Immunoaffinity purification of intact, metabolically active, cholinergic nerve terminals from mammalian brain

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1. A method for the immunoaffinity purification of cholinergic nerve terminals from mammalian brain was developed. 2. A sheep antiserum to *Torpedo* electric-organ synaptic membranes, previously shown to be specific for cholinergic terminals in mammalian brain, was incubated with crude mitochondrial fractions prepared from rat brain. 3. Cholinergic nerve terminals sensitized by this serum were purified from the mitochondrial fractions on a high-capacity cellulose immunoabsorbent bearing a mouse monoclonal anti-(sheep immunoglobulin G) antibody. 4. Adsorption of nerve terminals on to the immunoabsorbent was assessed by using a variety of enzyme markers and gave a maximum yield of 24% of choline acetyltransferase, whereas non-specific binding was less than 1.0% for all of the enzymes measured. 5. Cholinergic terminals were purified 26-fold from rat caudate nucleus, 30-fold from rat hippocampus and 38-fold from rat cerebral cortex. The terminals were shown to be intact, osmotically sensitive and metabolically active.

Studies of the detailed biochemistry of mammalian brain have been hindered by the lack of homogeneous tissue preparations. Most presently available preparations, e.g. tissue slices and synaptosomes, reflect the complexity of the brain, containing many diverse cells and cell fragments.

Immunoaffinity techniques have the required selectivity to permit the isolation of specific populations of cells and cell fractions from complex mixtures. They have, for example, been used in the isolation of plasma membranes (Luzio *et al.*, 1976; Luzio, 1977; Westwood *et al.*, 1979), Golgi membranes (Ito & Palade, 1978), synaptic vesicles (Matthew *et al.*, 1981), endosome-derived vesicles (Luzio & Stanley, 1983) and subpopulations of lymphocytes (de Kretser *et al.*, 1980). In most studies it was found that indirect techniques (i.e. those using a second anti-immunoglobulin immunoabsorbent) have been the most successful in the immunoaffinity isolation of subcellular particles.

In the present paper a sheep antiserum, raised to synaptic membranes purified from the purely

cholinergic electric organ of *Torpedo*, was used to sensitize cholinergic nerve terminals from rat brain. This antiserum had previously been shown in mammalian brain to bind to a ganglioside antigen (Chol-1) localized on specifically cholinergic terminals (Jones *et al.*, 1981; Richardson, 1981, 1983; Richardson *et al.*, 1982). The sensitized terminals were isolated on a high-capacity cellulose immunoabsorbent bearing a mouse monoclonal anti-(sheep IgG) antibody. A preliminary report of this work has been presented (Richardson & Luzio, 1983).

Experimental

Materials

[³H]Acetyl-CoA (772 Ci/mol) was from New England Nuclear, Southampton, U.K.; [1-¹⁴C]glutamate (59 Ci/mol), [U-¹⁴C]glucose (274 Ci/mol), L-3,4-dihydroxyphenyl[1-¹⁴C]alanine (5.4 Ci/mol) and [2-³H]AMP (19.3 Ci/mol) were from Amersham International, Amersham, Bucks, U.K. Nicotinamide-adenine dinucleotide 2',3'-cyclic phosphate was generously given by Dr. J. Bradbury of this Department. Cellulase (from *Trichoderma viride*) was obtained from Sigma, Poole, Dorset, U.K.

Abbreviations used: Chol-1, cholinergic antigen 1; IgG, immunoglobulin G; DOPA, L-3,4-dihydroxyphenylalanine; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

Antiserum, complement and immunoadsorbent

Sheep anti-(Chol-1) serum was raised to synaptic plasma membranes derived from the electric organ of *Torpedo marmorata*. Nerve terminals, prepared by the method of Israël *et al.* (1976) were suspended in 10mM-Hepes, pH 7.4, and the membranes pelleted by centrifugation at 17000g for 30 min. A sheep was immunized by multiple subcutaneous injections of an emulsion of the synaptic membranes (0.75 mg of protein) dissolved in 0.1% Triton X-100 with Freund's Complete Adjuvant.

Mouse monoclonal anti-(sheep IgG) antibodies were prepared by standard methods (Galfre & Milstein, 1981). Lymphocytes from immunized Balb/c mice were fused with P3-NSI/1-Ag4-1 myeloma cells. Three different positive hybrid cell lines were cloned twice, and the corresponding antibodies obtained by growth of cells as tumours in mice. One antibody, coded 375, had the highest titre and avidity (approx. 10^{11} M^{-1}) as determined by Scatchard analysis, and was used in all the present studies. The antibody was purified from ascites fluid by precipitation in 40%-satd. $(\text{NH}_4)_2\text{SO}_4$, followed by dialysis against 0.2M-borate (adjusted to pH 8.2 with 4M-NaOH) for 48 h. The dialysed monoclonal antibody was then coupled to cellulose as described by Hales & Woodhead (1980). The protein content of the immunoadsorbent was $245 \pm 45(5) \mu\text{g}/\text{mg}$ of cellulose.

Human blood taken from normal volunteers was allowed to clot, and the serum was stored at -70°C until required, as a source of complement.

Preparation of a crude mitochondrial fraction from rat brain

A crude mitochondrial fraction (fraction P_2) was prepared from various regions of rat brain largely by the method of Gray & Whittaker (1962), in 0.32M-sucrose containing 1mM-EDTA and 2.5mM-Hepes buffered to pH 7.4 with 4M-NaOH. Tissue (0.5–2 g, in 10 vol of sucrose medium) was homogenized by 12 up-and-down strokes in a Teflon/glass apparatus at 4°C . After centrifugation at 1000g ($r_{\text{av.}} = 8.26 \text{ cm}$) for 10 min at 4°C , the supernatant was re-centrifuged at 12000g ($r_{\text{av.}} = 8.26 \text{ cm}$) for 20 min at 4°C , and the resultant pellet (P_2) containing the nerve terminals was resuspended in sucrose medium.

Affinity purification of cholinergic nerve terminals

Samples of the P_2 fraction were incubated with a 1:40 dilution of anti-(Chol-1)-serum, or non-immune sheep serum, for 40 min at 4°C , and re-centrifuged at 12000g for 15 min. After three washes with sucrose medium, the pellet was resuspended in 4 ml of 45% (v/v) Percoll solution. The Percoll had been buffered to pH 7.4 with 1M-Hepes/NaOH, pH 7.4, and adjusted to 150mM-

NaCl with 2M-NaCl, before dilution to 45% with a Krebs-Ringer Hepes solution of the following composition (mM): NaCl, 140; KCl, 4; CaCl_2 , 2.6; MgCl_2 , 2; glucose, 5.5; Hepes, 5. After centrifugation at 10000g ($r_{\text{av.}} = 8.26 \text{ cm}$) for 5 min at 4°C , the nerve terminals were aspirated off, diluted 1:3 with the Krebs-Ringer Hepes solution and pelleted at 10000g for 2 min in an Eppendorf Microfuge. The pellet (equivalent to 25–75 mg of tissue) was incubated with various amounts of immunoadsorbent [which had previously been washed consecutively with (1 ml each) 1mM-HCl, 45% Percoll and Krebs-Ringer Hepes] in a total volume of 0.4 ml. After 40 min at 4°C , bound and free terminals were separated by centrifugation (10000g, 2 min in an Eppendorf Microfuge) through 0.5 ml of 45% Percoll, and the pellet was washed three times by centrifugation with 45% Percoll. Non-specific binding was determined by incubation of nerve terminals with a 1:40 dilution of non-immune sheep serum rather than anti-(Chol-1) serum.

Complement-mediated lysis

Complement-mediated lysis of cholinergic nerve terminals was as described by Richardson (1983). Briefly, P_2 fractions derived from 5–20 mg of tissue were incubated for 30 min at 4°C in a 1:40 dilution of sheep anti-(Chol-1) serum, centrifuged at 10000g for 2 min in an Eppendorf Microfuge, resuspended in a 1:15 dilution of human serum and incubated for 20 min at 32°C . Samples (0.25 ml) were centrifuged in an Eppendorf bench centrifuge, and the supernatants were assayed for released enzyme activities.

Hypo-osmotic lysis

Purified nerve terminals were suspended in 10mM-Hepes, pH 7.4, at 4°C . After 10 min, the suspension was adjusted to 0.15M-NaCl, centrifuged in an Eppendorf bench centrifuge, and the supernatants were assayed for released enzyme activities.

Respiration studies

Samples of P_2 fractions and purified cholinergic nerve terminals were incubated in a sealed tube at 37°C in 0.25 ml of Krebs-Ringer Hepes containing glucose (0.05–5.0 mM) and $5 \mu\text{Ci}$ of $[\text{U}-^{14}\text{C}]$ glucose (final radioactivity 4.0–183 Ci/mol). The evolved CO_2 was adsorbed in 0.2 ml of Hyamine 10X (1M in methanol), which was suspended in a micro-tube above the incubation medium. The incubations were stopped by the addition of 0.05 ml of 20% (w/v) trichloroacetic acid. After a further 30 min at 37°C , the micro-tubes were transferred to scintillation vials and counted for radioactivity

(^{14}C efficiency = 85%). $^{14}\text{CO}_2$ output was estimated on the assumption that it had one-sixth the specific radioactivity of the original glucose.

Electron microscopy

Purified nerve terminals were fixed in 2.5% (w/v) glutaraldehyde in 0.125 M cacodylate, pH 7.2, for 30 min at 20°C, washed with 0.125 M cacodylate, and the immunoabsorbent was digested with 62.5 units of cellulase (1 unit = 1 μmol of glucose released/h) for 4 h at 20°C. The remaining immunoabsorbent pellet was post-fixed in 2% OsO_4 , dehydrated and embedded in Spur's low-viscosity resin. Thin sections were cut with glass knives, stained with lead and uranyl acetate and examined in a Zeiss 109 electron microscope.

Assays

Choline acetyltransferase (EC 2.3.1.6) was assayed by the method of Fonnum (1975) and used as a marker for cholinergic nerve terminals. Glutamate decarboxylase (EC 4.1.1.15), a marker for 4-aminobutyrate-containing terminals, was assayed by a CO_2 -trapping method similar to that described by Fonnum *et al.* (1970), and DOPA decarboxylase (EC 4.1.1.28), a catecholaminergic marker, by the method of Sims *et al.* (1973). Lactate dehydrogenase (EC 1.1.1.27) was assayed by the method of Keiding *et al.* (1974) and used as a general cytoplasmic marker. Acetylcholinesterase (EC 3.1.1.7) was assayed by the method of Ellman *et al.* (1961), the myelin marker 2':3'-cyclic nucleotide phosphodiesterase (EC 3.1.4.37) by the method of Sogin (1976), and 5'-nucleotidase (EC 3.1.3.5) as described by Stanley *et al.* (1980). Cytochrome oxidase (EC 1.9.3.1) was used as a mitochondrial marker and assayed by the method of Cooperstein & Lazarow (1951). All enzyme assays were performed at 37°C, except for cytochrome oxidase, which was assayed at 20°C.

Total protein in subcellular fractions was measured by the method of Bradford (1976), and protein covalently coupled to cellulose was measured by the method of Lowry *et al.* (1951), with sheep IgG as standard. The protein content adsorbed on the immunoabsorbent was assayed after elution with 0.5 ml of 10 mM-HCl in 0.25 M-sucrose at 4°C for 5 min (Beck & Hales, 1975). After the immunoabsorbent was pelleted (10000g, 2 min in an Eppendorf centrifuge), the supernatant was diluted to 1 ml with 0.1% (w/v) SDS, and re-centrifuged at 10000g for 20 min. Supernatant (0.8 ml) was then mixed with 0.25 ml of 20% (w/v) trichloroacetic acid, centrifuged (10000g for 10 min) and the pellet was assayed for protein by the method of Udenfriend *et al.* (1972). Alternatively the pellet was subjected to SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970)

and the protein estimated by densitometry after staining by the method of Ansorge (1982).

Results

In initial experiments, immunoabsorbents consisting of polyclonal anti-(sheep IgG) antisera coupled to a variety of solid supports (Sephadex, Sepharose and cellulose) were prepared. Incubation of these immunoabsorbents with nerve-terminal preparations, previously coated with anti-(Chol-1) antiserum, did not result in the specific retention of detectable choline acetyltransferase activity on any of the immunoabsorbents.

One factor that may have contributed to this lack of binding was the low binding capacity of the polyclonal immunoabsorbents (less than 1 μg of sheep IgG bound/mg). Consequently an immunoabsorbent consisting of a mouse monoclonal anti-(sheep IgG) antibody coupled to cellulose was prepared. The binding capacity of this immunoabsorbent was $289 \pm 55(5) \mu\text{g}$ of sheep IgG/mg of cellulose.

The time course of binding of cholinergic nerve terminals to this immunoabsorbent, assessed by using choline acetyltransferase as a marker, is shown in Fig. 1. Uptake was maximal after 40 min,

Fig. 1. Time course of uptake of cholinergic nerve terminals on to immunoabsorbent

P_2 fractions derived from the cerebral cortex were preincubated with anti-(Chol-1) serum, washed, and then incubated with immunoabsorbent (1 mg). After various times, the bound and free terminals were separated by centrifugation through 45% (v/v) Percoll, and the immunoabsorbent was washed. Each point represents the mean $\pm$ S.E.M. for three determinations.

and 50% of maximal after approx 15 min. Increasing the amount of immunoabsorbent present served to increase the yield of choline acetyltransferase to 24%, as shown in Fig. 2. However, non-specific binding of choline acetyltransferase to the immunoabsorbent (i.e. that obtained with terminals incubated with non-immune sheep serum) approached 2% when 5 mg of immunoabsorbent was used, compared with a maximum of 0.3% with 1 mg. To keep an acceptably low value for the non-specific binding, and to obtain a rapid, reproducible, method for isolation of the terminals, all further adsorptions were performed for 40 min at 4°C with 1–1.5 mg of immunoabsorbent. The amount of P₂ fraction incubated with the immunoabsorbent was varied depending on the activity of choline acetyltransferase present. Thus, for brain regions of high activity, e.g. the caudate nucleus, approx. 200 µg of P₂ protein (equivalent to 25–40 mg of tissue) was used, whereas for the cerebral cortex approx. 400 µg of P₂ protein was used.

Table 1 shows the affinity purification of cholinergic nerve terminals from the P₂ fraction of the caudate nucleus, a region of the brain rich in cholinergic innervation. Choline acetyltransferase was purified 13-fold over the P₂ fraction, which is equivalent to a 26-fold purification from the original tissue, compared with the 3–4-fold purification obtained by subcellular fractionation by the method of Gray & Whittaker (1962). Acetylcholinesterase, lactate dehydrogenase and cytochrome oxidase were also purified with the choline acetyltransferase, but to a lesser extent. In con-

Fig. 2. Uptake of cholinergic nerve terminals on to immunoabsorbent

P₂ fractions derived from the cerebral cortex were preincubated with anti-(Chol-1) serum, washed, and then incubated with various amounts of immunoabsorbent. After 45 min the bound terminals were separated and washed. Each point represents the mean $\pm$ S.E.M. for three determinations.

Table 1. Affinity purification of cholinergic nerve terminals from rat caudate nucleus

P₂ fractions derived from rat caudate nucleus (equivalent to approx. 30 mg of tissue) were incubated with a 1:40 dilution of sheep anti-(Chol-1) serum or non-immune sheep serum (for non-specific binding), washed, and incubated with 1 mg of immunoabsorbent for 40 min at 4°C. After washing, the immunoabsorbent pellets were assayed as described in the Experimental section. Purification is expressed as the ratio of the specific activity in the purified terminals to that in the P₂ fraction. Results (per 100 mg of tissue) are expressed as means $\pm$ S.E.M., for the numbers of experiments shown in parentheses.

	P ₂ content	Non-specific binding	Purified terminals on immunoabsorbent	Purification	Yield (%)
Choline acetyltransferase (nmol/h) (21)	506 $\pm$ 67	1.0 $\pm$ 0.5	86.6 $\pm$ 9.2	13.0	14.6
Lactate dehydrogenase (µmol/h) (17)	145 $\pm$ 21	<0.2	6.9 $\pm$ 0.3	4.2	4.8
Glutamate decarboxylase (nmol/h) (6)	93.7 $\pm$ 3.5	<0.5	<0.5	0	<0.5
Cytochrome oxidase (µmol/h) (6)	14.3 $\pm$ 5.2	<0.1	1.0 $\pm$ 0.4	6.2	7.0
Acetylcholinesterase (µmol/h) (8)	56.3 $\pm$ 11.6	0.2 $\pm$ 0.1	4.3 $\pm$ 1.3	6.8	7.6
2':3'-Cyclic nucleotide phosphodiesterase (µmol/h) (8)	912 $\pm$ 85	<1.8	5.8 $\pm$ 2.0	0.6	0.6
Protein (µg) (6)	573 $\pm$ 73	1.2 $\pm$ 0.6	6.4 $\pm$ 1.0	—	1.1

trast, there was no detectable glutamate decarboxylase activity (a marker for terminals containing 4-aminobutyrate), and very little 2':3'-cyclic nucleotide phosphodiesterase activity, present in the purified terminal fraction. Table 1 also shows non-specific binding to the immunoabsorbent of the various enzymes assayed, which was never greater than 0.5%.

After this purification of cholinergic nerve terminals from the caudate nucleus, a similar attempt was made from a region of the brain with a much lower cholinergic content, the cerebral cortex. The results are shown in Table 2. The choline acetyltransferase activity of the cerebral-cortex P₂ fraction was less than 25% of that in the caudate nucleus. However, it is apparent that choline acetyltransferase, acetylcholinesterase, lactate dehydrogenase and cytochrome oxidase had again been purified. The choline acetyltransferase was purified 19-fold over the P₂ fraction, which is equivalent to a 38-fold purification from the original tissue. A 38-fold purification of the cholinergic terminals over glutamate decarboxylase was also achieved. No significant DOPA decarboxylase (a marker for terminals containing catecholamines) activity was found in the purified terminals. The major contaminant of the cortical preparation was the myelin enzyme 2':3'-cyclic nucleotide phosphodiesterase, which was purified

1.5-fold, with a yield of only 0.7%. Non-specific binding to the immunoabsorbent of all enzymes assayed was less than 1% (Table 2). Fig. 3 shows a number of intact nerve terminals present on immunoabsorbent after affinity purification from the cortex. They contain synaptic vesicles and mitochondria as well as having postsynaptic adhesions.

Cholinergic terminals were also prepared from the hippocampus, a region of the brain with a choline acetyltransferase content between that of the caudate nucleus and the cortex. Purification of choline acetyltransferase containing terminals from this region of the rat brain was 30-fold, with a yield of $11.5 \pm 0.7(5)\%$.

In Table 3 the choline acetyltransferase/lactate dehydrogenase ratio in purified cholinergic terminals is shown. This ratio was constant in cholinergic terminals from all three brain regions studied. The ratio was much larger than that observed in the P₂ fractions, but the same as the ratio of these activities released when the P₂ fraction was lysed with anti-(Chol-1) serum plus complement (Table 3). These results are consistent with the immunoaffinity procedure specifically purifying cholinergic terminals, and also implies that the purified terminals are intact in that they retain their original lactate dehydrogenase and choline acetyltransferase contents.

The intactness and metabolic activity of the

Table 2. *Affinity purification of cholinergic nerve terminals from rat cerebral cortex*

P₂ fractions derived from rat cortex (equivalent to approx. 70 mg of tissue) were incubated with a 1:40 dilution of sheep anti-(Chol-1) serum or non-immune serum (for non-specific binding), washed and incubated with 1 mg of immunoabsorbent for 40 min at 4°C. After washing, the immunoabsorbent pellets were assayed as described in the Experimental section. Purification is expressed as the ratio of the specific activity in the purified terminals to that in the P₂ fraction. Results (per 100 mg of tissue) are expressed as means $\pm$ S.E.M., for the numbers of experiments shown in parentheses.

	P ₂ content	Non-specific binding to adsorbent	Purified terminals on immunoabsorbent	Purification	Yield (%)
Choline acetyltransferase (nmol/h) (41)	165 $\pm$ 20	0.5 $\pm$ 0.2	15.6 $\pm$ 0.9	18.9	9.5
Lactate dehydrogenase (μ mol/h) (28)	145 $\pm$ 14	0.1 $\pm$ 0.1	1.1 $\pm$ 0.1	1.5	0.8
Glutamate decarboxylase (nmol/h) (13)	474 $\pm$ 69	<0.8	1.1 $\pm$ 0.4	0.5	0.2
DOPA decarboxylase (nmol/h) (6)	240 $\pm$ 65	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	0.8	0.4
Cytochrome oxidase (μ mol/h) (16)	11.8 $\pm$ 1.0	0.1 $\pm$ 0.1	0.4 $\pm$ 0.1	5.9	3.3
Acetylcholinesterase (μ mol/h) (8)	9.9 $\pm$ 0.8	0.1 $\pm$ 0.1	0.7 $\pm$ 0.1	14.1	7.1
2':3'-Cyclic nucleotide phosphodiesterase (μ mol/h) (8)	327 $\pm$ 36	1.3 $\pm$ 0.6	2.4 $\pm$ 0.6	1.5	0.7
5'-Nucleotidase (nmol/h) (20)	995 $\pm$ 105	<2.0	4.2 $\pm$ 1.6	0.8	0.4
Protein (μ g) (5)	640 $\pm$ 80	1.9 $\pm$ 0.6	3.2 $\pm$ 0.6	—	0.4

Fig. 3. *Electron micrographs of immunaffinity-purified cholinergic nerve terminals*
Mitochondria, synaptic vesicles and post-synaptic adhesions can be seen. The bars represent 0.25 μm.

Table 3. *Purification of cholinergic nerve terminals with respect to lactate dehydrogenase*
Cholinergic nerve terminals were purified from P₂ fractions derived from the three brain regions shown below. The complement-lysed ratio is that obtained after complement mediated lysis of P₂ fractions with anti-(Chol-1) serum under conditions which resulted in greater than 75% release of choline acetyltransferase. The values are means ± S.E.M., for the numbers of determinations in parentheses.

	10 ³ × Ratio choline acetyltransferase/lactate dehydrogenase		
	Caudate nucleus	Hippocampus	Cortex
P ₂	4.2 ± 0.4 (23)	2.3 ± 0.2 (6)	1.1 ± 0.1 (34)
Complement-lysed P ₂	14.5 ± 0.5 (6)	15.8 ± 1.8 (6)	14.9 ± 1.0 (6)
Cholinergic terminals	13.2 ± 1.0 (17)	14.5 ± 1.4 (4)	15.2 ± 1.0 (28)

purified nerve terminals was further investigated. Exposure of the nerve terminals to hypo-osmotic shock resulted in the release of $70.6 \pm 1.0(4)\%$ of the bound choline acetyltransferase and $96.5 \pm 0.9(4)\%$ of the lactate dehydrogenase showing that they were intact by the criterion of osmotic sensitivity. The metabolic competence of the purified terminals was assessed by measuring their ability to oxidize glucose to CO_2 . A linear double-reciprocal plot of extracellular glucose concentrations against CO_2 release was obtained, giving an apparent K_m for glucose of 0.25mM and a maximum rate of $180\text{nmol of CO}_2\text{ produced/min per mg of protein}$.

Discussion

Previous attempts to purify neurotransmitter-specific nerve terminals have been frustrated by the similarities in density and size of most terminals (Jones, 1975), and by the absence of known neurotransmitter-specific cell-surface antigens (Schachner, 1982), which would permit the application of immunoaffinity techniques. The production of the cholinergic-specific anti-(Chol-1) antiserum (Jones *et al.*, 1981), together with a high-capacity immunoabsorbent, has now permitted the isolation of cholinergic nerve terminals of very high purity.

The high capacity of the immunoabsorbent ($289\mu\text{g}$ of sheep IgG bound/mg of cellulose) is conferred both by the monoclonal nature of the second antibody and the high coupling ratio of antibody to cellulose. This capacity is more than 100 times that of those immunoabsorbents prepared by using polyclonal antisera with Sepharose or acrylamide bases. The high capacity confers two main advantages. Firstly it decreases the influence of binding competition between the labelled terminals and any free sheep IgG, and secondly permits the use of small amounts of immunoabsorbent. The latter has the advantage of decreasing non-specific binding to very low values (Tables 1 and 2). The use of a high-capacity immunoabsorbent therefore plays a crucial role in the purification of cholinergic nerve terminals, and may be generally useful in the purification of minor components of subcellular fractions. It permits the use of a polyclonal sensitizing antiserum against a specific membrane antigen and allows rapid purification from crude subcellular fractions. Such an approach is not possible with immunoabsorbents of lower capacity.

In the immunoaffinity purification of cholinergic terminals from rat brain, a maximum yield of 24% of the P_2 choline acetyltransferase was achieved. The P_2 fraction has previously been shown to contain approximately the same number

of nerve terminals as the original tissue (Clementi *et al.*, 1966). The purification of choline acetyltransferase (38-fold with respect to the cerebral cortex) was accompanied by a lesser purification of three other enzymes: acetylcholinesterase, lactate dehydrogenase and cytochrome oxidase. The slightly lower purification of acetylcholinesterase in both cortical and caudate nucleus preparations may be explained by the fact that, although present at all cholinergic synapses, the enzyme is also present elsewhere (Silver, 1974). The mitochondrial marker cytochrome oxidase was purified approx. 6-fold in the cortex and caudate nucleus preparation, with yields of 3% and 7% respectively. This implies that there is a significant concentration of nerve-terminal mitochondria into cholinergic terminals, which may reflect the absolute requirement of these terminals for acetyl-CoA for the synthesis of acetylcholine.

The myelin marker 2':3'-cyclic nucleotide phosphodiesterase represents the major contaminant in the cortical preparation, being purified 1.5-fold compared with fraction P_2 . However, Table 2 shows that over half this enzyme activity is due to non-specific binding. A preliminary partial purification of the P_2 fraction, e.g. on a sucrose density gradient (Gray & Whittaker, 1962), decreases the amount of myelin exposed to the immunoabsorbent and also the degree of contamination of the purified terminals (results not shown). Another plasma-membrane enzyme, 5'-nucleotidase, which has previously been shown to be absent from the presynaptic plasma membranes of cholinergic nerve terminals in rat cortex and hippocampus (Richardson, 1983), was not co-purified with the cortical cholinergic nerve terminals.

The purified nerve terminals were intact on the basis of osmotic sensitivity and the ratio of choline acetyltransferase to lactate dehydrogenase activity. In addition, they were shown to be capable of oxidizing glucose to CO_2 , with an apparent K_m for glucose of 0.25mM . This is higher than the value (0.08mM) reported by Clark *et al.* (1982) for a mixed population of nerve terminals. It is, however, closer to the value reported by the same group for the conversion of glucose into acetylcholine (0.15mM) by such a mixed population.

In conclusion, a relatively rapid (4h) method for the immunoaffinity purification of intact cholinergic nerve terminals from mammalian brain has been developed. The terminals have been purified up to 38-fold from the brain, with a maximum yield of 24%, and are both osmotically sensitive and metabolically active. They are consequently an ideal system for the detailed study of cholinergic biochemistry and pharmacology as well as constituting a model system for the study of presynaptic modulation.

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