The Distribution of Glutamate Decarboxylase and Aspartate Transaminase in Subcellular Fractions of Rat and Guinea-Pig Brain

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1. The subcellular distributions of glutamate decarboxylase and aspartate transaminase were studied in rat and guinea-pig brain. 2. Glutamate decarboxylase is localized in the synaptosome fraction. The mean density of the particles containing the enzyme is slightly greater than those derived from cholinergic neurones, though overlap is substantial. 3. The enzyme is readily released from synaptosomes by hypo-osmotic treatment, but in the presence of Ca^{2+} , Na^{+} and K^{+} it sediments with particulate material. 4. The release and binding of the enzyme to membrane fractions by Ca^{2+} were investigated. 5. Aspartate transaminase is present in brain as two isoenzymes with different kinetic properties. One isoenzyme is associated with the cytoplasm and the other with mitochondria.

Methods have been developed in this Laboratory for the subcellular fractionation of brain tissue that permit the isolation of detached presynaptic nerve terminals or synaptosomes (Hebb & Whittaker, 1958; Whittaker, 1959; Gray & Whittaker, 1962). The synaptosome fraction is rich in the bound forms of the putative central transmitters acetylcholine, noradrenaline and serotonin and in the enzymes that synthesize them (for literature review see Whittaker, 1965). The 'compartmentation' of certain of these components within the synaptosome has been studied by disrupting the latter in water (Whittaker, 1959; Johnson & Whittaker, 1963) and separating fractions containing soluble cytoplasmic constituents, synaptic vesicles, external membranes and intraterminal mitochondria by means of density-gradient centrifuging (Whittaker, Michaelson & Kirkland, 1964).

Recent work has been concerned with the possible role of certain amino acids (especially glutamate and γ -aminobutyrate) as transmitters. Subcellular distribution studies of the uncombined brain amino acids (Mangan & Whittaker, 1966) have shown that they are distributed in a manner somewhat similar to that of a cytoplasmic marker, in agreement with the results of Weinstein, Roberts & Kakefuda (1963) for γ -aminobutyrate and of Ryall (1964) for glutamate and γ -aminobutyrate. However, the absolute amounts of the pharmacologically active amino acids recovered in the synaptosome fraction are probably sufficient to

sustain a transmitter role (Krnjević & Whittaker, 1965), and the distribution of any transmitter between the synaptosome and supernatant fractions may simply reflect the way in which the neurones utilizing that transmitter are represented in the tissue sample under investigation (Whittaker, 1966).

There has been considerable disagreement about the subcellular distribution of the enzyme that forms γ -aminobutyrate, GAD† (L-glutamate 1-carboxy-lyase, EC 4.1.1.15). Albers (1960) and Shatunova & Sytinski (1964) found it in fractions operationally defined as mitochondrial, but Løvtrup's (1961) mitochondrial fraction was the least active of his fractions. Weinstein *et al.* (1963) localized it in the densest of their synaptosome fractions and suggested that it was present within the mitochondria of the synaptosomes. Salganicoff & De Robertis (1965) found GAD in their 'non-cholinergic' synaptosome fraction and advanced evidence that it was linked to the synaptic vesicles by Ca^{2+} . van Kempen, van den Berg, van der Helm & Veldstra (1965) thought that variations in the tissue concentration of homogenates and in the extent to which particulate GAD had been released from its occluded state before assay were the main causes of the discrepancies between the results of different workers. In subfractions of their mitochondrial fraction obtained by continuous-density-gradient centrifuging, peak activity was associated with particles equivalent in density to 1.2–1.3 M-sucrose, depending on the time of centrifuging, but

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† Abbreviations: GAD, glutamate decarboxylase; Asp-T, aspartate transaminase.

no morphological identifications were attempted. G. M. J. van Kempen (unpublished work cited by Whittaker, 1965) further found that GAD remained bound to readily sedimentable material after water treatment of the crude mitochondrial fraction. Balázs, Dahl & Harwood (1966) in a study of the subcellular localization of several enzymes involved in γ -aminobutyrate metabolism found that GAD was localized in continuous sucrose density gradients with a peak of activity in 1.1M-sucrose; this suggested that it was present in particles similar to those of Whittaker's (1959) fraction B (synaptosome fraction), but again no morphological studies were made.

Asp-T (aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1) is another enzyme concerned in glutamate metabolism. Most authors agree that the fractions with the highest activities are the crude mitochondrial and supernatant fractions (May, Miyazaki & Grenell, 1959; McArdle, Thompson & Webster, 1960; Sellinger, Rucker & Verster, 1964; Salganicoff & De Robertis, 1965; van Kempen *et al.* 1965; Balázs *et al.* 1966); however, the ratios of particle-bound to free supernatant enzyme reported vary from about 1:1 to 6:1. The lower ratios may have resulted partly from failure to release all the particulate enzyme from its occluded state (Sellinger & Rucker, 1963) and partly from using assay methods that are inadequate, because of product inhibition of the particulate enzyme. van Kempen *et al.* (1965) found that the distributions of GAD, Asp-T and lactate dehydrogenase activities in continuous-density-gradient subfractions of the crude mitochondrial fraction were all different, but were unable to decide whether this was due to the presence of different kinds of organelles each specific for a particular enzyme or to heterogeneity within one class of structure.

The subcellular distribution of GAD and Asp-T has now been re-examined by using the fractionation methods developed in this Laboratory. Particular attention has been paid to the morphology of the fractions, to the release of GAD by hypo-osmotic disruption and to the effect of ions on this process. Lactate dehydrogenase was used as a soluble cytoplasmic marker (Johnson & Whittaker, 1963) and fumarate hydratase as a readily releasable mitochondrial marker. A preliminary account of this work has been published (Fonnum, 1965).

METHODS

Preparation of subcellular fractions

Homogenates and primary fractions. Guinea-pig and rat cerebral cortex and rat forebrains were freed from white matter as completely as possible by dissection and scraping with a blunt scalpel. The tissue so obtained was homo-

genized in 0.32M-sucrose and the homogenates were separated into crude nuclear (P_1), crude mitochondrial (P_2), microsomal (P_3) and supernatant (S_3) fractions as described by Gray & Whittaker (1962). When preparations of synaptosomes were required for hypo-osmotic disruption, this procedure was modified: the crude nuclear fraction was washed only once and the combined supernatants centrifuged (g values cited are averages) at 10000*g* for 20 min. The mixture of synaptosomes and free mitochondria thus sedimented was washed by suspension in 0.32M-sucrose and resedimentation at 10000*g* for 30 min. The pellet was then suspended in water (3 ml./g. of original tissue) to give fraction P_2W and this was subjected to density-gradient separation as described below.

Subfractions. Fraction P_2 was separated into myelin (A), synaptosome (B) and mitochondrial (C) fractions as described by Gray & Whittaker (1962). It was also separated on discontinuous sucrose density gradients consisting of layers (4 ml./tube) of 0.8M-, 1.0M-, 1.1M-, 1.2M-, 1.3M- and 1.4M-sucrose kept for 1 or 18 hr. before use and on continuous linear gradients (0.8–1.6M-sucrose) (20 ml./tube). Amounts of fraction P_2 equivalent to 0.7–2.1 g. of tissue were centrifuged (53000*g* for 2.5–13 hr.) into these gradients in the Beckman model L-2 preparative ultracentrifuge with the SW25 swing-out rotor. The tube contents were separated after centrifuging by aspiration or tube puncture into six (discontinuous) or 12 fractions (continuous gradients), the sucrose contents of which were estimated from their volumes.

Hypo-osmotic fractions. Fraction P_2W prepared as described above was separated (5 ml./tube) into fractions containing soluble intraterminal cytoplasm (O), synaptic vesicles (D), external membrane fragments (E – G), incompletely disrupted synaptosomes (H) and intraterminal and somatic mitochondria (I) as described by Whittaker *et al.* (1964), except that centrifuging was continued for 2.5 hr. to obtain sharper separation.

Effect of ions on the binding of GAD to particulate fractions

Fraction P_2W : effect of ions. To 4 ml. of fraction P_2W was added with vigorous stirring 0.1 ml. of the salt solution to be examined. After 10 min. the suspensions were centrifuged at 100000*g* for 45 min. and the pellets so obtained suspended in 50 mM-sodium phosphate buffer, pH 6.5. The distribution of enzyme between supernatant and pellet was determined after activation with Triton X-100 (for details see below).

Hypo-osmotic fractions: effect of Ca^{2+} ions. Fractions D , E , G and I were each diluted with an equal volume of water and centrifuged at 100000*g* for 45 min. to sediment particulate material. The pellets were resuspended in 3.5 ml. of 10 mM-tris-HCl buffer, pH 7.4; to 1.5 ml. of suspension [equivalent to about 0.75 g. (fraction I), 1.0 g. (fractions E and G) or 1.5 g. (fraction D) of original tissue] was added 2.5 ml. of fraction O (equivalent to about 0.75 g. of original tissue) that had been recentrifuged to remove any traces of particulate material, 0.16 ml. of 100 mM- $CaCl_2$ and 0.1 ml. of aqueous pyridoxal phosphate (1 mg./ml.) to protect the enzyme. The suspensions were recentrifuged as before and the activity of each pellet was assayed after activation with Triton X-100. Suspensions without added $CaCl_2$ and fraction O served as controls for endogenous

GAD. Results are expressed as the percentage of added GAD bound/mg. of pellet protein.

protein or protein nitrogen, assuming a nitrogen content of 14%.

Assays

GAD. This enzyme was estimated at 37° by two methods. When GAD was estimated manometrically (Roberts, Younger & Frankel, 1951), the incubation vessel contained 150 μ moles of L-glutamate adjusted to pH 6.2 with NaOH, 100 μ g. of pyridoxal phosphate and 150 μ moles of sodium phosphate buffer, pH 6.2, in a final volume of 3 ml. When GAD was estimated radiochemically (Sisken, Sano & Roberts, 1961), the incubation vessel contained 75 μ moles of L-glutamate, 0.2 μ C of DL-[1-¹⁴C]glutamate, 100 μ g. of pyridoxal phosphate and 150 μ moles of sodium phosphate buffer, pH 6.2, also in 3 ml. The evolved CO₂ was taken up in 1 ml. of m-Hyamine in methanol. The reaction was stopped by the addition of 0.3 ml. of N-HCl with continued shaking for 90 min. to complete the adsorption of CO₂. The radioactivity was counted in a Packard Tri-Carb scintillation counter in 0.5% (w/v) 2,5-diphenyloxazole and 0.02% (w/v) 1,4-bis-(4-methyl-5-phenoxazol-2-yl)-benzene in toluene. The enzyme lost activity on storage and was consequently always assayed on the day the fractions were prepared.

Asp-T. Enzymic activity was measured by the method of Karmen (1955) as described by Boyd (1961). Total Asp-T activity was measured at 25° in 3 ml. of 0.1 M-sodium phosphate buffer, pH 7.4, containing 200 μ moles of L-aspartate, 10 μ moles of 2-oxoglutarate, 0.3 μ mole of NADH₂ and 8 mg. of crystalline malate dehydrogenase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany), previously freed from (NH₄)₂SO₄ by dialysis at 0° for 24 hr. Mitochondrial Asp-T was measured similarly, but at pH 6.0 and with 6.6 μ moles of aspartate.

Other enzymes. Lactate dehydrogenase was estimated as described by Whittaker *et al.* (1964), choline acetyltransferase by the method of Fonnum (1966) with 8 mM-MgCl₂ in the reaction mixture, and fumarate hydratase by that of Racker (1950) except that the reaction mixture of 3 ml. contained 100 μ moles of sodium L-malate and 150 μ moles of sodium phosphate buffer, pH 7.4, at a temperature of 25°.

Units of enzyme activity. The units of enzyme activity employed are: GAD and choline acetyltransferase, μ moles of substrate transformed/hr.; others, μ moles of substrate transformed/min.

Release of occluded enzymes. To ensure release of any occluded enzyme in subcellular fractions, these were treated with 0.1 vol. of 10% (w/v) Triton X-100 before assay, except for choline acetyltransferase, which was released with 0.1 vol. of 5% (w/v) Nonex 501 (British Drug Houses Ltd., Poole, Dorset).

Acetylcholine. Bound acetylcholine in fractions was assayed by a modification (Whittaker *et al.* 1964) of the leech microassay technique of Szerb (1962) after release by heating them at pH 4 and 100° for 10 min. Interference by sucrose after heat treatment was corrected by appropriate sucrose blanks or avoided by diluting the fractions with an equal volume of water, centrifuging them at 100000g for 50 min. and resuspending the sedimented material in acidified diluted mammalian Locke solution.

Protein nitrogen. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as a standard, and expressed as mg. of

Other techniques

Gel electrophoresis. Fractions were diluted with 0.07 M-sodium barbitone buffer, pH 8.6, treated with Triton X-100 as described above to release bound enzyme and centrifuged at 100000g for 15 min. to remove particulate material. Samples of the supernatant (2 μ l.) were subjected to agar-gel electrophoresis at 25 v/cm. for 30 min. at 0–4° as described by Boyd (1966), but with 0.07 M-sodium barbitone buffer. Asp-T was localized by a modification (J. W. Boyd) of the method of Decker & Rau (1963) by covering the gel with a 0.8% (w/v) agar-gel mixture containing 10 mM-2-oxoglutarate, 40 mM-L-aspartate, 1.8 mg. of 6-benzamido-7-methoxy-m-toluidine diazonium chloride/ml. (Warner-Chilcott Laboratories, Morris Plains, N.J., U.S.A.) and 8 mM-sodium phosphate buffer, pH 7.9. A blue colour developed in areas of enzyme activity as a result of the reaction of the diazonium salt with oxaloacetic acid.

Electron microscopy. Subfractions prepared by centrifuging fraction P₂ from rat forebrain into continuous sucrose density gradients for 2.5 hr. were examined in the electron microscope. The details of preparation and the results obtained are given in the Appendix (Whittaker, 1968).

RESULTS

Distribution of GAD in subcellular fractions of guinea-pig cortex

The distribution of GAD was first studied in fractions from guinea-pig cortex, since guinea-pig brain has been the tissue most commonly used in previous work in this Laboratory.

Primary fractions and subfractions. The results obtained are summarized in Table 1. It is seen (column 4) that activity is mainly recovered in the crude mitochondrial (P₂) and high-speed supernatant (S₃) fractions, the ratio of particulate to supernatant enzyme being about 3:1. On subfractionating fraction P₂ into myelin (A), synaptosomes (B) and mitochondria (C), the highest activity and specific activity were found in the synaptosome fraction. The distribution of GAD closely resembles that of acetylcholine and choline acetyltransferase (Hebb & Whittaker, 1958): the proportion of GAD recovered in the purified mitochondrial fraction (C) is somewhat higher, indicating that this enzyme may be preferentially localized in the denser synaptosomes; however, the statement of Salganicoff & De Robertis (1965) that the main GAD activity in the rat is associated with particles denser than 1.2 M-sucrose could not be substantiated in the guinea pig.

Hypo-osmotic fractions. The 'compartmentation' of GAD within the synaptosomes was next studied. Table 1 shows that GAD behaved like a soluble enzyme, most of the activity being found in fraction O, where the soluble cytoplasmic constituents are located. A small second peak of

Table 1. *Distribution of GAD and protein nitrogen in subcellular fractions of guinea-pig cerebral cortex*

For abbreviations, nomenclature of fractions and definition of unit see the text. The number of experiments averaged was three, unless stated otherwise in parentheses; two to four animals were used in each experiment. The homogenates from which fractions P_1 , P_2 , P_3 and S_3 were derived had (mean values): GAD, 27.7 units/g.; protein N, 15.5 mg./g. Fraction P_2W from which fractions $O-I$ were derived had (mean values): GAD, 7.5 units/g.; protein N, 4.3 mg./g. The values in column 6 were obtained by dividing those in column 4 by the corresponding values in column 5. Results for choline acetyltransferase and acetylcholine are taken from Hebb & Whittaker (1958), Whittaker (1959) and Whittaker *et al.* (1964).

Fraction	Distribution (% of total recovered material)				Relative specific activity of GAD
	Choline acetyltransferase	Acetylcholine	GAD	Protein N	
P_1	14	12*	17 (4)	25	0.68
P_2	41	50*	55 (4)	43	1.28
P_3	14	8*	2 (4)	8	0.25
S_3	30	30*	26 (4)	24	1.08
Recovery (% of homogenate)	99	91	86 (4)	106	—
A	25	33	6	17	0.35
B	74	67	77	58	1.33
C	1	0	17	25	0.68
Recovery (% of fraction P_2)	100	96	89	108	—
O	73	2	69	26	2.65
D	8	30	3	6	0.50
E	2	8	1	4	0.25
F	5	5	4	11	0.36
G	5	11	2	9	0.22
H	4	35	7	25	0.28
I	3	9	14	19	0.73
Recovery (% of fraction P_2W)	83	81	95	112 (2)	—

* Total acetylcholine (eserine present).

Table 2. *Distribution of GAD, other enzymes and protein nitrogen in the primary subcellular fractions from rat forebrain homogenates*

For abbreviations, nomenclature of fractions and definition of units see the text. Mean values are given $\pm$ s.d. when four or more experiments were involved.

Component	No. of expts. averaged	Concn. in homogenate (units/g. of tissue)	Distribution in fractions (% of total recovered material)				Recovery (% of homogenate)
			P_1	P_2	P_3	S_3	
GAD	4	52 $\pm$ 4	9 $\pm$ 3	56 $\pm$ 5	4 $\pm$ 1	31 $\pm$ 6	86 $\pm$ 6
Total Asp-T	4	72 $\pm$ 12	15 $\pm$ 4	61 $\pm$ 5	3 $\pm$ 1	21 $\pm$ 2	98 $\pm$ 15
Mitochondrial Asp-T	2	30	23	72	2	3	93
Fumarate hydratase	5	14 $\pm$ 1	16 $\pm$ 7	77 $\pm$ 7	4 $\pm$ 1	3 $\pm$ 1	96 $\pm$ 17
Protein N	4	13 $\pm$ 4	20 $\pm$ 5	47 $\pm$ 7	9 $\pm$ 3	24 $\pm$ 2	100 $\pm$ 13

activity was associated with partially disrupted synaptosome (H) and mitochondrial (I) fractions. Most of the activity in the latter fraction could have been due to contamination with 'dense' synaptosomes. It is noteworthy that very little GAD was found in the synaptic-vesicle-rich fraction D or the membrane fractions E , F and G . The small

activity in fraction D can readily be explained by diffusion of enzyme from fraction O during the run. This finding is not in agreement with the view of Salganicoff & De Robertis (1965) that in the rat GAD is bound to vesicles.

In view of these discrepancies, and the low GAD activity of guinea-pig cortex, the main studies,

including those of Asp-T distribution, were carried out with rat brain as described in the following sections.

Distribution of GAD, Asp-T and marker enzymes in subcellular fractions of rat brain

Primary fractions. The distribution of GAD and Asp-T in the primary subcellular fractions of rat forebrain is given in Table 2. The activity of GAD in rat forebrain homogenates is seen to be approximately twice that of guinea-pig cortex. Separate experiments showed that this was a genuine species difference and not due to the exclusion of non-cortical tissue from the guinea-pig homogenates. The distribution of the enzyme follows the same pattern as in the guinea pig. The distribution of the mitochondrial marker fumarate hydratase shows that the small amounts of GAD activity in fractions P_1 and P_3 could have resulted from contamination of these fractions by particles that mainly sediment in fraction P_2 .

Total Asp-T activity (measured at pH 7.4 and 66mM-substrate concentration) showed a distribution similar to that of GAD, except that the particle/supernatant ratio was somewhat higher. When, however, the assay conditions were altered to pH 6 and 2.2mM-aspartate, conditions known to favour the mitochondrial isoenzyme in liver (Boyd, 1961), the activity paralleled that of fumarate hydratase, suggesting that Asp-T assayed under these conditions is a valid mitochondrial marker in brain as in liver.

Density-gradient fractions. Fig. 1 is a composite diagram summarizing the results of a number of experiments in which fraction P_2 prepared from rat forebrain and cortex was separated on density gradients under various conditions of loading and duration of centrifuging. Figs. 1(d), 1(h) and 1(i) give the distribution of GAD and marker components in single experiments with continuous gradients in which centrifuging was carried out for 2.5, 5 and 13 hr. respectively. Figs. 1(a)–(c) and 1(e)–(g) give the massed results of all experiments lasting 2.5 and 5 hr. respectively and show the degree of consistency obtained. The conditions varied somewhat from experiment to experiment; these are shown, with the protein nitrogen distributions, where measured, in Table 3. The appearance, after centrifuging, of the tubes containing continuous gradients resembled that figured by van Kempen *et al.* (1965): a water-clear top zone corresponding to the position of the parent fraction was followed by a band of ivory-coloured particulate material in the 0.8M-sucrose region; below this there was a broad opalescent band terminating in the 1.5M-sucrose region. Morphological studies of the various fractions [for details see the Appendix

Fig. 1. Distribution of acetylcholine and enzymes in fractions of crude rat brain synaptosome preparations after separation on density gradients for 2.5, 5 and 13 hr. For conditions see Table 3. The large diagrams (d, h and i) relate to single experiments; each of the smaller diagrams (a–c and e–g) contain the massed results for a single component and thus indicate the degree of replication obtained from one experiment to another. Diagrams (a)–(c) relate to 2.5 hr. separations, (e)–(g) to 5 hr. separations. Each symbol (∇ , $\circ$, $\times$, Δ , $\bullet$, ∇ , Δ , $\square$ and $\blacksquare$) indicates a single experiment. Abbreviations: ACh, acetylcholine; Ch-T, choline acetyltransferase; Fum, fumarate hydratase; LDH, lactate dehydrogenase; mito Asp-T, mitochondrial Asp-T. Recoveries were in the range 80–100%.

Table 3. *Conditions of preparation and protein nitrogen contents of density-gradient fractions derived from crude mitochondrial fractions from rat brain*

The load (column 5) is the weight of tissue from which the fraction was prepared. Gradients were continuous (C) or discontinuous (D); the latter were prepared 1 hr. before use except in the experiment asterisked, when the gradient stood for 18 hr. before use. In the experiments with continuous gradients, these ran from 0.8 M- to 1.6 M-sucrose; 12 fractions (corresponding to 0.06-0.07 M increments in sucrose concentration) were separated. In the experiments with discontinuous gradients, layers of 0.8 M-, 1.0 M-, 1.1 M-, 1.2 M-, 1.3 M- and 1.4 M-sucrose were separated into six fractions each corresponding to 0.1 M increments. Thus the fraction numbers are not comparable in the two types of experiment. For further details see the text.

Tissue	Duration of run (hr.)	Type of gradient	Expt. symbol (as in Fig. 1)	Load (tube (g.))	Distribution of protein N in fraction (% of total recovered)										
					1	2	3	4	5	6	7	8	9	10	11
Forebrain	2.5	C	▶	1.8	—	—	—	—	—	—	—	—	—	—	—
		C	○	1.7	22.4	7.5	7.5	9.4	9.1	12.2	10.0	9.1	5.9	7.0	—
		C	×	2.1	24.0	9.7	8.3	8.1	9.1	8.9	8.6	8.1	6.3	5.1	4.2
		C	▲	1.0	8.3	8.4	9.3	11.0	12.1	12.4	9.7	9.5	9.1	5.0	5.3
Cortex	5.0	D*	●	1.0	15.1	15.9	27.8	23.4	10.6	7.0	—	—	—	—	—
		D	▽	1.1	17.0	16.0	24.6	25.2	9.8	7.1	—	—	—	—	—
		D	△	1.1	17.9	11.3	17.6	25.2	14.0	14.0	—	—	—	—	—
		D	□	1.2	—	—	—	—	—	—	—	—	—	—	—
Forebrain	13.0	C	■	0.7	—	—	—	—	—	—	—	—	—	—	—
		C	None	—	—	—	—	—	—	—	—	—	—	—	—

(Whittaker, 1968)] showed, as expected, that the material in the top ivory-coloured band consisted largely of myelin fragments; the broad intermediate zone was rich in synaptosomes and the terminal zone consisted almost entirely of mitochondria. The myelin band was well separated under conditions of light loading. The uppermost synaptosome fractions were contaminated with occasional myelin fragments; in addition they contained numerous empty membranous sacs similar in size to synaptosomes. The densest fractions contained numerous free mitochondria, but the intermediate zone was remarkably homogeneous. However, small (0.1–0.2 μ diam.) membrane fragments contaminated all fractions.

After centrifuging for 2.5 hr., GAD was distributed throughout the gradient with a peak corresponding to about 1.1 M-sucrose (Fig. 1a). Its activity was very low in the regions containing myelin and mitochondria and its distribution corresponds to that of synaptosomes. Lactate dehydrogenase had a similar distribution, except that the curve was somewhat broader owing to the relatively high activity of the lightest, myelin-containing, fractions; the activity of all these fractions is attributable to entrapped cytoplasm, of which the axonal fragments in the myelin-rich fractions contain considerably less than the synaptosomes (Marchbanks, 1967). By contrast, the peak of fumarate hydratase activity appeared, as expected, in the zone richest in free mitochondria (1.35–1.44 M-sucrose); there is also a shoulder in the synaptosome region attributable to the intra-terminal mitochondria. Asp-T, measured under conditions favouring the mitochondrial enzyme, has a distribution similar to that of fumarate hydratase, but total Asp-T is bimodally distributed with peaks in the synaptosome and mitochondrial regions of the gradient. The former is attributable both to the cytoplasmic and intraterminal mitochondrial enzymes.

More prolonged centrifuging causes a shift of the GAD peak to lower regions of the gradient: to a level corresponding to 1.15 M-sucrose after centrifuging for 5 hr. and to 1.2 M-sucrose after 13 hr. This shift may be caused by the progressive osmotic dehydration of the synaptosomes, resulting from the prolonged exposure to hyperosmotic sucrose.

The distribution of acetylcholine and choline acetyltransferase, markers for synaptosomes derived from cholinergic neurones, was remarkably similar to that of GAD. However, the densest synaptosome fractions had a consistently lower content of cholinergic markers than of GAD. Separation of synaptosomes that must be presumed to contain GAD from those derived from cholinergic neurones was greatest in the experiments with rat cortex after centrifuging for 5 hr.; the fractions having

Table 4. *Distribution of GAD, other enzymes and protein nitrogen in subfractions of fraction P₂W from rat forebrain*

For abbreviations, nomenclature of fractions and definition of units see the text. All recoveries were close to 100%. Mean values are given $\pm$ s.d. when four or more experiments were involved.

Component	No. of expts. averaged	Concn. in fraction P ₂ W (units/g.)	Distribution in fractions (% of total recovered material)							Recovery (% of fraction P ₂ W)
			O	D	E	F	G	H	I	
GAD	5	15.5 $\pm$ 5.3	66 $\pm$ 9	3 $\pm$ 1	3 $\pm$ 1	4 $\pm$ 1	3 $\pm$ 1	13 $\pm$ 7	8 $\pm$ 3	103 $\pm$ 13
Lactate dehydrogenase	6	16.4 $\pm$ 4.4	51 $\pm$ 10	5 $\pm$ 2	4 $\pm$ 1	5 $\pm$ 2	10 $\pm$ 3	14 $\pm$ 5	11 $\pm$ 3	98 $\pm$ 11
Total Asp-T	5	28.0 $\pm$ 8.9	16 $\pm$ 2	1 $\pm$ 1	1 $\pm$ 1	4 $\pm$ 2	4 $\pm$ 2	14 $\pm$ 5	60 $\pm$ 9	108 $\pm$ 9
Mitochondrial Asp-T	5	15.9 $\pm$ 3.2	1 $\pm$ 0	1 $\pm$ 1	1 $\pm$ 1	2 $\pm$ 1	3 $\pm$ 1	15 $\pm$ 5	77 $\pm$ 5	102 $\pm$ 6
Fumarate hydratase	2	9.3	1	0	0	0	2	18	79	93
Protein N	6	3.3 $\pm$ 0.8	21 $\pm$ 7	5 $\pm$ 1	5 $\pm$ 1	14 $\pm$ 4	11 $\pm$ 2	20 $\pm$ 6	24 $\pm$ 4	100 $\pm$ 10

maximum choline acetyltransferase activity had densities corresponding to 1.0–1.1 M-sucrose whereas those having maximum GAD activity floated in 1.1–1.2 M-sucrose. However, the overlap of activities was virtually complete and no clear-cut separation of a ‘cholinergic’ from a ‘non-cholinergic’ fraction occurred as described by Salganicoff & De Robertis (1965). The results thus resemble those obtained for acetylcholine and 5-hydroxytryptamine by Michaelson & Whittaker (1963).

In Fig. 1 distributions are expressed as percentages of the total recovered activity. The protein nitrogen contents of the various fractions (Table 3) were such that the patterns of distribution would not have been basically different had the results been expressed as relative specific activities.

Intraterminal compartmentation. When synaptosomes were submitted to hypo-osmotic disruption and the disrupted material was separated on a density gradient according to the technique of Whittaker *et al.* (1964), GAD behaved like a soluble cytoplasmic constituent as in the guinea pig (Table 4). The most active fraction was thus fraction *O*, but there was a small peak lower down the gradient in fraction *H*, containing incompletely disrupted synaptosomes. The distribution was essentially the same as that of lactate dehydrogenase, though slightly less lactate dehydrogenase was released than GAD owing to the adsorption of the more basic lactate dehydrogenase isoenzymes on particulate material (Fonnum, 1967). By contrast, the mitochondrial markers, fumarate hydratase and mitochondrial Asp-T, were recovered, as expected, mainly in the mitochondrial pellet (fraction *I*). Total Asp-T had a distribution that could be accounted for by the combined contributions of enzymes localized in the soluble fraction and in mitochondria.

Characterization of Asp-T isoenzymes

It is apparent from Tables 2 and 4 and Fig. 1 that the subcellular distribution of total Asp-T is different from that of the mitochondrial enzyme, signifying the presence of a second, soluble, isoenzyme in the supernatant (*S*₃) and the synaptosome cytoplasmic (*O*) fractions. Fig. 2 (last two lanes) shows electrophoretograms of the mitochondrial fraction *I* and the cytoplasmic fraction *O* from disrupted synaptosomes; the more slowly moving α -isoenzyme is localized exclusively in the soluble cytoplasm and the faster-moving β -enzyme in mitochondria. The parent material (fraction *P*₂W) and intact synaptosomes (first five lanes) contain both isoenzymes; however, the material from fractions 3 and 5 of a 2.5 hr. density-gradient run, representing the less dense of the synaptosome fractions, are richer in the cytoplasmic isoenzyme,

Fig. 2. Enzymograms showing separation of soluble cytoplasmic (α) and mitochondrial (β) isoenzymes of Asp-T. The predominance of the soluble isoenzyme in the lighter synaptosome fractions (3 and 5) from a continuous density gradient and of the mitochondrial isoenzyme in the heavier fractions (8 and 10) should be noted. In subfractions from synaptosomes disrupted by suspension in water, the α -isoenzyme is, as expected, the sole form in the soluble cytoplasmic fraction (O) and the β -isoenzyme the sole form in the mitochondrial fraction (I). Both enzymes are present in the parent fractions (P_2 and P_2W). For details of preparation of fractions for electrophoresis see the text. The small dots are air bubbles in the gel.

Table 5. *Michaelis constants of the mitochondrial and cytoplasmic Asp-T isoenzymes from rat forebrain*

Sources of enzymes were: cytoplasmic, fraction O; mitochondrial, fraction I. The latter fraction was suspended in distilled water and sedimented at 100000g for 15 min. to remove any remaining traces of occluded cytoplasm.

Source of enzyme	K_m for substrate (mM)	
	Aspartic acid	2-Oxoglutaric acid
Cytoplasm	5.3	0.13
Mitochondria	0.44	1.0

whereas fractions 8 and 10, containing the denser synaptosomes and considerable mitochondrial contamination [see Table 1 in the Appendix (Whittaker, 1968)], have a much higher proportion of the mitochondrial isoenzyme. The Michaelis constants of the two enzymes are different, as shown in Table 5; the actual values are close to those obtained by Boyd (1961) for rat liver.

Solubility of GAD under different conditions

Experiments with fraction P_2W . As mentioned above, GAD is readily released from synaptosome

fractions by hypo-osmotic treatment. Such treatment also causes a fall in ionic concentration and pH, which might have affected the distribution of GAD as it does that of choline acetyltransferase (Fonnum, 1967). Salganicoff & De Robertis (1965) have suggested that GAD is linked to synaptic vesicles by Ca^{2+} . Accordingly, the solubility of GAD was investigated in the presence of various ions. As shown in Table 6, when synaptosomes are disrupted either by treatment with ether (Table 6, conditions I and II) or by suspension in hypo-osmotic sucrose (condition III), GAD is released *pari passu* with the soluble cytoplasmic marker lactate dehydrogenase. However, on suspension in hypo-osmotic sodium chloride or calcium chloride (condition IV), the release of GAD into the supernatant is considerably less than expected. This is not primarily due to a stabilizing effect of the ions on the external synaptosome membrane, since, if sodium chloride or calcium chloride is added after hypo-osmotic disruption (condition V), the release of lactate dehydrogenase is actually greater than in the control, as previously noted (Fonnum, 1967), but the release of GAD is again less than would be expected. The GAD must have been reconverted into a sedimentable form by the addition of the ions, and calcium chloride is more effective in bringing this about than is sodium chloride.

Addition of ions to fraction O that had first been centrifuged at 100000g for 1 hr. to remove all traces of particulate matter caused no sedimentation of the GAD present in this fraction. The sedimentation of GAD in the presence of membranous material and ions must therefore be due to binding to particulate material and not simply to aggregation or salting-out of the soluble enzyme.

Experiments with hypo-osmotic fractions. In order to determine the component structures of fraction P_2W on which GAD was adsorbed in the presence of Ca^{2+} or Na^+ attempts were made to fractionate material disrupted in 20mM-calcium chloride. As previously noted (Fonnum, 1967), calcium chloride had a strongly coacervating effect on the particulate material and this was mostly recovered in fraction H. The distribution of lactate dehydrogenase between fractions O and H was about 1:1, whereas that of GAD was 1:15. With 30mM-sodium chloride in place of 20mM-calcium chloride, the ratios were 3:1 for lactate dehydrogenase and 1:1 for GAD. Thus both enzymes are partially entrained in membrane material that sediments to the level of the H band, but this effect is more noticeable with GAD than with lactate dehydrogenase and more noticeable with Ca^{2+} than with Na^+ .

In another approach, fractions D, E, G and I were prepared in the usual way and their ability to bind soluble GAD at pH 7.4 in the presence of

Table 6. *Release of GAD and lactate dehydrogenase from a washed synaptosome fraction from rat forebrain homogenates*

Experimental details are as follows: treatments I–IV, the synaptosome fraction was prepared as described in the text for fraction P_2W except that the washed pellet was suspended in 3 ml. of water or medium/amount of pellet derived from 1 g. of tissue, immediately centrifuged at 100000g for 30 min. and the enzyme activity determined in the supernatant and pellet; treatment V, as described in the text. Recoveries were 80–100%. Enzyme activities either relate to single experiments or are mean values (numbers of experiments in parentheses) $\pm$ s.d. (for means of three or more experiments). In column 6, — indicates 'not estimated'.

	Composition of medium		Enzyme released into supernatant (% of total recovered)	
	Solute	Concn. (mm)	GAD	Lactate dehydrogenase
I. Suspension in sucrose followed by addition of ether	Sucrose	320	80 $\pm$ 2 (3)	77 $\pm$ 9 (3)
II. Suspension in sucrose–NaCl followed by addition of ether	Sucrose	320	62 (2)	96 (2)
	NaCl	30		
III. Suspension in sucrose	Sucrose	320	1	2
		150	11	10
		100	40	32
IV. Suspension in medium	NaCl	30	23	55 (2)
	CaCl ₂	20	4 $\pm$ 2 (3)	31 $\pm$ 6 (4)
V. Suspension in water followed by addition of ions as indicated	—	—	64 $\pm$ 8 (8)	57 $\pm$ 7 (4)
		NaCl	69	85
		75	42 (2)	80 (2)
		150	29 $\pm$ 2 (3)	74 (2)
		KCl	41	—
		150	28 (2)	—
	CaCl ₂	0.25	64	—
		0.50	54 (2)	—
		1	32 (2)	—
		2	27 (2)	—
		3	24 (2)	73
		5	24 (2)	73
		10	15	68

Table 7. *Binding of GAD to rat brain membrane fractions by calcium chloride*

Experimental details are given in the text. The results are expressed as mean values $\pm$ s.d. (numbers of experiments in parentheses).

Fraction	Percentage of added GAD bound/mg. of protein of resuspended pellet
<i>D</i>	8.5 $\pm$ 2.8 (4)
<i>E</i>	2.6 $\pm$ 1.6 (5)
<i>G</i>	6.0 $\pm$ 2.9 (5)
<i>I</i>	2.9 $\pm$ 1.3 (4)

4 mm-calcium chloride was examined as described in the Methods section. GAD was bound by all the fractions tested; however, when the percentage bound was expressed per mg. of membrane protein, which is a rough measure of the affinity of GAD for the various fractions, fraction *D* had the highest

Table 8. *Effect of different media on the release of GAD bound to membranes by Ca²⁺ ions*

To the hypo-osmotically disrupted rat brain synaptosome fraction P_2W was added CaCl₂ (final concn. 2.5 mm) and the suspension centrifuged at 100000g for 45 min. The pellets were resuspended to the original volume in different media at pH 7, again centrifuged at 100000g for 45 min. and the enzyme activity was determined in the supernatant and pellet. The values relate either to single experiments or are mean values (numbers of experiments in parentheses) $\pm$ s.d. (more than two experiments).

Medium	GAD recovered in the supernatant (% of total recovered)
Water	12 $\pm$ 4 (3)
320 mm-Sucrose	1
5 mm-Oxaloacetate	32 (2)
5 mm-Citrate	51 (2)
5 mm-EDTA (potassium salt)	52
1% (w/v) Triton X-100	79 (2)

value, followed by fraction *G* (Table 7). Since the proportion of the total protein nitrogen recovered in fraction *D* is quite small (soluble and particulate protein nitrogen together is only about 5% of that in fraction *P₂W*; Table 4), the proportion of the total GAD bound to the vesicles in the presence of 4mM-calcium chloride must be quite small.

Reversal of GAD binding. The link between particulate matter and GAD in the presence of calcium chloride was very firm (Table 8). Only a small proportion of bound GAD was released when the pellet was washed with water or sucrose (line 1). Agents forming complexes with Ca^{2+} , such as citrate, oxalate or EDTA (potassium salt), only partially reversed the binding in the relatively low concentrations tested. The detergent Triton X-100, which disperses membranous structures, released the enzyme nearly completely.

DISCUSSION

Is GAD present within the synaptosome? The results presented here show that particulate GAD is localized in both guinea-pig and rat brain homogenates in an osmotically sensitive particle containing cytoplasm, whose sedimentation characteristics are identical with those of synaptosomes.

The distribution in the primary and density-gradient fractions (Tables 1 and 2 and Fig. 1) is similar to that of two other presumed synaptosome constituents, acetylcholine and choline acetyltransferase. In the fractions prepared by continuous-density-gradient centrifuging, those that were richest in GAD were also rich in synaptosomes and were negligibly contaminated with myelin or free mitochondria [see the Appendix (Whittaker, 1968)]. The possibility remains that GAD might be attached to the many unidentified small membrane fragments that contaminate even the most homogeneous of these fractions: however, Marchbanks (1967) has shown that the osmotically sensitive space in synaptosome preparations correlates well with the space calculated from the area of synaptosome profiles in electron micrographs of such preparations; further, the occluded state of the enzyme in untreated particulate fractions and its release therefrom by ether, hypo-osmotic treatment and detergents are phenomena fully consistent with a synaptosomal location.

The recovery of the greater part of the particulate GAD activity in the crude mitochondrial fraction is again consistent with an occurrence in synaptosomes, since these have sedimentation properties similar to those of mitochondria in iso-osmotic media. It is also in agreement with the findings (though not necessarily the interpretations) of other workers using broadly similar techniques

(Albers, 1960; Weinstein *et al.* 1963; Shatunova & Sytinski, 1964; Salganicoff & De Robertis, 1965; van Kempen *et al.* 1965). Løvtrup's (1961) results are interesting in that he (correctly) found his mitochondrial fraction to have a low GAD activity: as shown by his electron micrographs (Løvtrup & Zelander, 1962), his procedure of sedimenting his mitochondria within a relatively narrow range of integrated centrifugal fields (2.1×10^4 – $10.5 \times 10^4 g$ -min.) and repeatedly washing them produces a relatively homogeneous fraction (compare also Stahl, Smith, Napolitano & Basford, 1963); a few synaptosomes are visible in his micrographs, but they are badly damaged and have probably lost their GAD to the supernatant. His method of preparing his other particulate fractions (not examined morphologically) would, however, have caused considerable contamination with synaptosomes, thus accounting for their GAD activity.

Although many variations in loading, duration of run and the type of gradient were studied, no separation of rat forebrain synaptosomes into clearly defined 'cholinergic' and 'non-cholinergic' fractions was obtained. There was a tendency in all experiments for the denser fractions to be richer in GAD than in cholinergic components. This tendency was most marked with cortex and at 5 hr. with a fairly lightly loaded continuous gradient (Fig. 1*h*). The separations were similar to those obtained with serotonin and acetylcholine by Michaelson & Whittaker (1963).

An important factor to be taken into account when comparing the distributions of choline acetyltransferase, lactate dehydrogenase and GAD in the density gradient is the tendency of the first two of these to be adsorbed on membranes under conditions of low ionic strength (Fonnum, 1967), a phenomenon particularly evident with rat choline acetyltransferase. Since the least dense synaptosome fractions are the ones most heavily contaminated with membrane fragments morphologically similar to those occurring in fraction *G*, the hypo-osmotic fraction most effective in binding choline acetyltransferase, the possibility exists that some of the choline acetyltransferase in the upper part of the gradient is not synaptosomal, but is soluble acetyltransferase adsorbed on membrane fragments. Significantly, the distributions of choline acetyltransferase and acetylcholine were not identical, though they should have been if both were exclusively localized in synaptosomes derived from cholinergic neurones. The distribution of acetylcholine, though not quite coincident with that of GAD (Fig. 1*h*), is closer to it than that of choline acetyltransferase at 5 hr. For this reason, and because of the technical difficulties in performing accurate assays in fractions containing high and variable sucrose concentrations, the separations

previously obtained (Salganicoff & De Robertis, 1965) may be more apparent than real.

Electron-microscopic studies [for details see the Appendix (Whittaker, 1968)] have similarly failed to demonstrate any clear-cut separation of synaptosomes into morphologically distinct types. Nafstad & Blackstad (1966) have shown that there are significant differences in the proportion of cytoplasm occupied by mitochondria in nerve terminals in various locations in the hippocampus. This provides a basis for possible differences in physical properties that might permit separation of detached hippocampal nerve terminals by density-gradient centrifuging. However, evidence is lacking that such differences exist throughout the central nervous system or that they are to be correlated with particular transmitters. It is therefore hardly to be expected that any clear-cut separation of preparations of synaptosomes derived from large areas of the brain could be achieved by density-gradient centrifuging alone. However, if GAD is a specific constituent, in a limited region, of mitochondrion-rich nerve endings, the presence of synaptosomes derived from these endings in the denser fractions might be sufficient to explain the observed trend in density-gradient distribution.

Intraterminal localization of GAD. If a synaptosomal localization of GAD is accepted the question then arises as to whether it is a soluble constituent of the synaptosomal cytoplasm or membrane-bound or both. The results of hypo-osmotic and other treatment suggest that GAD, like choline acetyltransferase, is a free cytoplasmic constituent of nerve endings. The possibility that GAD might have been liberated from mitochondria during homogenization or hypo-osmotic disruption is rendered unlikely by the fact that fumarate hydratase, an enzyme known to be readily released from ox heart mitochondria (Backmann, Allmann & Green, 1966), remains associated with brain mitochondria during these procedures.

In the presence of Na^+ , K^+ and particularly Ca^{2+} , a considerable proportion of the enzyme sediments with particulate material, perhaps with Ca^{2+} as a result of the formation of a phospholipid- Ca^{2+} -enzyme complex. The synaptic-vesicle fraction showed the highest affinity for the soluble enzyme, but accounted for only a small part of the total amount of enzyme bound by the various fractions under these conditions. The finding that the adsorbed enzyme is not easily released by water, sucrose or even agents forming complexes with Ca^{2+} suggests that very little of the free GAD in the disrupted synaptosome preparations could have originated as the result of the breakdown of a readily releasable, Ca^{2+} -bonded, vesicular GAD as proposed by Salganicoff & De Robertis (1965).

Localization of Asp-T. The finding that this

enzyme is present in brain, as in liver (Boyd, 1961), in two forms differing in location and kinetic properties necessitates a reappraisal of earlier results. The main source of error is the underestimation of the mitochondrial enzyme. To obtain maximum activity this must be released by a detergent before assay (Boyd, 1961; Sellinger & Rucker, 1963; Brunngraber, Aguilar & Occomy, 1963), a high concentration of 2-oxoglutarate must be used and the oxaloacetate formed must not be allowed to accumulate, as this inhibits the mitochondrial enzyme preferentially (Boyd, 1962). These conditions are not adequately met by the colorimetric method used by van Kempen *et al.* (1965) and Salganicoff & De Robertis (1965); this explains the higher proportions of soluble to bound enzyme and lower values for homogenates found by these workers.

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APPENDIX

The Morphology of Fractions of Rat Forebrain Synaptosomes Separated on Continuous Sucrose Density Gradients

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Several attempts have been made to separate synaptosomes (detached presynaptic nerve terminals) into chemically and morphologically distinct fractions by means of density-gradient centrifuging. Variants of the discontinuous sucrose gradients originally employed for this purpose by Hebb & Whittaker (1958) have been used (De Robertis, de Iraldi, Arnaiz & Salganicoff, 1962; Weinstein, Roberts & Kakefuda, 1963); continuous gradients prepared by allowing diffusion to occur in an initially discontinuous gradient (Michaelson & Whittaker, 1963) or by means of a gradient maker (van Kempen, van den Berg, van der Helm & Veldstra, 1965; Fonnum, 1965, 1968; Balázs, Dahl & Harwood, 1966) have also been tried. Morphological studies of the fractions have been mainly qualitative; they have not so far indicated that any clear-cut separation into morphologically distinct types of synaptosome has been achieved.

In the present study, carried out in connexion

with the main paper (Fonnum, 1968), fractions of synaptosomes derived from rat forebrain and

EXPLANATION OF PLATE 1

(a) Portion of a light synaptosome fraction from the middle of a sucrose density gradient (fraction I6, block A; for sucrose concentration see Table 1), showing a region where empty membranous profiles resembling synaptosome 'ghosts' are concentrated. Profiles 1, 2 and 3 show intermediate stages between synaptosome and 'ghost' profiles. (b) Portion of the same fraction (block B) showing well-preserved synaptosomes. Note post-synaptic adhesion (thick arrow) and large vesicles among smaller ones (thin arrows). A third block (C) gave similar profiles. About half the particles in the field cannot be identified as synaptosome profiles, but most of these are quite small. Black patches resembling profile 1 and very small vesiculated bodies (profile 2) are found in serial sections to be grazing sections of larger synaptosomes (C. D. Voorhorst, personal communication). Magnification (a and b) $\times 30000$.