SEPARATION OF ADRENERGIC AND CHOLINERGIC SYNAPTOSOMES FROM IMMATURE RAT BRAIN

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1. Introduction

The modern quantitative molecular neurobiology had its inception with the isolation and characterization of synaptosomes in the laboratories of Whittaker and of DeRobertis [1, 2]. These pinched off terminal boutons of axons, frequently still retaining subsynaptic and postsynaptic elements, constitute the most appropriate starting material for the purification of the various molecules and supramolecular aggregates confined by and defining the synapse. Synaptosomes are usually isolated by a combination of rate and equilibrium sedimentation techniques on sucrose or Ficoll gradients. They consist of a mixed population varying, for instance, in the identities of their chemical transmitters. Clearly, inquiries into synaptosomal structure, function, and biogenesis would be facilitated if this population could be subfractionated into defined types according to the transmitters and the cognate enzymes they contain. This communication shows that synaptosomes from cerebral cortices of 15 day old rats can be separated on Ficoll gradients into two fractions, P_2B_2 and P_2B_3, enriched in and presumably derived from adrenergic and cholinergic nerve endings, respectively. Different types of synaptosomes will be operationally defined by the transmitters they contain:

a) "cholinergic" – contain acetylcholine as a transmitter;

b) "adrenergic" – contain norepinephrine, dopamine, or putative transmitters such as histamine, tyramine, serotonin, etc.

2. Materials and methods

2.1. *Materials*

Enzyme grade sucrose (Schwartz-Mann). Ficoll (Pharmacia) was dialyzed 72 hr against three chages of glass-distilled water, concentrated with an Amicon UM-10 ultrafilter, and the stock solution frozen until use. All Ficoll solutions were made 0.32 M in sucrose and buffered with 5 mM HEPES, pH 7.45. Concentrations were determined from refractive indices, and the pH routinely checked immediately before use. Other biochemicals were from Sigma or Pierce. All solutions except Ficoll stock solutions were made with deionized water and kept cold or frozen until used. [1-^{14}C]acetylcholine iodide, (4.5 mCi/mmole), [1,2-^{14}C]choline chloride (6.2 mCi/mmole), and 3,4-dihydroxyphenyl-[1-^{3}H]diethylamine (dopamine) (10 Ci/mmole) were obtained from New England Nuclear. Male Sprague-Dawley rats, 14–16 days old, were from Harlan Industries, Cumberland, Indiana.

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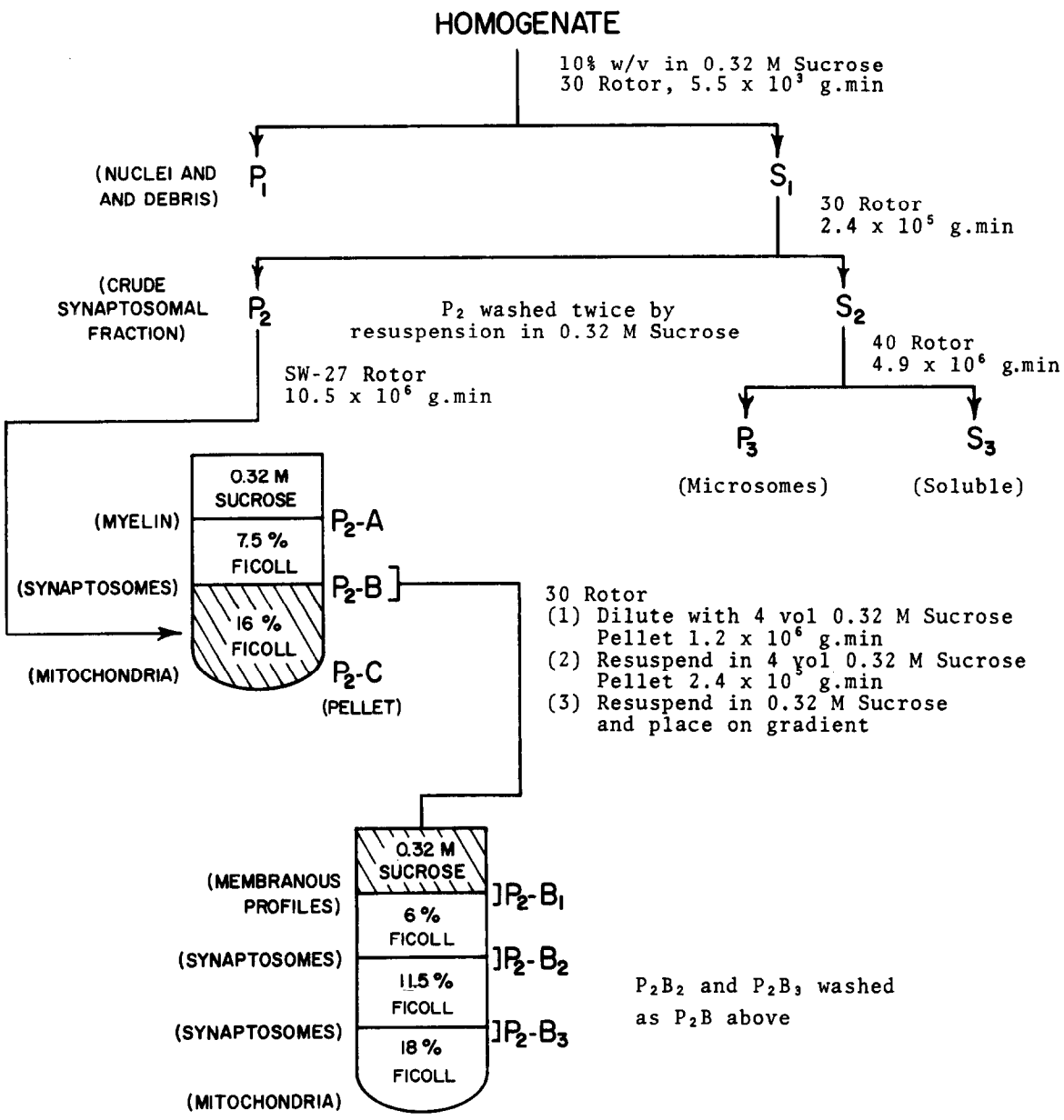
Abbreviations:

ACH, acetylcholine; AChE, acetylcholinesterase; ChAc, choline acetyltransferase; NaK-ATPase, Na^+-K^+-activated, ouabain-sensitive adenosine triphosphatase; COMTase, catechol-O-methyl transferase; DBH, dopamine β-hydroxylase; *g*·min, relative centrifugal force at midpoint of tube multiplied by centrifugation time in minutes; GABA, γ-amino-butyric acid; HEPES, *N*-2-hydroxyethylpiperazine- *N'*-2-ethanesulfonic acid.



Scheme 1.

2.2. *Preparation of subcellular fractions*

All operations unless noted were performed at 4–6°. Beckman rotors were used for the centrifugation procedures as outlined in scheme I. The only washing step in the original procedure was a single wash of P_2.

Stunned rats were decapitated and the cerebral cortices removed and placed in ice-cold 0.32 M sucrose. Tissue was homogenized in fresh 0.32 M sucrose (5 ml/brain) by 20 hand strokes in a Teflon-glass homogenizer at 0.2 mm clearance.

2.3. *Tissue slice incubations*

Cerebral cortices prepared as described above were sliced with a McIlwain chopper and preincubated 15 min at 30° in 9 ml Ringer's solution with oxygenation [3]. One ml Ringer's solution, containing 10 μCi [^{3}H]dopamine and 6 μCi [^{14}C]choline with sufficient cold carriers to bring the final concentration in the incubation medium of each component to 1×10^{-6} M, was added per brain and incubated an additional 15 min. Experiments using [^{14}C]acetylcholine used 13 μCi per brain with a final concentration of 1×10^{-6} M. Incorporation was stopped by pelleting the slices for 10 sec in a clinical centrifuge, resuspending in an equal volume of ice-cold Ringer's and repelleting. Slices were then washed once with cold 0.32 M sucrose by pelleting for 3900 $g \cdot$min in a 30 rotor before homogenization as described above. Eserine sulfate and nialimide (final conc. 1×10^{-4} M of each) were added with the label and at all resuspension steps during fractionation to inhibit cholinesterase and monoamine oxidase, respectively.

2.4. *Analytical procedures*

Aliquots for various assays were removed at each step in the separation procedure and pelleted for 22×10^5 $g \cdot$min in a 30 rotor. Fractions from Ficoll gradients were diluted with 3.5 vol of 0.32 M sucrose before pelleting. Samples for enzyme assays were resuspended in 0.32 M sucrose and stored at −20° until assayed. Glucose-6-phosphatase and COMTase were assayed after no more than one freeze–thaw cycle, and no enzyme was assayed after more than two freeze–thaw cycles.

Specific activity is expressed as μmoles/min per μg protein. Relative specific activities are defined as:

$$\frac{\text{Specific activity of fraction}}{\text{Specific activity of reference fraction}}$$

Standard procedures were used to assay for the following: protein [4, 5]; inorganic phosphate [6]; serotonin [7]; acetylcholine [8]; norepinephrine and dopamine [9]; cytochrome *c* oxidase (EC 1.9.3.1) [10]; AChE (EC 3.1.1.7) [11]; COMTase (EC 2.1.1.6) [12, 13]; ChAc (EC 2.3.1.6) [14]; DBH (EC 1.14.2.1) [15].

Acid phosphatase (EC 3.1.3.2) was assayed with *p*-nitrophenyl-phosphate at 37° in 100 mM citric acid buffer, pH 4.8, containing 0.1% Triton X-100 and 2 mM $MgCl_2$. Final substrate concentration was 2 mM, and the reaction was stopped by the addition of 2 vol of 0.15 M NaOH containing 1.45% Triton X-100. Absorbance was read at 400 nm.

Na-K-ATPase (EC 3.6.1.4) was assayed by determination of inorganic phosphate released in the presence or absence of 1 mM ouabain at 37°. The reaction buffer contained 100 mM Tris, pH 7.6, 4 mM $MgCl_2$, 1 mM EDTA, 140 mM NaCl, and 14 mM KCl. The reaction was initiated by the addition of ATP to a final conc. of 5 mM.

5′-Nucleotidase (EC 3.1.3.5) was assayed by determination of inorganic phosphate released from 5′-AMP at 37°. The reaction mixture contained 50 mM Tris, pH 7.7, and 2 mM $MgCl_2$. The reaction was initiated by addition of 3 mM 5′-AMP. Glucose-6-phosphatase (EC 3.1.3.9) activity was measured at 30° in 100 mM sodium cacodylate buffer, pH 6.0, containing 4 mM EDTA and 2 mM KF. Reaction was initiated by addition of glucose-6-phosphate to a final conc. of 2.5 mM.

3. Results

3.1. *Fractionation scheme*

A series of preliminary experiments with continuous iso-osmotic Ficoll gradients indicated the feasibility of separating two fractions of synaptosomes distinguished on the basis of their prelabeling *in vitro* with [^{3}H]dopamine and [^{14}C]choline. The technique was modified by the use of discontinuous grandients and yielded the scheme outlined (scheme 1). The two key steps are the use of a flotation gradient for the isolation of crude synaptosomes (P_2B) and the separation of two discrete populations of these particles banding sharply at the 6%–11.5% (P_2B_2) and the 11.5–18% (P_2B_3) Ficoll interfaces.

3.2. *Uptake of transmitters*

By this technique we have isolated synaptosomes that exhibit a preferential localization of [^{3}H]dopamine relative to [^{14}C]choline or [^{14}C]acetylcholine taken up by tissue slices. The results of typical experiments are shown in the two right-hand columns of table 1. We note the highly preferential uptake of dopamine relative to choline by fraction P_2B_2 as compared to P_2B_3. The corresponding ratio of uptake of dopamine to acetylcholine is lower (1.57 *vs* 4.37) but such a

Table 1
Distribution of transmitter substances in subcellular fractions from cerebral cortex of 15 day old rats.

Fraction	Norepinephrine		Serotonin		Dopamine (D)		Acetylcholine (A)		[^{3}H]Dopamine[a] / [^{14}C]-Choline	[^{3}H]Dopamine / [^{14}C]Ac. Chol.
	TC $\times 10^3$	SC $\times 10^3$	TC $\times 10^3$	SC $\times 10^3$	TC $\times 10^3$	SC $\times 10^3$	TC $\times 10^3$	SC $\times 10^3$		
P_2	464 ± 12	1.6	–	–	29 ± 3	0.87	9.3 ± 1.5	4.3	(1.0)	(1.0)
P_2B	343 ± 5	7.6	16.6 ± 1.3	4.6	24 ± 3	0.91	6.4 ± 0.8	8.5	1.3	1.0
P_2B_2	257 ± 4	17.6	2.1 ± 0.1	5.4	15 ± 1	1.73	1.0 ± 0.0	12.5	3.5	1.4
P_2B_3	51 ± 2	2.6	1.3 ± 0.1	2.4	5.6 ± 0.6	0.48	3.6 ± 0.5	34.8	0.8	0.9
P_2B_2/P_2B_3	5.04	6.8	1.6	2.1	2.7	3.6	0.27	0.36	4.4	1.5

TC = μg/g original tissue; SC = μg/μg protein. Average values from three separate runs are tabulated with standard deviations. These data are based on fractions obtained from our original procedure which included no washing steps except a single wash of P_2.

[a] Relative to the ratio in the crude mitochondrial (P_2 fraction); this fraction has taken up 5.3 nmoles of dopamine and 1.2 nmoles of acetyl choline in the first, 9.7 nmoles of dopamine and 0.59 nmoles of choline (all uncorrected for the total amount of protein used in the experiment) in the second set of experiments.

result would be consistent with the fact that cholinergic synaptosomes are capable of active transport only of choline and utilize it as the precursor of acetylcholine *in vivo* [6]. In all these experiments the amounts of dopamine taken up per gram of starting material are approximately the same, and its recovery and the recoveries of total counts at the various stages of subfractionation of the homogenate are ⩾90%.

Table 2
Comparison of marker enzymes in synaptosomal fractions.

	Specific activity relative to P_2	
	P_2B_2	P_2B_3
Enzymes associated with transmitters		
Choline acetyltransferase	1.3	2.9
Catechol-*O*-methyltransferase	5.5	0.45
Dopamine β-Hydroxylase	6.0	0.14
Negative marker enzymes		
Cytochrome *c* oxidase	0.53	0.58
Acid phosphatase	0.45	0.50
Glucose-6-phosphatase	< 0.02	< 0.02
Plasma membrane enzymes		
Na-K-ATPase	2.8	2.2
5'-Nucleotidase	3.1	2.4
Acetylcholinesterase	3.0	3.1

3.3. *Localization of transmitters*

A more direct proof of the fractionation of two classes of synaptosomes is provided by measuring their specific contents of actual transmitters. As is evident from the data in the first four columns of table 1 the adrenergic transmitters (dopamine, norepinephrine and serotonin) are concentrated in the less dense synaptosomes (P_2B_2), and acetylcholine in the more dense (P_2B_3) synaptosomes. More than 67% of transmitters found in the crude mitochondrial fraction (P_2) are found in the crude synaptosomal fraction (P_2B), and of this amount, more than 75% is recovered in P_2B_2 plus P_2B_3. The one exception is serotonin for which the recoveries are low, a result that may be correlated with the lower selectivity in the apparent distribution.

3.4. *Enzyme assays*

Further information can be obtained by assay of enzymes which are (a) required for the metabolism of specific transmitters within the synaptoplasm, (b) characteristic of synaptic plasma membranes, and (c) specific for cell fractions that may be included in the fraction of interest, either intrinsic to synaptosomes such as mitochondria and lysosomes [17], or as contaminants, such as membranes of the endoplasmic reticulum, Golgi, and other plasma membranes.

The distribution of such marker enzymes is summarized in table 2, which shows their enrichment relative to P_2, the crude mitochondrial fraction, as

reference. In terms of the types of markers cited above the enzymatic data are summarized as follows:

(a) Three enzymes frequently mentioned as associated with specific transmitter, ChAc, COMTase and DBH, are recovered preferentially in the synaptosomal fractions; their relative distribution parallels that previously established for the transmitters acetylcholine and norepinephrine.

(b) Enzymes that are now commonly accepted as markers for the synaptosomal (and other) plasma membranes are all enriched, approximately to the same extent (40% of the total activity from P_2 with an increase in specific activity of about 2.8-fold) but appear equipartitioned between the two populations.

(c) The level of contamination by free mitochondria, lysosomes and endoplasmic reticulum appears satisfactory based on the values of the RSA for the marker enzymes used in various reference fractions (see scheme 1) as follows: for mitochondria, cytochrome oxidase in fraction P_2C – 1.43; for lysosomes, acid phosphatase in fraction P_2C – 1.38; for endoplasmic reticulum, glucose-6-phosphatase in fraction P_2A – 2.0 and in fraction P_2B_1 – 4.67.

The enzyme data refer to preparations that include the additional washing steps for fractions P_2, P_2B, P_2B_2 and P_2B_3, as shown in scheme 1. These steps result in a quantitative improvement in the purity of the synaptosome fractions.

4. Discussion

This method for the isolation of two distinct synaptosomal populations depends on (a) the use of young (~ 15 days old) rats to minimize all problems introduced by the presence of myelin in significant quantities; (b) two equilibrium banding steps in isoosmolar gradients of Ficoll, the first by flotation, the second by sedimentation; (c) the collection of fractions exclusively from the boundaries indicated (with some attendant loss in yield) and (d) extensive washing at the steps indicated.

Although Alberici et al. [18] showed a somewhat similar distribution of COMTase on Ficoll gradients, that of other markers was either not studied or, in the case of ChAc, separation was not achieved, probably because of short sedimentation times (30 min) insufficient to reach isopycnicity. DeRobertis [19] has claimed that it is possible to separate P_2B on sucrose gradients into "cholinergic" and "noncholinergic" fractions. Examination of the data presented shows, however, that while the heavier "noncholinergic" fraction is indeed low in acetylcholine and enriched in GABA-related enzymes, the lighter "cholinergic" one appears to be composed of a mixed population, containing catecholamines, histamine and acetylcholine in large amounts, as well as mitochondria as shown by the elevated level of succinate dehydrogenase. In fact Snyder et al. [20] have provided good evidence that synaptosomes labeled by GABA uptake co-sediment with others labeled with radioactive catecholamines upon prolonged sedimentation, but are present in a lighter portion of the gradient, in apparent disagreement with DeRobertis. Snyder et al. [20] have also shown, in agreement with our data on Ficoll gradients, that synaptosomes labeled with norepinephrine, dopamine and tyramine all appear to co-sediment in sucrose gradients.

Synaptosomes capable of storing catecholamines have recently been isolated from rat brain hypothalamus on density gradients of colloidal silica by Lagercrantz and Pertoft [21]. They were found in two subpopulations, one banding at a density of 1.03 g/ml and the other (of synaptosomes containing mitochondria) at 1.055 ± 0.010 g/ml. The peaks corresponding to the latter fraction banded broadly in sucrose in the region of 1.16 g/ml. No data were provided on other types of transmitter or on any of the apposite enzymes.

The use of brains of immature rats (15 days old) was suggested by the fact that myelinization in the developing rat cerebral cortex is initiated at about 15 days of age [22]. Consequently contamination by myelin has been essentially eliminated from the present fractionations. Maturation of synapses in the rat brain, however, also is not complete at 15 days [23] and the present results cannot as yet be generalized to the mature rat cerebral cortex.

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