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STUDIES OF SUB-CELLULAR DISTRIBUTION OF γ-AMINOBUTYRIC ACID AND GLUTAMIC DECARBOXYLASE IN MOUSE BRAIN*

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Abstract—A study has been made of the γaminobutyric acid (γABA) content and L-glutamic acid decarboxylase (GAD) activity of various subcellular fractions prepared from homogenates of mouse brain in a sucrose density gradient. Portions of the fractions studied chemically also were examined morphologically by electron microscopy. The largest proportions of the sedimentable γABA and GAD were found to be associated with fractions consisting predominantly of nerve-ending fragments. The distribution of γABA and GAD did not coincide among these fractions, which were shown by electron microscopy to be morphologically heterogeneous.

AMONG vertebrates the enzyme, glutamic decarboxylase, and the reaction product, γ-aminobutyric acid, have been found solely in the central nervous system. This enzyme, in conjunction with the enzymes γ-aminobutyric-α-ketoglutaric transaminase and succinic semialdehyde dehydrogenase, is responsible for the metabolic pathway: glutamate → γ-aminobutyrate → succinic semialdehyde → succinate.[1] Therefore in the central nervous system, but not in other vertebrate tissues, there exists a metabolic shunt around the α-ketoglutarate oxidase system of the tricarboxylic acid cycle. It has been estimated that this shunt may account for as much as 40% of the oxidative metabolism of the brain.[2]

At the present time it is not clear whether the shunt operates in neurons or glia, or whether certain of the metabolic steps may occur in the glia and other steps in the neuronal elements. In an attempt to clarify these points, studies have been carried out on an anatomical and histological level. The results[3–6] have suggested that γABA, GAD, and the γ-aminobutyric-α-ketoglutaric transaminase may be associated with nerve-cell bodies rather than with axis cylinders or myelin. However, there is still no conclusive proof that some or all of the enzymes are in part or completely in the intimately associated glial elements of the areas studied. The possibility that the decarboxylase and transaminase may be present in different cell types or different intracellular sites has been suggested to account for the finding that hydroxylamine and aminooxyacetic acid are potent inhibitors *in vitro* of the decarboxylase and transaminase, whereas only the transaminase is inhibited in the brains of treated animals.[7]

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On a subcellular level it has been reported that the entire metabolic shunt can be carried out by a mitochondrial fraction.[8] On the other hand Lovtrup[9] found very little GAD in a mitochondrial preparation of rat brain.

Another aspect of the problem, which may be of importance from a metabolic and functional standpoint, is the report that as much as 60% of the γABA can be recovered from brain homogenates in a sedimentable form,[10] and that under proper conditions the subcellular particulates of brain homogenates are capable of accumulating exogenously added 1-^{14}C-γ-aminobutyric acid in the absence of any apparent energy source.[11] Here again, little is known about the cellular and subcellular localization.

In recent years it has become evident that brain homogenates may not only yield information pertaining to subcellular distribution but also to neuronal localization. Gray and Whittaker[12] and De Robertis *et al.*[13] have reported that "mitochondrial" fractions of brain homogenates are rich in complex cellular fragments which they have interpreted as presynaptic nerve endings and that the nerve-ending fragments can be separated from various subcellular particles by density-gradient centrifugation.

Since cellular and subcellular localization is of importance in the formulation of hypotheses concerning the functional significance of γABA and the metabolic shunt in which it may participate, an investigation of the distribution of γABA and GAD in brain homogenates was undertaken.

METHODS

Brain preparations. Forty-five milliliters of a 10% mouse brain homogenate was prepared in 0·8 M sucrose. The homogenate (40 ml) was centrifuged at 800 × *g* for 10 min and the pellet washed twice with a total volume of 15 ml of the sucrose solution. The combined supernatant and washings were centrifuged at 20,000 × *g* for 30 min and a pellet was obtained which was subfractionated by means of density gradient centrifugation.

The choice of 0·8 M sucrose for obtaining the 20,000-*g* pellet was based on reports[12,13] which indicated that myelin fragments do not sediment in this concentration of sucrose. The 20,000-*g* pellet would therefore be expected to be relatively devoid of this material. In a preliminary experiment there was no significant difference in the amount of sedimentable γABA collected in these pellets in 0·4 or 0·8 M sucrose, a finding that justified the use of the 0·8 M sucrose in subsequent experiments.

The subfractionation of the 20,000-*g* pellet was based on the preliminary finding that this fraction contained four to five times more γABA than the 800-*g* pellet, and approximately 2·5-fold more γABA than could be obtained by centrifuging the supernatant of the 20,000-*g* pellet at 80,000 × *g* for 150 min. On a per milligram protein basis the 800-*g* pellet was least enriched with respect to γABA. The 20,000-*g* and 80,000-*g* pellets were approximately equivalent. Since other reports[12, 13] suggest that the 20,000-*g* pellet should be very heterogeneous it appeared that a subfractionation of this material should ultimately yield a subcellular component which was richest in γABA per milligram protein and which might also account for the major share of the sedimentable γABA.

The 20,000-*g* pellet was transferred to a 25-ml cylinder with 0·8 M sucrose to give a total volume of 10 ml. Fifteen ml of 2 M sucrose was then added to give a final concentration of 1·5 M sucrose. The pellet was dispersed in the medium by brief

homogenization. Five-ml aliquots were placed in each of four tubes which are used with the No. 40 head of a Spinco preparative ultracentrifuge; 6 ml of 1·4 M sucrose was layered on top of each tube. The tubes were then centrifuged for 90 min at 80,700 × *g*. The material that concentrated at the surface of the tube in this preliminary density separation is designated as T1. This procedure served to remove the particles from any residual soluble components that remained at the bottom of the tube. The T1 fractions were combined and resuspended in a total volume of 10 ml of 1·4 M sucrose. Aliquots of 2 ml were placed in each of three tubes for the Spinco SW39 swinging-bucket rotor and 1 ml each of 1·3, 1·2, and 1·0 M sucrose was layered on top. Centrifugation for 90 min at 115,000 × *g* resulted in the formation of a film of particles at the surface and a broad band of particles at each of the three interface positions (Fig. 1). Five fractions were removed successively by aspiration from the surface;

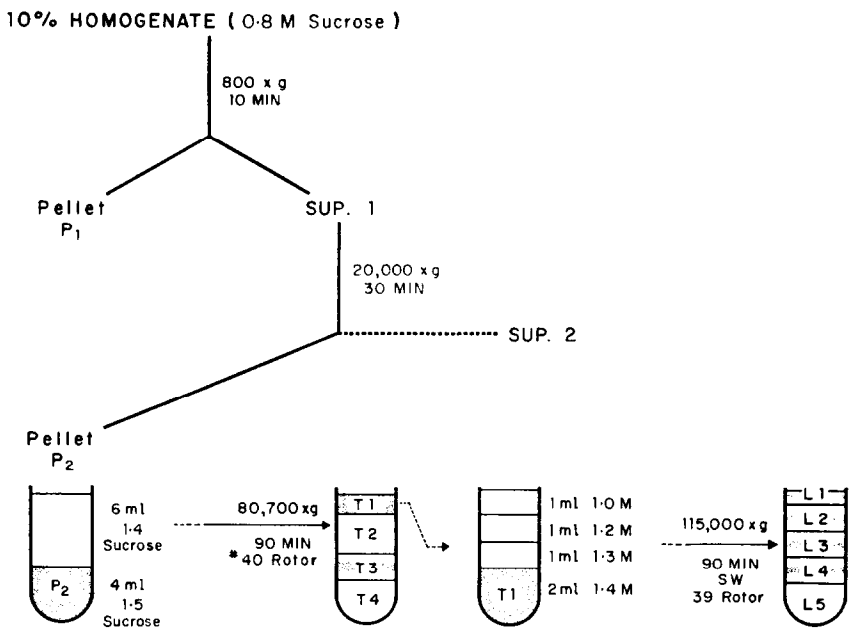

FIG. 1. Fractionation procedure.

equivalent materials from the three tubes were combined. Aliquots of these fractions were used for assays and electron microscopy. Residual portions of the original homogenate and preliminary centrifugation fractions were saved for analysis.

Proteins were determined by the method of Lowry, *et al.*[14] *Glutamic decarboxylase* activity was determined essentially according to Sisken *et al.*[15] To 0·36 ml of substrate, which contained 0·266 μc glutamic acid-1-^{14}C, 75 μM glutamic acid, 15 μg pyridoxal phosphate, and 0·055 M potassium phosphate buffer (pH 6·4), there was added 0·4 ml of a particular fraction. The $^{14}CO_2$ formed during a 30-min incubation at 37° was trapped in Hyamine base and counted in a Packard Tri-Carb liquid scintillation counter. In early experiments a second assay was carried out on each fraction using

0·2 ml of material plus 0·2 ml of the appropriate sucrose solution, in order to ascertain linearity of the reaction rate under the conditions employed.

Determinations of γABA were carried out as follows: 1-ml aliquots of the fractions were acidified with 0·01 N HCl to pH 4 to 5, diluted to about 6 ml with water, and heated in a boiling water bath for 3 min. The protein precipitate was removed by centrifugation. Some of the residual cloudiness was removed by forming an emulsion with an equal volume of chloroform and breaking the emulsion by centrifugation. The clarified aqueous phase, pH 4 to 5, was placed on a 5 × 0·9 cm column of Dowex 2-X8 (OH^- form). The column was washed with 30 ml of water and then eluted with 1 N acetic acid (30 ml).[16] The eluate which was now free of sucrose was dried by means of a fan and infrared heat. Three to four additions of water (each approximately 1 ml) were required to remove the last traces of acetic acid. The γABA content of this material was determined enzymatically as described by Baxter.[17] This procedure yielded recoveries of 95 to 100% when known amounts of γABA were added to brain homogenates.

Electron microscopy. To a 0·2-ml aliquot of a fraction there was added 0·2 ml of 1% osmic acid in veronal acetate buffer, pH 7·4, and the material fixed at 4° for 30 min. Centrifugation in a Coleman microfuge for 15 min, 15,000 × *g*, yielded a pellet which was then dehydrated in alcohol and embedded in an epoxy resin. Sections were stained with lead hydroxide in a nitrogen atmosphere for 30 min. The electron microphotographs were obtained with a Hitachi HU 10 electron microscope.

RESULTS

In these experiments there was four to five times more γABA and nine to eleven times more GAD in the 20,000-*g* pellet than in the 800-*g* pellet. On a per milligram protein basis the 20,000-*g* pellet contained approximately 1·6 times more γABA than the 800-*g* pellet and 2·8 times more GAD activity.

Centrifugation of the 20,000-*g* pellet in the 1·4 to 1·5 M sucrose gradient resulted in the flotation to the surface of 65 to 70% of the protein, γABA, and GAD. A relatively small band of particles remained at the 1·4 to 1·5 M interface. There was little or no purification of γABA or GAD on a per milligram protein basis either in the particles at the surface or at the interface. The final density gradient yielded a partial resolution of particulate-bound γABA and GAD and some degree of purification.

Tables 1 and 2 show the distribution of γABA and GAD, respectively, in the fractions obtained from the final density gradient centrifugation. The peak concentration and quantity of γABA was consistently in the L2 fraction (see Table 1). On a per milligram protein basis two maxima occur, one in the L2 and the other in the L5 fraction. The absence of particulates in the L5 region suggests that this second maximum represents γABA which is released during the process of rehomogenizing the T1 fraction in preparation for the final gradient. Therefore the maximum purity of particle-bound γABA as well as the largest quantity is associated with the L2 fraction.

The distribution of GAD (Table 2) differed from that of γABA. The enzyme was principally associated with the L2 and L3 fractions. A slightly larger quantity was consistently recovered in the L3 fraction, which also had a maximum concentration on a per milligram protein basis. This is in sharp contrast to the γABA distribution which dropped almost in half from the L2 to the L3 fraction.

From a morphological aspect the distribution of particles on the final density gradient has a marked similarity to the density gradient distribution reported by De Robertis *et al.*[13] These similarities are: (1) nerve-ending fragments containing relatively few or no mitochondrial inclusions are most richly concentrated in the lower sucrose density range (L3 fraction, Fig. 2); (2) nerve endings that are rich in mitochondrial inclusions are concentrated in slightly higher densities of sucrose (L3 fraction, Fig. 3); (3) free mitochondria, in spite of extensive swelling, equilibrate

TABLE 1. DISTRIBUTION OF γABA IN SUBCELLULAR FRACTIONS OF MOUSE BRAIN HOMOGENATE

Fraction		Experiment 1		Experiment 2		Experiment 3	
		γABA (μg)	μg γABA / mg protein	γABA (μg)	μg γABA / mg protein	γABA (μg)	μg γABA / mg protein
1 g brain wet wt.	(O)	422	3·04	398	2·9	361	2·8
800-*g* pellet	(P1)	20	0·9	17	0·9	5	0·4
20,000-*g* pellet	(P2)	104	1·4	85	1·5	66	1·2
20,000-*g* supernatant	(S2)	284	5·7	263	5·1	234	4·8
Subfractions of P2	(T1)	52·7	1·5	57·5	1·5	39·5	1·3
	(T2)	6·0	1·3	5·9	1·4	2·1	0·5
	(T3)	15·0	2·4	7·2	1·2	4·2	0·6
	(T4)	17·7	3·7	12·0	2·8	10·3	2·4
Subfractions of T1	(L1)	5·1	1·3	10·7	1·5	2·2	0·32
	(L2)	22·3	2·1	20·5	2·1	15·1	1·37
	(L3)	12·4	1·3	12·6	1·4	6·0	0·67
	(L4)	7·6	1·3	8·4	1·3	0·9	0·27
	(L5)	6·7	2·6	8·5	4·2	4·6	3·54

TABLE 2. DISTRIBUTION OF GAD IN SUBCELLULAR FRACTIONS OF MOUSE BRAIN HOMOGENATE*

Fraction		Experiment 2		Experiment 3		Experiment 4	
		GAD	GAD / mg protein	GAD	GAD / mg protein	GAD	GAD / mg protein
1 g brain wet wt.	(O)	1,027	7·6	1,291	10·0		
800-*g* pellet	(P1)	79	4·6	44	3·5		
20,000-*g* pellet	(P2)	725	12·5	490	9·1		
20,000-*g* supernatant	(S2)	535	10·3	603	12·4		
Subfractions of P2	(T1)	351	9·0	302	9·7	254	8·4
	(T2)	40	9·7	47	10·1		
	(T3)	55	9·4	67	9·9		
	(T4)	28	6·7	37	8·6		
Subfractions of T1	(L1)	17	2·4	16	2·3	5	1·8
	(L2)	84	8·7	94	8·6	44	5·6
	(L3)	99	11·2	109	12·4	57	8·8
	(L4)	30	4·7	39	11·9	46	8·3
	(L5)	8	3·9	12	9·2	15	7·5

* The GAD activity is expressed as counts/min × 10^{-2}/hr incubation/g wet weight of brain

in a higher density of sucrose than do the nerve-ending fragments (L2 fraction, Fig. 2).

The interpretation of the following detailed morphological description of the fractions is based on the concurring and independent interpretations which have been given in the literature by Gray and Whittaker[12] and De Robertis *et al.*[13]

Electron micrographs of subfraction L2 (Fig. 2) indicate that it is predominantly composed of isolated nerve endings (ne), some of which have an adhering postsynaptic membrane (psm), others containing the postsynaptic web (sw). The nerve endings are identified by the numerous synaptic vesicles (sv). In this fraction most of the nerve endings were relatively free from enclosed mitochondria (mi). Very few free mitochondria were visible, and those that were present were invariably small and condensed and showed little or no signs of swelling. In addition, a number of presumed postsynaptic membranes (sm) can be seen independently from the nerve endings. This fraction also contains a number of membranous fragments (mic) which are very similar to the membranes demonstrated in the microsomal fraction derived from brain.[18]

Subfraction L3 (Fig. 3) contained a large number of nerve endings (ne) rich in mitochondrial inclusions (mi). A number of small compact mitochondria and a few swollen mitochondria contaminated this fraction in addition to presumably microsomal membranes. One postsynaptic membrane is pointed out on the micrograph which, if seen in isolation from the adjoining presynaptic element, would not be considered to be any different from much of the microsomal membrane material demonstrated by Hanzon and Toschi.[18] It is thus evident that much of the membranous contamination of these fractions may not necessarily have originated from the endoplasmic reticulum.

Fraction L4 (Fig. 4) contained a little of all the previously described elements but was overwhelmingly composed of numerous isolated mitochondria which exhibited varying degrees of swelling. The fact that this fraction has so little of the GAD activity verifies the finding of Lovtrup[9] and adds weight to his report by showing that on a per milligram protein basis as well as in terms of total enzyme this represents a highly impoverished fraction. However, this may be an artifact due to the very swollen state of the mitochondria.

The high content of GAD and γABA in the L2 and L3 fractions suggests that both may be components of the presynaptic nerve endings. One possible explanation for the differential distribution of GAD and γABA may be that γABA is produced in the mitochondria of the nerve ending but is stored or bound in the cytoplasm or on the limiting membranes or vesicles of the nerve endings. The lower γABA content of the mitochondria-rich nerve endings would then be a reflection of the proportionally smaller storage or binding space in these neurons. Studies by Weinstein *et al.*[19] on the binding of exogenous ^{14}C-γABA are compatible with this interpretation. One factor at first seems incompatible with a mitochondrial localization of the decarboxylase; that is the finding that the highly purified mitochondrial fraction, L4, would be expected to have the greatest activity on a total and per milligram protein basis, which is not the case. This discrepancy may somehow be related to the loss of decarboxylase from these swollen mitochondria. An alternative possibility is that the free mitochondria are predominantly of glial origin and may differ from neuronal mitochondria in not containing glutamic decarboxylase.

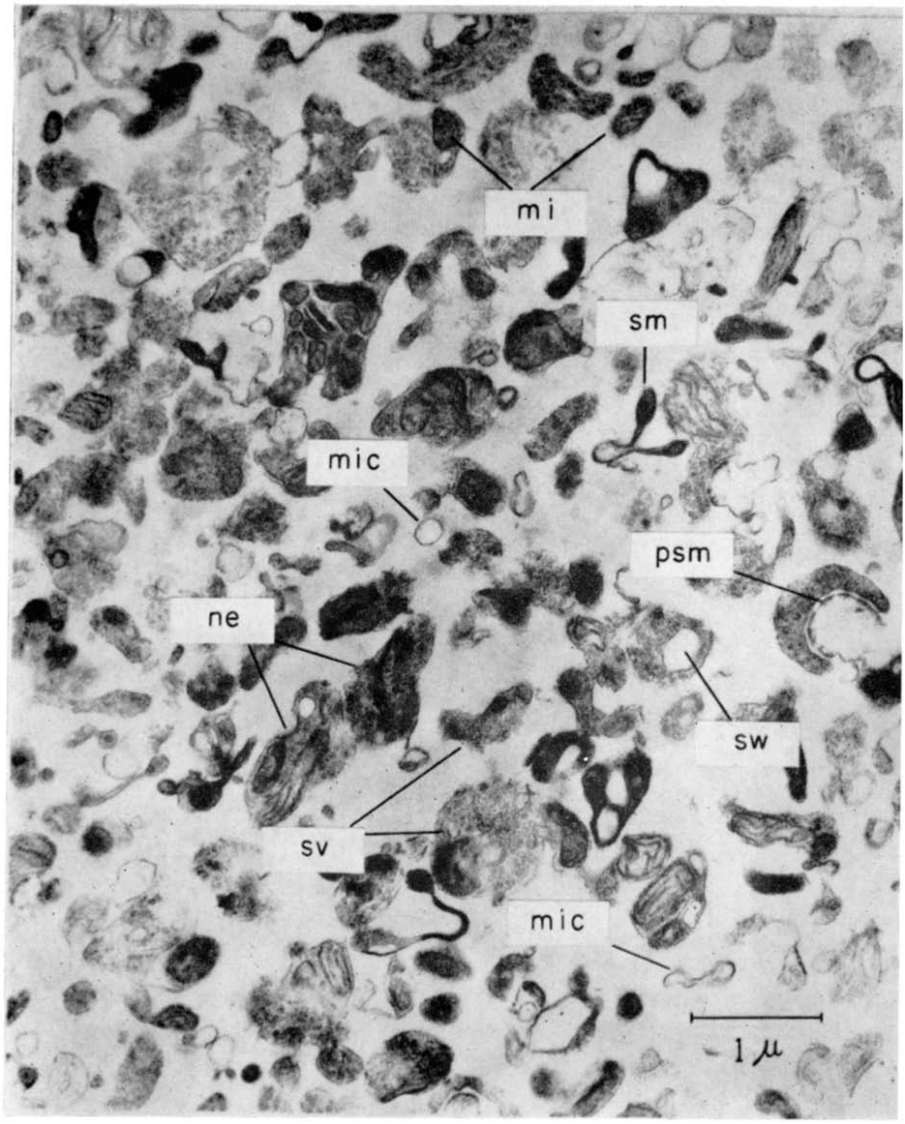

FIG. 2. Electron micrograph of fraction L2 showing numerous pinched-off nerve endings; mi, mitochondria; mic, microsomal membrane; ne, presynaptic nerve ending; psm, postsynaptic membrane; sw, postsynaptic web; sm, isolated postsynaptic membrane; sv, synaptic vesicles.

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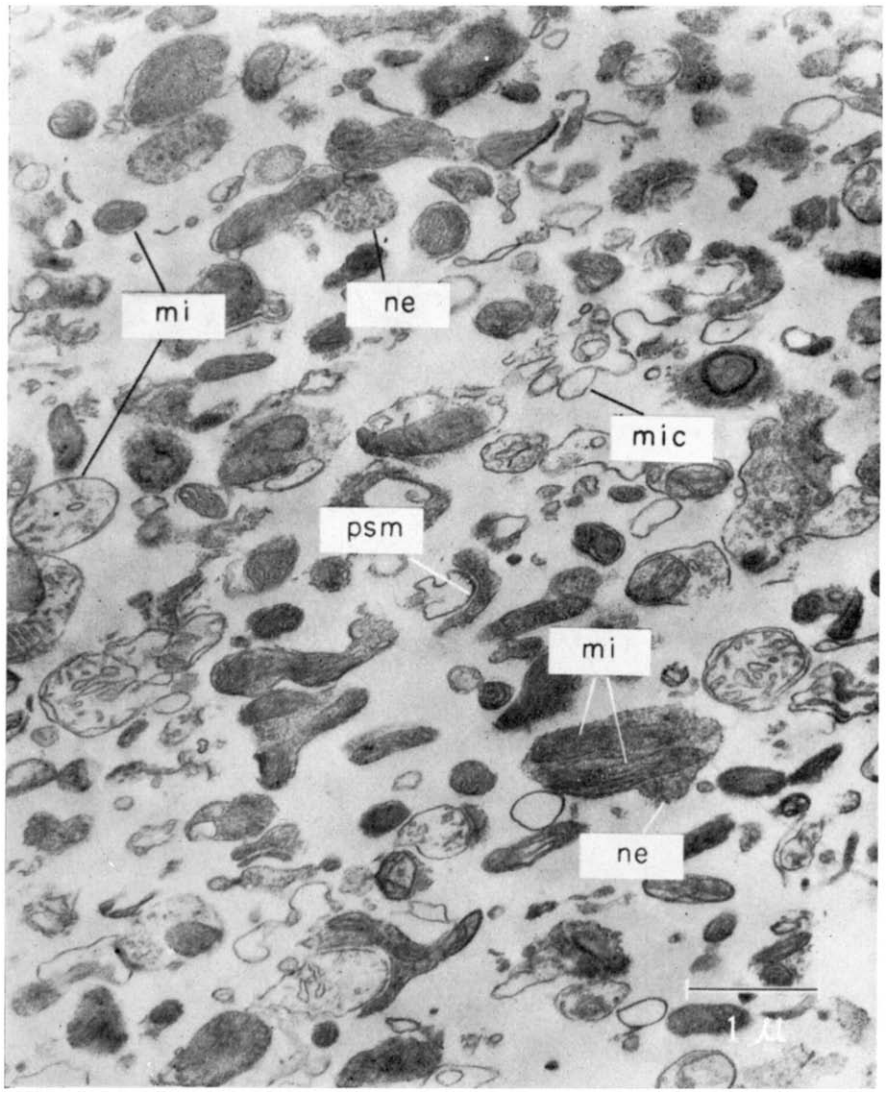

FIG. 3. Electron micrograph of fraction L3 showing numerous nerve endings (ne) which are rich in mitochondrial content (mi). Compact and swollen free mitochondria (mi) can also be seen; mic, microsomal membrane; psm, postsynaptic membrane.

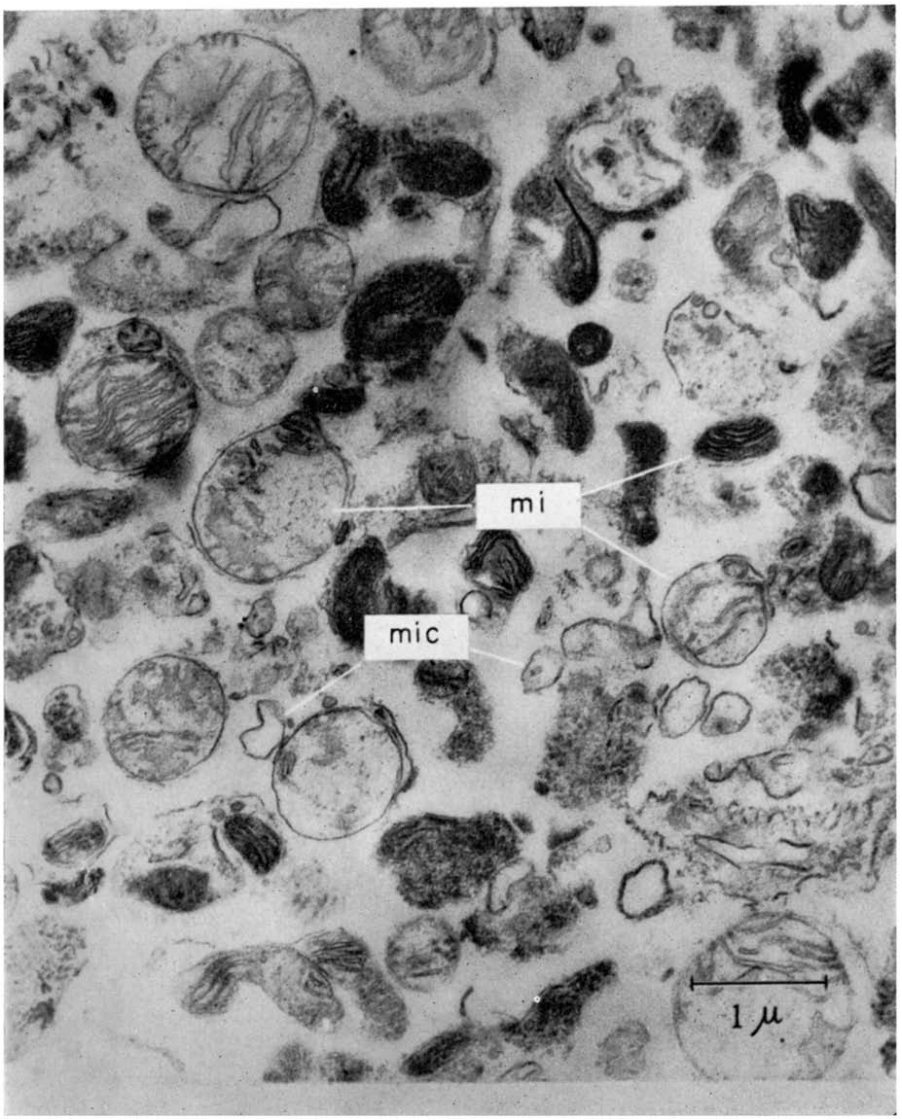

FIG. 4. Electron micrograph of fraction L4 showing numerous free mitochondria with different degrees of swelling; mi, mitochondria; mic, microsomal membrane.

In conclusion, γABA and GAD are associated with presynaptic fractions which are of neuronal origin. However, nothing can be said about the possibility that γABA and GAD may also be indigenous to glial elements.

In view of the large quantities of γABA that apparently are not associated with mitochondria it seems reasonable to postulate that the operation of the γABA metabolic shunt is not dependent solely upon the γABA within the confines of the mitocondria.

Future studies are planned to analyze neuronal and glial cells separately in order to determine whether or not the operation of the γABA metabolic shunt and binding or storage of γABA are confined to neuronal elements.

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