 5 (Fig. 2) and fraction 6. A relatively smaller proportion of the particles was made up of nerve endings in fraction 7 (Fig. 3) and fewer still were found in fraction 8. From fractions 5–8 there was a progressive relative increase in particles which were round or oval in shape, exhibiting variable electron density, and usually having a well-defined limiting membrane. These particles, which fit the description of microsomes,[13] were already noted in fraction 4 but were hardly detectable in fractions 9 and 10. From fraction 7 on there was a progressive increase in the appearance of myelin structures,[11, 12] which became the almost exclusive constituents of fractions 9 and 10 (Fig. 4). In the microsomal preparations, fractions 1 and 2 were devoid of particles, and fraction 3 had insufficient material for embedding. The morphology of the particles in fraction 4 (Fig. 5); nos. 5 and 6 (Fig. 6); nos. 7 and 8 (Fig. 7) was similar. In all of them the major constitutents appeared to be the round to oval particles already described as microsomes in the crude mitochondrial subfractions. Differences in size, shape, and electron density seen among the microsomal particles could well have depended on the plane of section. In this series there could not be found electron-dense ribosomes (100–150Å) either attached to the limiting membrane of the microsomes or free from them. In addition to the microsomes, a number of membranous strands could be seen which were relatively more abundant in fractions from the higher sucrose densities than in those from the lower densities. They may be fragments of cell membranes, but at times they appeared to have a double membrane structure suggestive of a collapsed microsome. Fraction 10 (Fig. 7), and to a lesser extent fraction 9, showed particles which clearly differed from the microsomal elements in that they were limited by a strongly osmiophilic circumference and often contained spherical inclusions of a similar type. A few of these particles could be said to be myelinoid because of the lamellar arrangement of the electron-dense membrane structures. None of the microsomal subfractions contained any particles which could be identified as mitochondria or nerve endings.

Results with subfractions of the crude mitochondrial and microsomal suspensions

Consistent biochemical patterns were found in each of three separate experiments with the crude mitochondrial suspensions. The results of the chemical measurements (total protein, total γABA, and radioactivity in each fraction of the density gradient) are presented in Table 1 and Fig. 8. Table 2 and Fig. 9 show the average results of two duplicate density gradient experiments carried out on microsomal particles. It can be seen from a comparison of Figs. 8 and 9 that the distribution of protein, total γABA,

TABLE 1. "CRUDE MITOCHONDRIAL" SUSPENSIONS

	Sucrose Molarity	Protein (mg/ml)	Radioactivity (cpm/ml)	Total γABA (μg/ml)	Ratios Radioactivity (cpm/mg prot.)	Ratios Total γABA (μg/mg prot.)	Spec. activity (cpm/μg γABA)
Resuspension in 2 M sucrose		18·8	66,000	29·2	3,510	1·6	2·260
Material submitted to gradient		17·5	62,000	27·5	3,540	1·6	2,260
Material collected from gradient*		16·3	61,800	27·3	3,790	1·7	2,260
Recovery		94%	100%	99%			
Gradient fractions 1	2	0·30	28,300	8·4	94,300	28·0	3,400
2	2–1·4	1·27	12,300	3·8	9,700	3·0	3,200
3	1·4–1·3	2·37	3,300	3·1	1,400	1·3	1,100
4	1·3–1·2	4·27	9,800	5·3	2,300	1·2	1,900
5	1·2–1·1	7·43	19,600	9·7	2,600	1·3	2,000
6	1·1–1·0	4·70	13,300	6·7	2,800	1·4	2,000
7	1·0–0·9	1·32	3,700	2·1	2,800	1·6	1,800
8	0·9–0·8	0·75	1,300	0·9	1,700	1·2	1,400
9	0·8–0·7	0·96	600	0·7	600	(0·7)†	(900)†
10	0·7–0·35	1·11	400	0·3	400	(0·3)†	(1,300)†

* Since 0·75 ml of material was submitted to the gradient (per tube) and 0·5-ml fractions were collected, the content of 1 ml of material (i.e. its concentration) was calculated by adding the concentrations of all 10 fractions and multiplying the totals by 0·67.

† Because of the small amounts of γABA, radioactivity, and protein the validity of these values is in doubt.

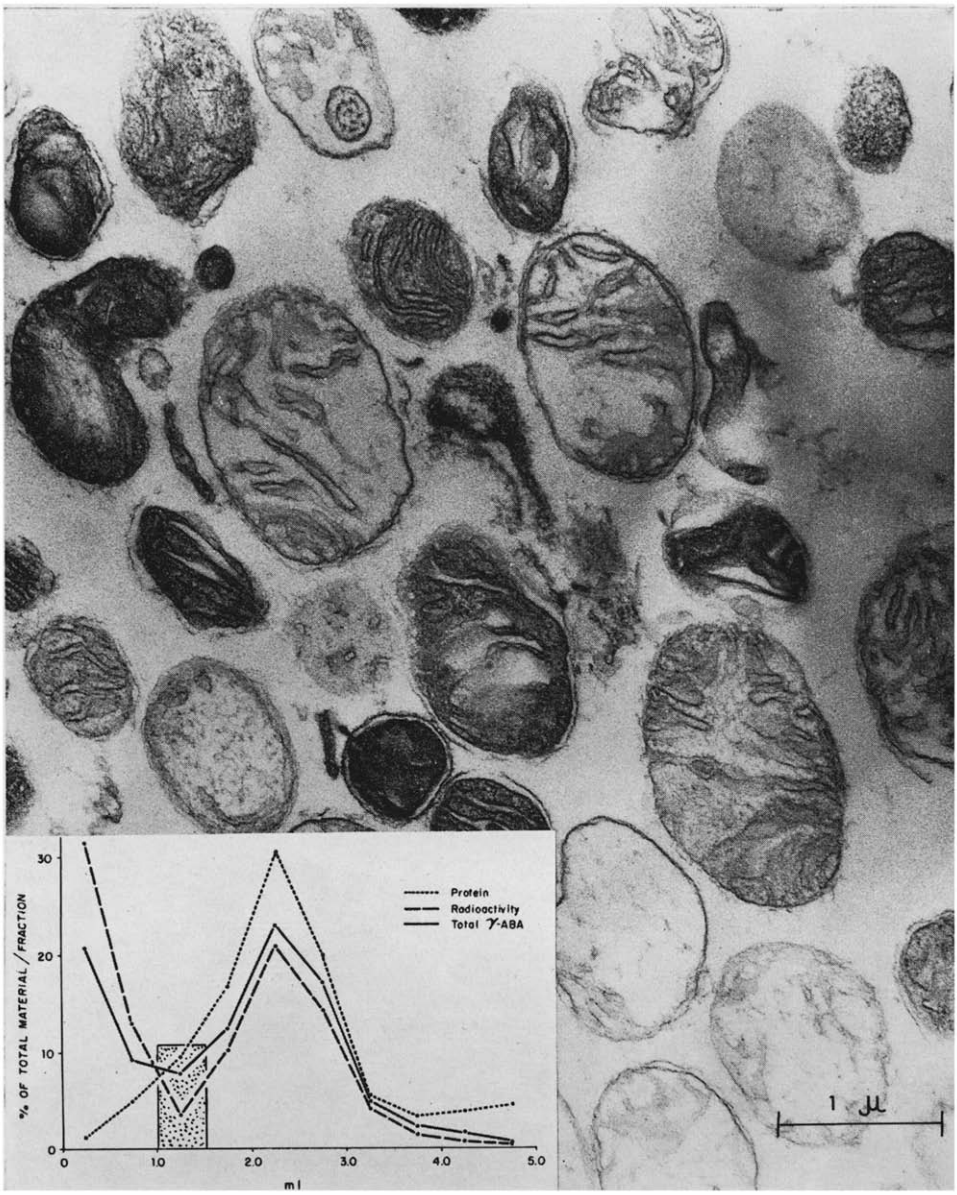

FIG. 1. Mitochondrial gradient fraction 3, consisting principally of mitochondria.

facing page 1216 B.P.

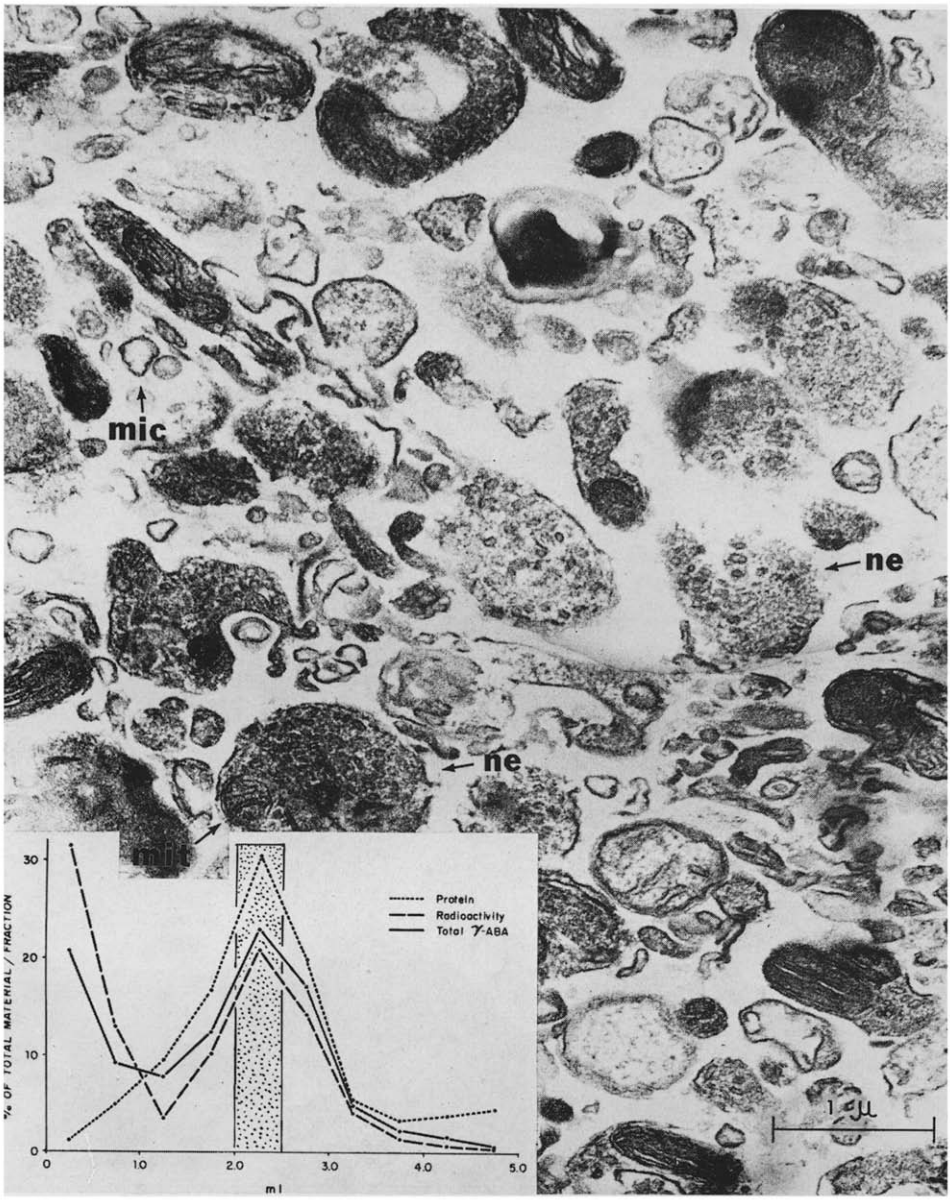

FIG. 2. Mitochondrial gradient fraction 5, consisting principally of nerve-ending particles (ne), many of which contain mitochondria. Microsomes (mic) are present in the fraction.

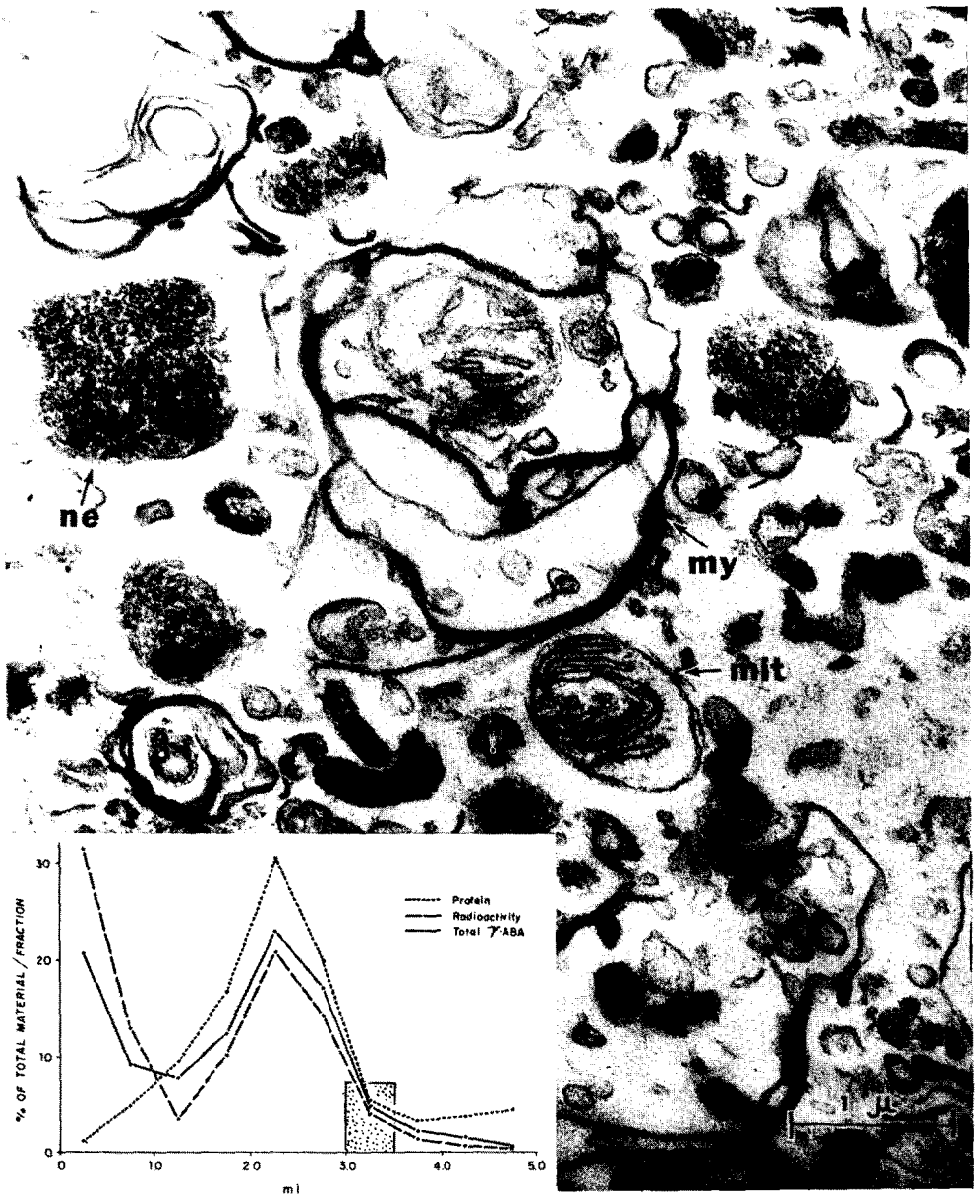

FIG. 3. Mitochondrial gradient fraction 7, containing all previously identified structures in addition to myelin (my).

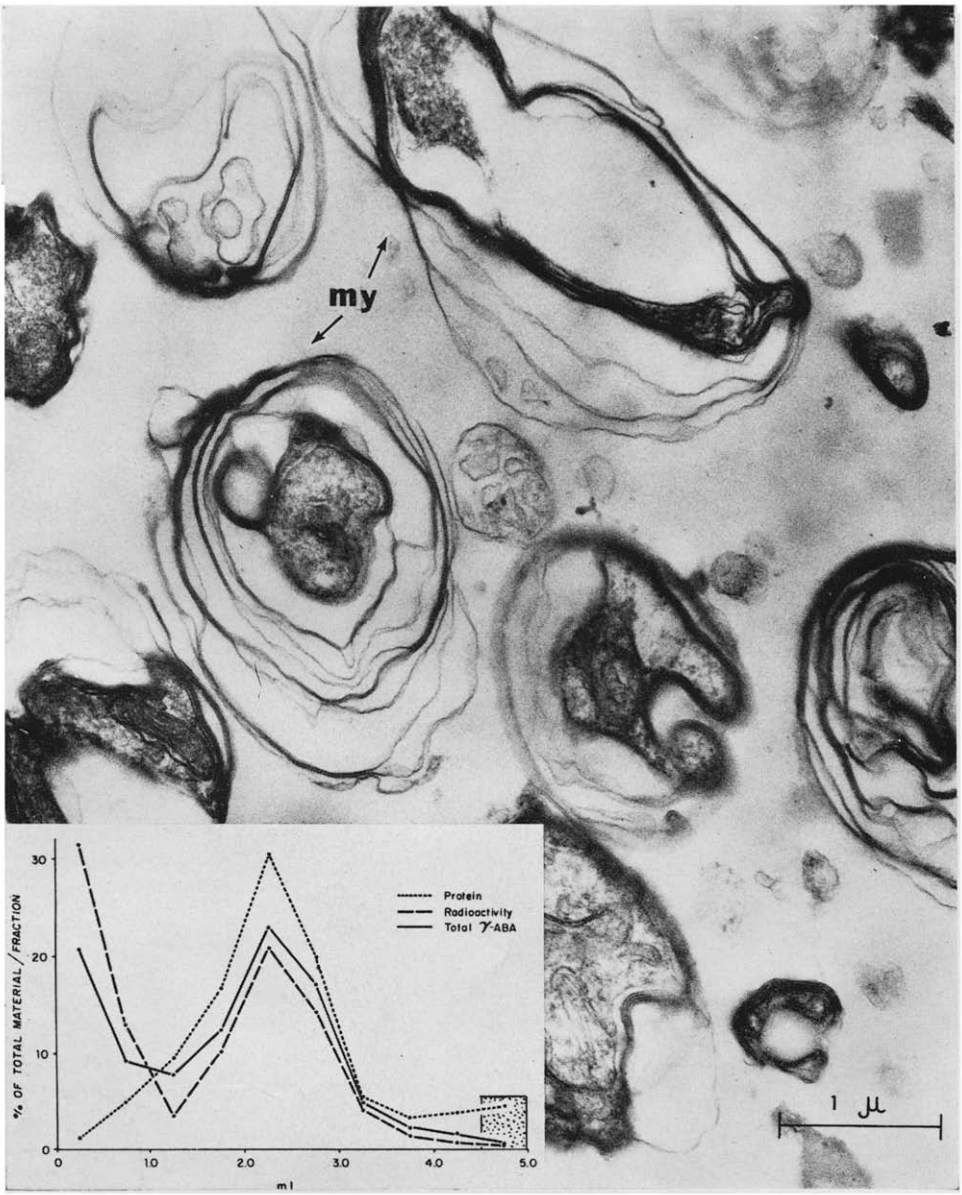

FIG. 4. Mitochondrial gradient fraction 10, consisting principally of myelin (my).

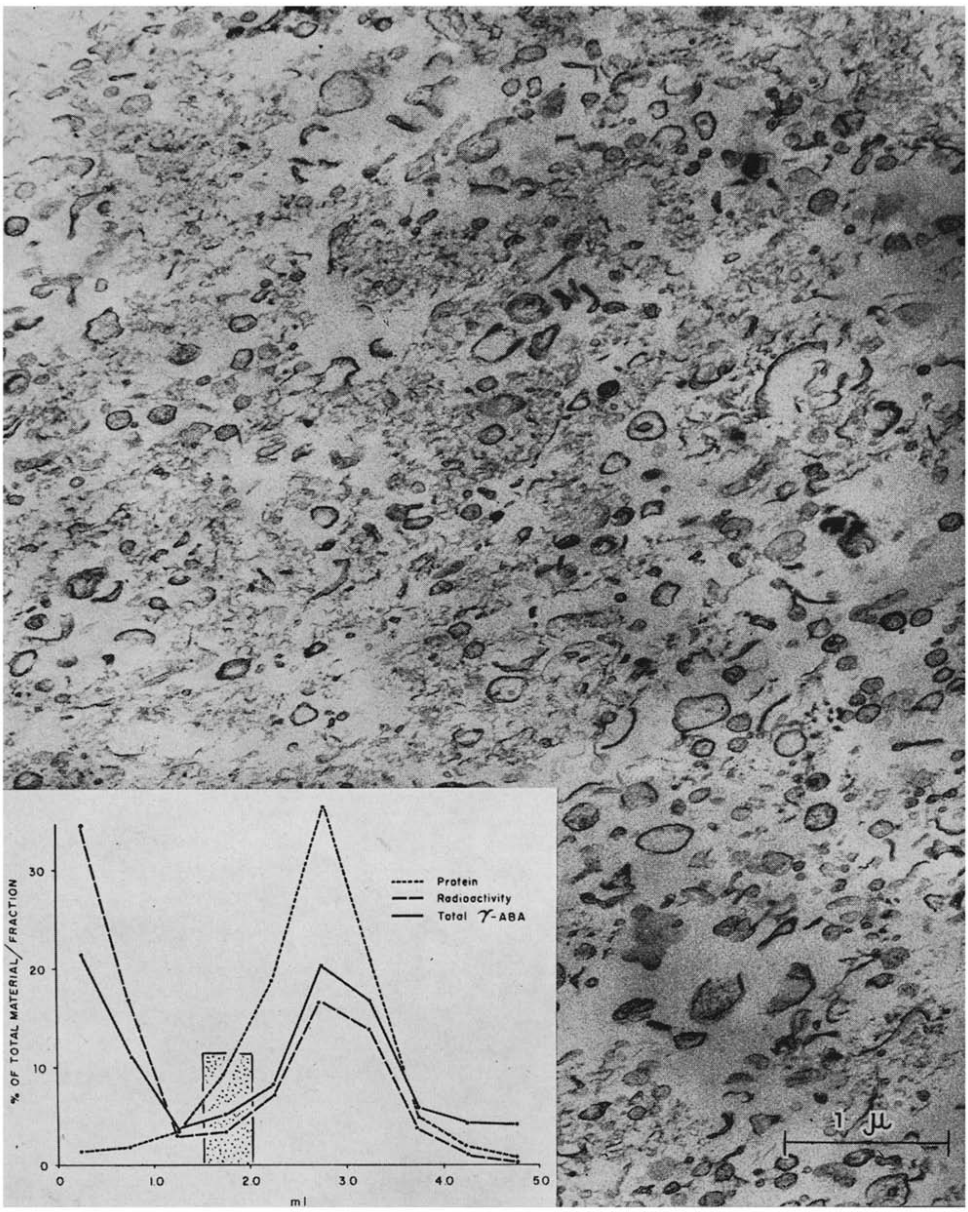

FIG. 5. Microsomal gradient fraction 4, consisting principally of microsomes.

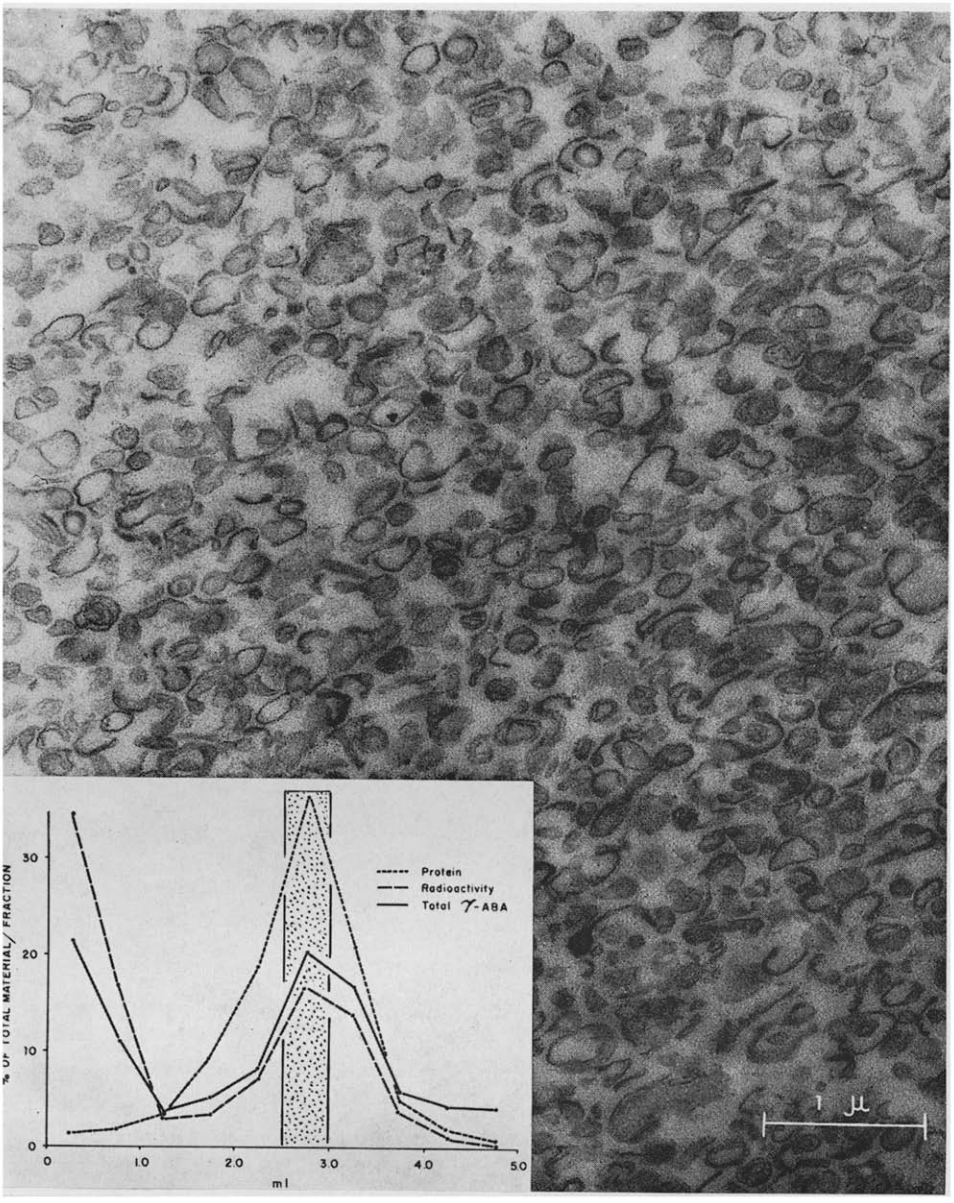

FIG. 6. Microsomal fraction 6, consisting principally of microsomes.

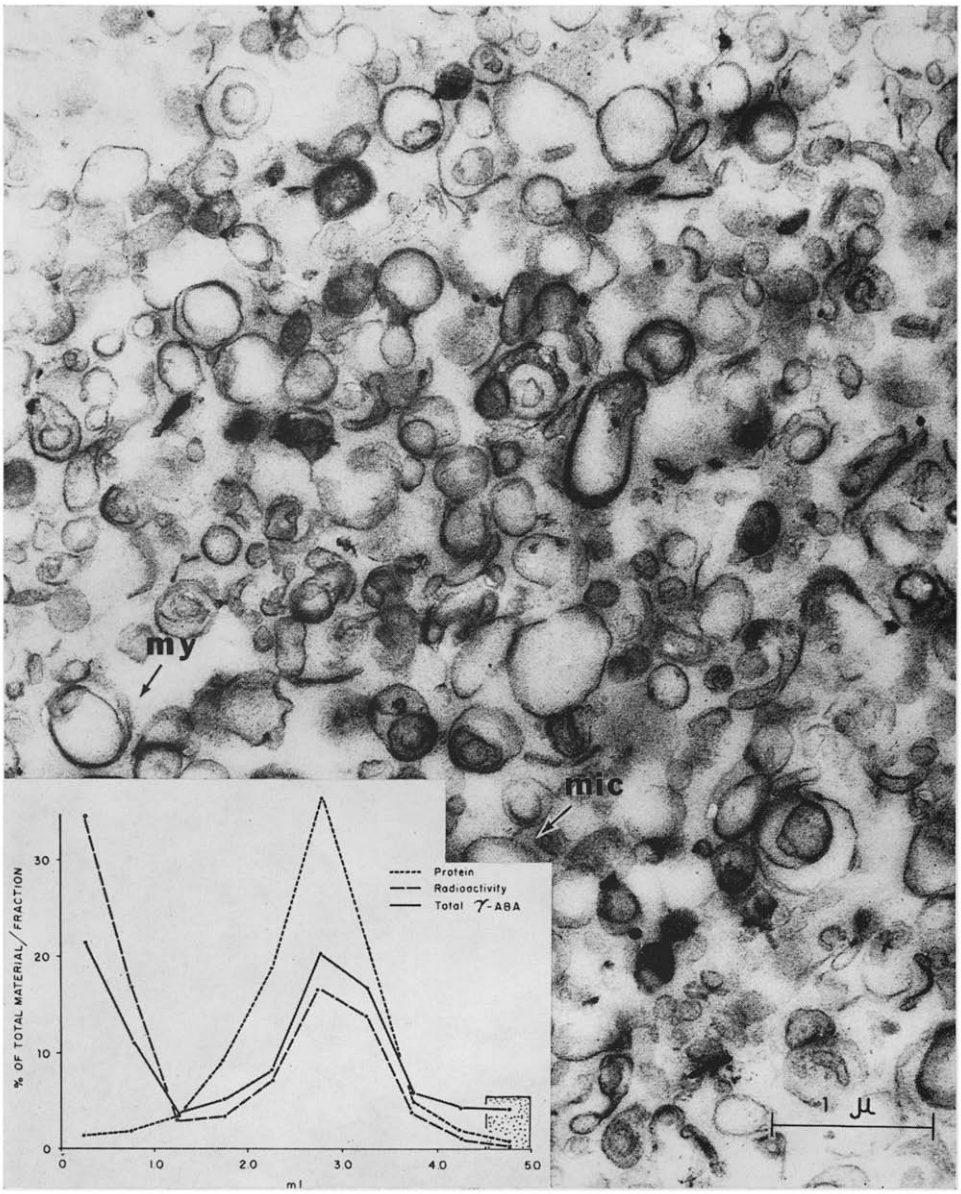

FIG. 7. Microsomal fraction 10, consisting of microsomes (mic) and small myelin (my) structures.

and radioactivity followed somewhat similar patterns in both preparations, the microsomal material showing a general shift of maximal values of the measured variables towards the lower densities. Protein, which was virtually absent in the bottom fractions (where the sample had been placed), rose with the decreasing densities to a peak (crude mitochondrial fraction 5, and microsomal fraction 6), then decreased progressively to low or negligible levels. Radioactivity and γABA showed substantially the same distribution as the protein in all fractions except the high sucrose density fractions (1 and 2), where they were strikingly high in comparison with the low protein levels.

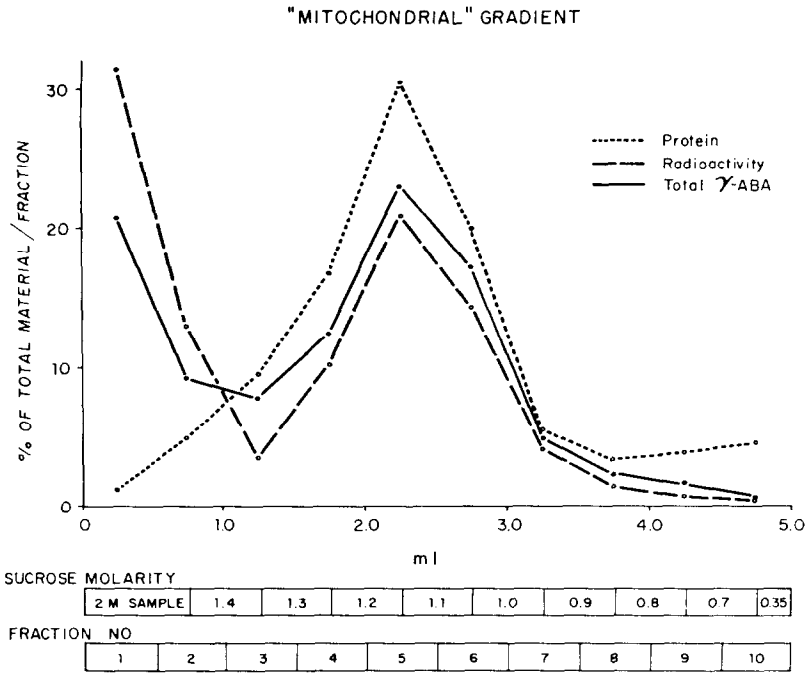

FIG. 8. Mitochondrial gradient. Per cent of total γABA, radioactivity, and protein in the mitochondrial gradient fractions.

Three major regions can be distinguished in both gradients: (a) the low density fractions (crude mitochondrial, 8, 9, and 10); microsomal (9 and 10) have little protein, γABA, or radioactivity. Therefore, myelin (Fig. 4) and the myelinoid particles (Fig. 7) are of little if any relevance with regard to the ability of the unfractionated material to associate with γABA. (b) The central fractions (crude mitochondrial, 4–7, and microsomal, 4–8) contained most of the protein and a large proportion of the γABA and the radioactivity. Thus, γABA and radioactivity were associated with particulate matter such as microsomes (Figs. 5 and 6) and nerve endings plus microsomes (Figs. 2 and 3). (c) The high density fractions (1, 2, and 3) appear to have most of their γABA and radioactivity in a free form as shown by the scarcity of particles in microsomal fractions 1, 2, and 3 and crude mitochondrial fraction 1. γABA of a low specific activity was associated with mitochondria (fractions 2 and 3, Fig. 1) prepared from the crude mitochondrial fraction.

TABLE 2. "MICROSOMAL" SUSPENSIONS

			Protein	Radioactivity	Total γABA	Radioactivity	Ratios Total γABA	Spec. activity
			(mg/ml)	(cpm/ml)	(μg/ml)	(cpm/mg prot.)	(μg/mg prot.)	(cpm/μg γABA)
Resuspension in 2 M sucrose			13·6	79,400	17·3	5,800	1·27	4,600
Material submitted to gradient			11·8	70,500	15·3	5,900	1·29	4,600
Material collected from gradient*			12·8	71,500	16·3	5,600	1·27	4,400
Recovery			108%	101%	106%			
		Sucrose molarity						
Gradient fractions	1	2	0·28	36,500	5·2	130,000	18·6	7,000
	2	2–1·4	0·39	17,700	2·7	45,400	6·9	5,600
	3	1·4–1·3	0·77	3,000	0·8	3,900	1·0	3,800
	4	1·3–1·2	2·16	4,000	1·3	1,850	0·6	3,100
	5	1·2–1·1	4·10	8,400	2·2	2,050	0·5	3,800
	6	1·1–1·0	6·40	17,900	5·0	2,800	0·8	3,600
	7	1·0–0·9	3·60	14,200	4·0	3,940	1·1	3,600
	8	0·9–0·8	1·00	4,200	1·4	4,200	1·4	3,000
	9	0·8–0·7	0·40	1,000	0·9	2,500	(2·2)†	(1,100)†
	10	0·7–0·35	0·20	175	0·8	900	(4·0)†	(200)†

* Calculations as in Table 1.
† See footnote to Table 1.

These data indicated that the resuspension of the original pellets in 2 M sucrose and their subsequent fractionation in the density gradient, while resulting in a considerable loss of radioactivity and γABA, still allowed much of it to remain with the particulate matter of the central fractions. Further evidence for this conclusion was sought by diluting aliquots of all fractions with equal volumes of water (to decrease the density), centrifuging for 30 min at 105,000 *g* in the cold, and measuring the radioactivity of the supernatant fluids. While the osmotic effect of such treatment might have been expected to produce release of radioactivity from the particles, as much as 40 per cent of the counts was still found to be associated with the pellets (Table 3).

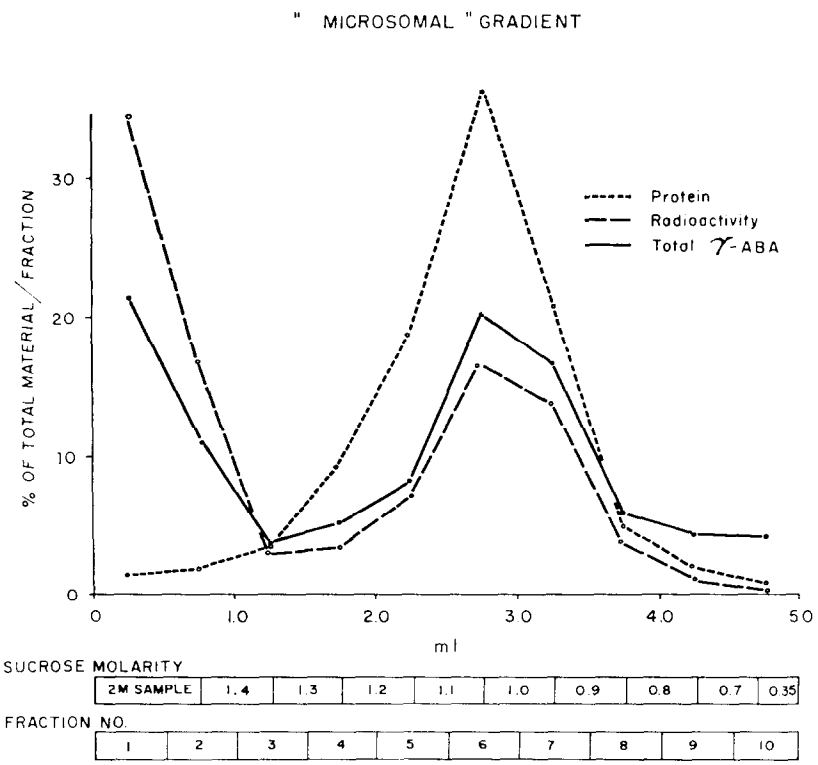

FIG. 9. Microsomal gradient. Per cent of total γABA, radioactivity, and protein in the microsomal gradient fractions.

As shown in Tables 1 and 2, the free γABA of the bottom fractions had a higher specific activity than that of the unfractionated material. Conversely, the specific activity of the γABA associated with particles (fractions 4–7) was lower than that of the unfractionated material. The large amounts of free γABA found in both preparations after the gradient subfractionation could only have resulted from a release of γABA previously associated with the particulate matter or from a breakdown of the particles during the course of the experiment. It could not be attributed to free γABA which had been occluded in the unwashed pellet, since calculations indicated that this can be no more than 5% of the total γABA associated with any of the unwashed pellets. If there were only one isotopically labeled pool in the saline-incubated particles, either of the

TABLE 3. BOUND RADIOACTIVITY IN DENSITY GRADIENT FRACTIONS*

Fraction no.:	1	2	3	4	5	6	7	8	9	10
A. "Crude mitochondrial" preparation										
Collected fraction (cpm/ml)	28,500	13,500	4,200	14,900	22,100	11,600	3,100	1,150	570	380
Supernatant material (cpm/ml)	30,500	14,000	3,950	12,100	14,000	7,100	2,100	820	330	115
Bound radioactivity (%)			(6)†	19	37	39	32	29	(42)†	(70)†
B. "Microsomal" preparation										
Collected fraction (cpm/ml)	43,000	20,200	3,840	6,500	13,400	21,000	15,500	5,600	1,200	120
Supernatant material (cpm/ml)	40,000	18,700	3,640	5,300	10,500	14,600	9,900	4,200	675	50
Bound radioactivity (%)	(7)†	(7)†	(5)†	18	25	30	36	25	(44)†	(57)†

* Each collected fraction was diluted with an equal volume of distilled H_2O and centrifuged in the cold for 30 min at 105,000 *g*. The counts in the supernatant materials were multiplied by 2 to correct for the initial dilution.

† See footnote to Table 1.

above processes would have yielded free γABA of the same specific activity as that of the unfractionated material and left unaffected the specific activity of the γABA associated with the particles. Therefore, the above findings establish for both preparations that more than one pool of γABA was associated with particulate matter after incubation in the presence of Na^+ and prior to the subfractionation procedure. They are consistent with the interpretation that the subsequent losses occurred selectively at the expense of one pool (or pools) relative to another (or others).

According to the model described in the Introduction,[10] the high specific activity pool (REP) established in the presence of Na^+ would be expected to leave the particles under the Na^+-free conditions of the gradient. This pool would thus be the major, if not the only, source of the high specific activity γABA free in the bottom fractions. The low specific activity (SEP) would largely remain trapped in the particles in the Na^+-free medium and thus would account for the considerable amounts of radioactivity which remained associated with the particles of the central fractions. For crude mitochondrial preparations (for which the model was originally derived) the gradient data of Table 1 are quantitatively in agreement with these expectations. The free radioactivity of fractions 1 and 2 had approximately 45% of the total radioactivity of the original unfractionated particles and the calculated free γABA* was about 25 per cent of the total γABA. The REP in unfractionated, but otherwise similar, preparations has been calculated[10] to contain approximately half the radioactivity and one quarter of the γABA associated with the particles. Although a comparable detailed analysis of the γABA pools in microsomal preparations has not yet been carried out, all the evidence thus far available[8] has pointed to a strong similarity in the ability of the two preparations to associate with γABA. The extension to both preparations of the model with REP and SEP seems justified by the finding in the microsomal gradient (Table 2) that the γABA of high specific activity which has been released (fractions 1 and 2) and the γABA of low specific activity (fractions 4–8) which has been retained by the particles is entirely analogous to what has been demonstrated in the crude mitochondrial gradients. One of the suggestions made in the model was that the slowly equilibrating isotope had once been bound to Na^+-dependent carrier sites and subsequently released into the interior of the particle, which has a high content of endogenous γABA.[10] Consistent with this interpretation was the observation that the particulate fractions retaining significant amounts of radioactivity (fractions 4–7 of both preparations) were without exception relatively rich in endogenous γABA. Moreover, the specific activity was practically constant (Tables 1 and 2), even when the γABA to protein ratios varied from fraction to fraction (cf. "microsomal" fractions 4–7).

Special consideration should be given to fraction 3 of the crude mitochondrial gradient. This fraction consisted almost exclusively of mitochondria, and the specific activity (Table 1) was lower than that of the adjacent fractions 2 and 4. This fact indicates that the γABA content of this fraction cannot be attributed to diffusion of

* An estimate of the mitochondrial contribution to the γABA of fraction 2 can be made on the assumption that the γABA and protein of fraction 3 is entirely mitochondrial and that, therefore, the ratio of γABA protein (1·3 μg/mg protein) of that fraction represents that of the mitochondria themselves. Thus, from the protein content of fraction 2 (1·27 mg/ml) the mitochondrial γABA of the fraction would be $1{\cdot}3 \times 1{\cdot}27 = 1{\cdot}65$ μg/ml and its nonmitochondrial, or free, γABA, $3{\cdot}8 - 1{\cdot}65 = 2{\cdot}15$ μg/ml or about 4% of the total γABA in the gradient.

soluble γABA from the bottom of the gradient tube nor to contamination of the fraction by nerve-ending particles. Thus it must be deduced that the mitochondria contain endogenous γABA, but it cannot be said with any certainty if the isotope in this fraction is truly associated with the mitochondria. Some unpublished preliminary experiments with relatively purified mitochondria suggest that the isotope probably enters mitochondria, and that they may contain Na^+-dependent binding sites.*

The gradient data established the existence of the SEP in various particulate fractions. The carrier-mediated diffusion model states that the movement of isotope into the SEP is mediated by Na^+-dependent binding sites which exist in the membrane of the particle. Thus the particles that can establish a SEP should also exhibit a Na^+-dependent binding of the isotope. As a means of testing this hypothesis crude mitochondrial suspensions were treated as in the previous experiments, with the exception that nonradioactive instead of radioactive γABA was present during the saline incubation. After the gradient separation, the fractions were diluted with two volumes of a NaCl-buffered solution (so prepared as to yield the same final concentrations of NaCl and buffer as in the earlier saline suspension), ^{14}C-γABA was added (0·75 μg/ml final concentration), and incubation was carried out again for 30 min at 0–4°. At the end of the incubation, radioactivity was measured in the saline suspensions as well as in the supernatant fluids derived from them by 15-min centrifugation at 15,000 *g* (see Methods). The protein and γABA contents at various stages of the procedure were comparable with those in the previous experiments. Table 4 shows the results, averaged from two experiments. Significant levels of bound radioactivity could be detected in fractions 4 to 7; little, if any, radioactivity was found to be accumulated by particles from either the bottom (fractions 1–3) or the top (fractions 9 and 10), in spite of the fact that the protein contents were comparable with those of fractions showing binding. When the levels of radioactivity bound by fractions 4–7 were calculated on a protein basis, they were found to be similar.

DISCUSSION

The ability to associate with endogenous and exogenous γABA is found in various particles (microsomes, nerve endings, and mitochondria). Microsomal fractions 4–8 were described in the preceding sections as being largely composed of two classes of particles, the closed vesicular microsomes and membranous strands. The increases of radioactivity and γABA per mg protein in fractions 4–7, together with the persistence of approximately the same specific activity throughout, point to a progressive increase in these fractions of the number of γABA-retaining particles relative to the particles that do not retain γABA. Examination of the electron micrographs showed that the vesicular microsomes also tended to increase in relative amounts progressively from fractions 4–7, while the membranous strands decreased. The recognition that most of the γABA of fraction 3 from the crude mitochondrial mixture is associated with particles, coupled with the morphological homogeneity of these particles, leaves no doubt about free mitochondria being another class of γABA-retaining particles. Preliminary unpublished data suggest that the mitochondria may have the ability to incorporate isotopically labeled γABA into an endogenous pool. The particles

* Kuriyama,Weinstein and Roberts unpublished data.

TABLE 4 ^{14}C-γABA BINDING ABILITY OF THE DENSITY GRADIENT FRACTIONS FROM A "CRUDE MITOCHONDRIAL" SUSPENSION

Average of 2 experiments.

Fraction no.:	1	2	3	4	5	6	7	8	9	10
^{14}C-γABA binding (%)	(0·8)†	(1·1)†	(1·4)†	9·5	13·5	10·8	3·5	(2·5)†	(0·6)†	(0·5)†
Bound radioactivity (cpm/ml)			(420)†	2,850	4,050	3,240	1,050			
Protein in saline incubate (3× diluted fractions) (mg/ml)			0·70	1·25	2·25	1·40	0·47			
Bound radioactivity per unit protein cpm/mg)			(600)†	2,280	1,800	2,315	2,234			

† See footnote to Table 1.

in fractions 4 or 7 of the crude mitochondrial gradient show similar specific activities and similar γABA to protein ratios. This would suggest that the fractions are predominantly composed of a single class of particles. The morphological studies demonstrate considerable heterogeneity and thus make it possible to entertain an alternative conjecture that in these fractions there are different γABA-retaining particles which maintain a constant proportionality to each other; but even in this case it would appear likely that the uniform chemical characteristics are due to the dominance of the nerve-ending particles. This is a particle which previously has been shown[5, 6] to contain endogenous γABA. It was previously suggested that some, if not all, of the endogenous γABA is retained within the particles in a soluble nonbound form. This agrees with the previous findings[9] that hypotonicity and a variety of treatments which are known to break such particles prevented the accumulation of ^{14}C-γABA by the particles.

These results demonstrate the presence of endogenous γABA within the mitochondria, microsomes and, most probably, nerve endings. A Na^+-dependent carrier-mediated movement of ^{14}C-γABA across a membrane into an endogenous γABA pool is indicated for microsomes and most probably indicated for nerve-ending particles. No such conclusion can be drawn from our data on mitochondria.

REFERENCES

1. E. ROBERTS and E. EIDELBERG in *International Review of Neurobiology*, C. C. PFIEFFER and J. R. SMYTHIES, Eds., vol. 2, p. 279. Academic Press, New York (1960).
2. E. ROBERTS, Ed. *Inhibition in the Nervous System and γ-Aminobutyric Acid.* Pergamon Press, New York (1960).
3. K. A. C. ELLIOT and H. H. JASPER, *Physiol. Rev.* **39**, 383 (1959).
4. K. A. C. ELLIOT and N. M. VAN GELDER, *J. Physiol. (Lond.)* **153**, 423 (1960).
5. H. WEINSTEIN, E. ROBERTS and T. KAKEFUDA, *Biochem. Pharmac.* **12**, 503 (1963).
6. R. W. RYALL, *J. Neurochem.* **11**, 131 (1964).
7. N. F. SHATUNOVA and I. A. SYTINSKY, *J. Neurochem.* **10**, 701 (1964).
8. S. VARON, H. WEINSTEIN and E. ROBERTS, *Biochem. Pharmac.* **13**, 269 (1964).
9. K. SANO and E. ROBERTS, *Biochem. Pharmac.* **12**, 489 (1963).
10. H. WEINSTEIN, S. VARON, D. R. MUHLEMAN and E. ROBERTS, *Biochem. Pharmac.* **14**, 273 (1965).
11. E. G. GREY and V. P. WHITTAKER, *J. Anat.* **96**, 79 (1962).
12. E. DE ROBERTIS, A. P. DE IRALDI, G. R. DE LORES ARNAIZ and L. SALGANICOFF, *J. Neurochem.* **9**, 23 (1962).
13. V. HANZON and G. TOSCHI, *Exp. Cell. Res.* **21**, 332 (1960).