Biochemical Pharmacology, Vol. 22, pp. 2079–2084. Pergamon Press, 1973. Printed in Great Britain.

COMPARISON OF THE ENZYMATIC AND COLCHICINE BINDING PROPERTIES OF SUBCELLULAR FRACTIONS OF RAT BRAIN

V. J. NICKOLSON* and H. VELDSTRA†

Department of Biochemistry, State University, Wassenaarseweg 64, Leiden, The Netherlands

(*Received* 21 *December*, 1972, *accepted* 16 *February* 1973)

Abstract—Capacity for colchicine binding and activities of lactate dehydrogenase, monoamine oxidase and cholinesterase were determined in subfractions of adult rat brain. Colchicine binding activity was mainly found in cytoplasmic fractions. The fractions containing membranes bound little colchicine. Activity and specific activity of colchicine binding were roughly comparable with the corresponding values of lactate dehydrogenase. Colchicine binding in particulate fractions therefore is probably due to contamination with cytoplasmic protein. Polyacrylamide gel electrophoresis revealed some similarity between the protein patterns of the subsynaptosomal particulate fractions and showed that colchicine binding protein is not present in synaptic vesicles.

DURING the last few years it has been suggested that neurotubules play some role in synaptic transmission. This suggestion was based upon the inhibitory effect of colchicine on a variety of secretory processes,[1–10] and on the binding of colchicine by membranous fractions of rat brain.[11–14] Particulate fractions of rat brain, especially those containing microsomal and nerve-ending membranes, as well as synaptic vesicles, bind colchicine.[11–12] The presence of neurotubular protein in nerve endings of young mice[13] and in synaptic vesicle preparations of rat and mouse brain[14] has been demonstrated. To establish whether colchicine binding by synaptic membranes is due to contamination with cytoplasmic protein we compared the colchicine binding capacity of subcellular fractions of rat brain with the activities of the following enzymes: monoamine oxidase (localized in mitochondrial membranes[15]), cholinesterase (mainly localized in presynaptic and postsynaptic membranes[16]) and lactate dehydrogenase (a cytoplasmic enzyme[17,18]). The protein patterns of the various fractions were compared by acrylamide gel electrophoresis.

MATERIALS AND METHODS

Fractionation. Male Wistar rats (180–220 g) were killed by a blow on the back of the neck. Brains were removed and cerebral hemispheres dissected. Noncortical material was removed (cf. Whittaker and Sheridan[19]) and the remaining cortical grey matter was homogenized in 20 vol. of 0·32 M sucrose with a Braun (model C) homogenizer fitted with a Teflon pestle (clearance 0·25 mm). The homogenate was centrifuged for 10 min at 1000 *g* to remove nuclei and cell debris (P_1). The supernatant (S_1) was then centrifuged at 10,000 *g* for 30 min (P_2, S_2). The sucrose concentration of the pellet

* Present address: Medical Biological Laboratory TNO, Lange Kleiweg 139, Rijswijk, Z.H., The Netherlands.

† To whom requests for reprints should be sent.

(P_2), containing synaptosomes and mitochondria (cf. Gray and Whittaker[20]) was reduced to 0·1 M with distilled water (adjusted to pH 7 with Tris) in order to lyse the synaptosomes. The suspension was passed repeatedly through a hypodermic needle (diameter 0·45 mm) and centrifuged for 30 min at 20,000 *g* to remove mitochondria, undisrupted synaptosomes and large membrane fragments (P_2'). Six ml of the resulting supernatant (S_2') were layered onto a discontinuous sucrose density gradient (cf. Whittaker and Sheridan[19]) which consisted of 2 ml 2·0 M, 4 ml 0·8 M, 8 ml 0·6 M, 8 ml 0·4 M and 8 ml 0·25 M sucrose and had been standing for 1 hr at 4°. The gradient was centrifuged in a Spinco SW 27 rotor for 2·5 hr at 82,000 *g*. During subsequent fractionation the gradient was scanned at 280 nm with a Gilford spectrophotometer. Five fractions (I–V) were collected. All procedures were carried out at 4°.

Sucrose concentrations were determined by measuring the refractive index with an Abbe refractometer.

Protein was measured as described by Lowry *et al.*[21] using crystallized bovine serum albumin as a standard.

Cholinesterase (ChE) was determined by the method of Reed *et al.*[22] using [acetyl-^{3}H]choline (sp. act. 4·55 mCi/mmole, the Radiochemical Centre, Amersham) as a substrate. The radioactive acetate formed was adsorbed onto Amberlite CG-120 resin, eluted with ethanol and counted in a scintillation medium consisting of dioxane (1000 ml), naphthalene (100 g), 2,5-diphenyloxazole (PPO, 7 g) and 1,4-di-[2-(5-phenyloxazolyl)]-benzene (POPOP, 100 mg).

Monoamine oxidase (MAO, EC 1.4.3.4) was measured by the method of Kraml[23] with kynuramine dihydrobromide (Sigma) as a substrate. The fluorescence of the 4-hydroxyquinoline formed was measured with an Aminco Bowman spectrofluorimeter.

Lactate dehydrogenase (LDH, EC 1.1.1.27) was determined by the method of Kornberg[24] using pyruvate and NADH (Boehringer) as substrate and coenzyme. The oxidation of NADH was measured automatically with a Gilford spectrophotometer by following the extinction at 340 nm for 4 min.

Colchicine binding was determined as described previously.[25] Before being tested, fractions were diluted two-fold with 20 mM sodium phosphate buffer pH 6·5, containing 20 mM $MgCl_2$, 2 mM GTP (Serva) and 6 mM dithiothreitol (Calbiochem).

Neurotubular subunit protein was purified by the batch method of Weisenberg *et al.*[26]

Protein precipitating from a soluble supernatant of rat brain between 40 and 50 per cent ammonium sulphate was adsorbed onto DEAE-cellulose (Serva, W. Germany), washed twice with 0·5 M KCl, eluted with 0·8 M KCl, dialyzed and lyophilized.

Polyacrylamide gel electrophoresis was carried out following the procedure of Lim and Tadayyon.[27] All fractions were diluted with water so that the final sucrose concentration was 0·1 M. Particulate fractions (P_1, P_2, I–IV) were centrifuged for 1 hr at 100,000 *g* in a Spinco 50 Ti rotor. Pellets were solubilized in a medium consisting of 50 mM K_2CO_3, 8 M urea, 10 per cent (v/v) mercaptoethanol and 5 per cent (v/v) Triton X-100.

The soluble proteins in the fractions S_2, S_2' and V were concentrated ten times by addition of dry Sephadex G-25 (coarse). The fluid which remained after swelling of the Sephadex was collected by centrifugation and mixed with the solvent described above. The final protein concentration in all samples was 1 mg/ml. Electrophoresis

was carried out on a polyacrylamide gel system consisting of a 1·75 × 0·24 per cent stacking gel in acetate buffer pH 5·9 and a 7·0 × 0·20 per cent separation gel in acetate buffer pH 2·7. Gels contained 5 M urea and 0·25 per cent Triton X-100. The anode buffer (pH 4·0) contained 110 mM glycine, 16 mM acetic acid and 3 M urea. The cathode buffer consisted of 4·5 M acetic acid which was adjusted to pH 2·7 with KOH.

Stacking and separation were carried out at 4 and 8 mA/cm^2 respectively. Protein bands moved to the cathode. Gels were fixed and stained with 1 per cent amido black in 7 per cent acetic acid and destained by transverse electrophoresis in 0·2 per cent acetic acid.

Electron microscopy. Samples were fixed in 2·5 per cent glutaraldehyde in 100 mM sodium phosphate buffer pH 7·2 and centrifuged for 1 hr at 100,000 *g* in a Spinco 50 Ti rotor. Pellets were washed, post-fixed in 1 per cent osmium tetroxide in the same buffer, dehydrated in ethanol series and embedded in Epon.

Fixation and dehydration were carried out at 4°. Ultrathin sections were cut on an LKB Ultratoms III with glass knives.

Sections were stained with uranyl acetate and lead acetate in water and examined in a Philips EM 200 electron microscope. Negative staining preparations were made on formvar-coated copper grids and stained with uranyl acetate.

RESULTS

Figure 1 shows the optical density (280 nm) pattern of the gradient. On the basis of this pattern the gradient was divided into 5 fractions, the mean sucrose concentrations of which are shown in Table 1. Electron microscopy reveals that fractions I and II contain membrane fragments (Figs. 2 and 3). Fraction III contains synaptic vesicles, small membrane fragments and some coated vesicles (Fig. 4) whereas fraction IV consists of morphologically pure synaptic vesicles (Fig. 5). Fraction V, in which the

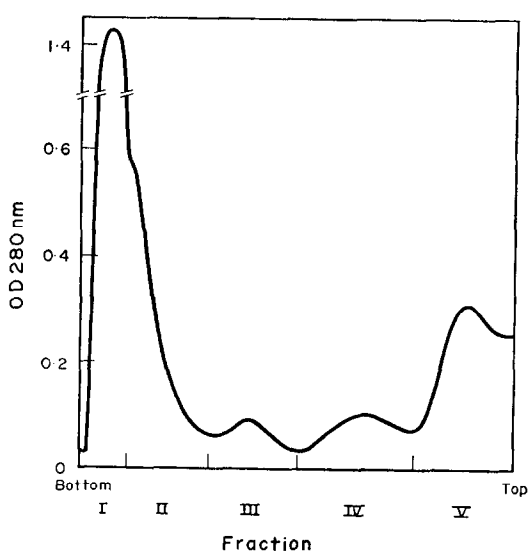

FIG. 1. Optical density (280 nm) pattern of the gradient. Fractions were collected as indicated in the figure.

nerve ending cytoplasm is found, shows no membranous structures. To characterize the fractions enzymatically, the activities of MAO, ChE and LDH were determined in addition to protein content and colchicine binding activity.

TABLE 1. CONCENTRATION OF SUCROSE IN THE FRACTIONS OF THE GRADIENT. MEAN VALUES ± S.E.M. FIGURES IN BRACKETS DENOTE NUMBER OF EXPERIMENTS

Fraction	Sucrose concn (M)
I	1·15 ± 0·07 (3)
II	0·71 ± 0·02 (3)
III	0·53 ± 0·02 (3)
IV	0·38 ± 0·00 (3)
V	0·15 ± 0·00 (3)

Tables 2 and 3 give the distribution and relative specific activities (RSA) of MAO, ChE, LDH and colchicine binding for the primary fractions (P_1–S_2') and the fractions of the gradient (I–V). The distribution of colchicine binding activity is similar to the distribution of LDH but different from the distribution of the membrane bound enzymes MAO and ChE. This indicates that colchicine binding protein is localized in the cytoplasm in a soluble form. This finding is consistent with the observation that neurotubules immediately depolymerize into colchicine binding subunit protein when rat brain is homogenized in 0·32 M sucrose.[28] The fact that the amount and the RSA of colchicine binding found in the nerve ending cytoplasm (fraction V) is lower than the corresponding values for LDH may be accounted for by the observation that nerve endings almost never contain neurotubules[29] and therefore may contain little

TABLE 2. DISTRIBUTION OF PROTEIN, MAO, ChE, LDH AND COLCHICINE BINDING ACTIVITY IN THE PRIMARY FRACTIONS (P_1–S_2') AND THE GRADIENT FRACTIONS (I–V).* VALUES ARE EXPRESSED AS MEAN PERCENTAGES ± S.E.M. OF THE TOTAL AMOUNT RECOVERED.† FIGURES IN BRACKETS DENOTE NUMBER OF EXPERIMENTS

Fraction	Protein	MAO	ChE	LDH	Colchicine binding
P_1	17·2 ± 1·2 (2)	21·2 ± 2·5 (2)	9·0 ± 5·0 (2)	8·3 ± 1·8 (2)	14·7 ± 8·3 (2)
S_2	31·7 ± 0·7 (2)	5·9 ± 0·3 (2)	25·5 ± 1·1 (2)	57·3 ± 5·1 (2)	63·1 ± 12·5 (2)
P_2'	39·7 ± 3·9 (2)	70·7 ± 2·1 (2)	53·5 ± 7·7 (2)	17·3 ± 1·2 (2)	19·9 ± 0·7 (2)
S_2'	10·6 ± 1·3 (2)	3·3 ± 0·3 (2)	12·1 ± 1·7 (2)	17·0 ± 3·0 (2)	7·0 ± 1·0 (2)
I	1·1 ± 0·1 (3)	1·9 ± 0·1 (3)	5·8 ± 1·4 (2)	1·0 ± 0·2 (3)	0·6 ± 0·1 (3)
II	0·5 ± 0·0 (3)	1·1 ± 0·1 (3)	2·7 ± 0·3 (2)	0·5 ± 0·0 (3)	0·2 ± 0·1 (3)
III	0·3 ± 0·1 (3)	0·2 ± 0·0 (3)	0·8 ± 0·2 (2)	0·6 ± 0·1 (3)	0·2 ± 0·0 (3)
IV	1·2 ± 0·2 (3)	0·1 ± 0·0 (3)	0·4 ± 0·1 (2)	1·6 ± 0·1 (3)	0·2 ± 0·1 (3)
V	7·3 ± 1·2 (3)	0·1 ± 0·0 (3)	2·6 ± 0·1 (2)	14·1 ± 2·0 (3)	5·2 ± 1·4 (3)

* The corresponding fractions of 3 gradients were pooled in each experiment and determinations were done in duplicate.

† (92·9 ± 4·8 per cent for the primary fractions and 87·7 ± 4·6 per cent for the fractions of the gradient.

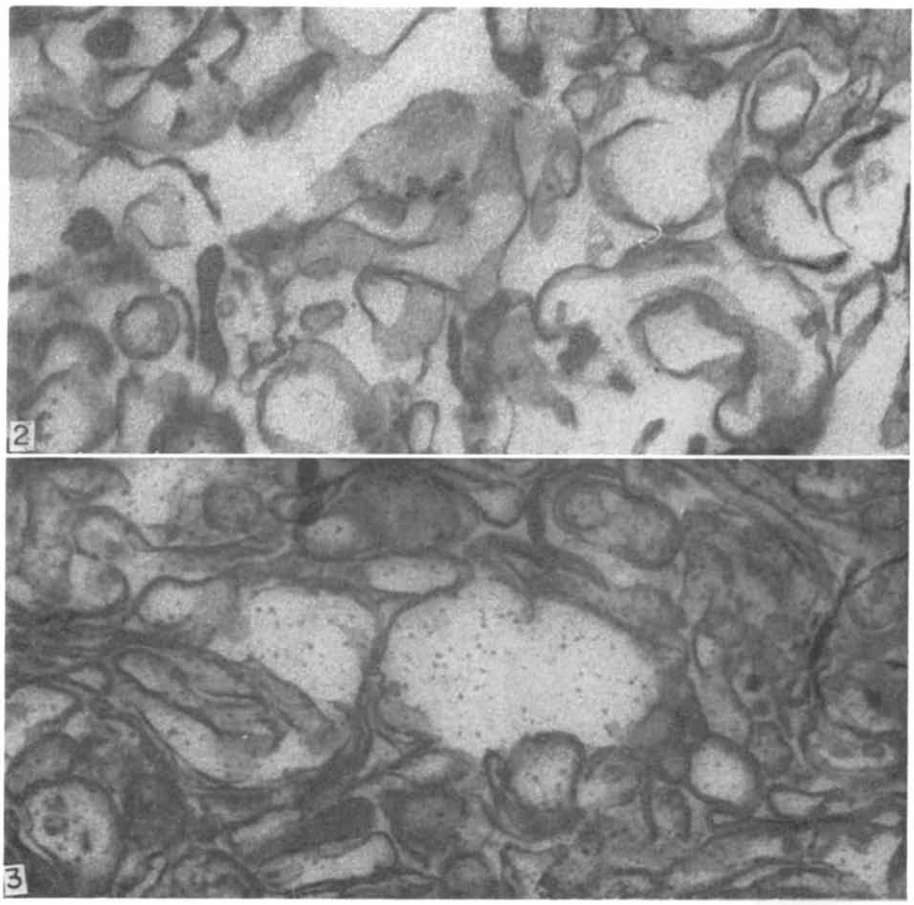

FIG. 2. Electron micrograph of the material in fraction I. (× 56,400)

FIG. 3. Electron micrograph of membranous material in fraction II. (× 56,400)

B.P.—*facing page* 2082

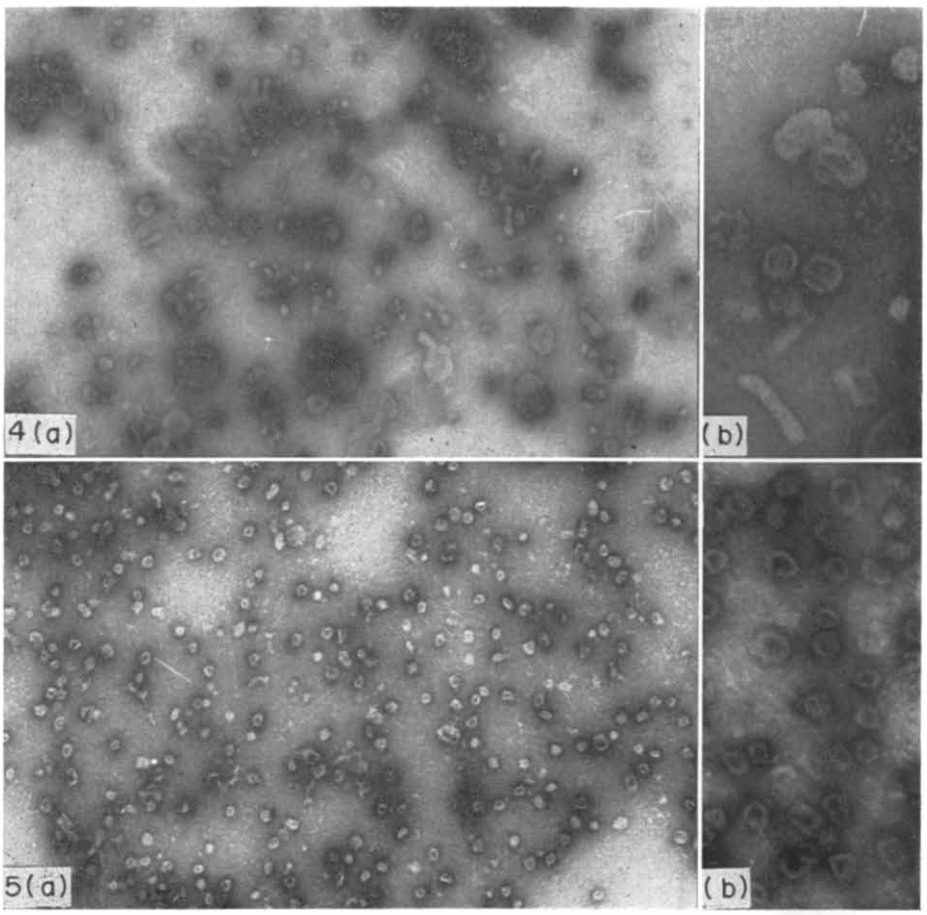

FIG. 4. Fraction III, negatively stained with uranylacetate. Note the presence of coated vesicles, elongate vesicles, "smooth" synaptic vesicles and membrane fragments. [Magnification (a) ×29,860; (b) ×84,800]

FIG. 5. Fraction IV, negatively stained with uranylacetate. Almost pure synaptic vesicles are visible. [Magnification (a) ×29,760; (b) ×84,800].

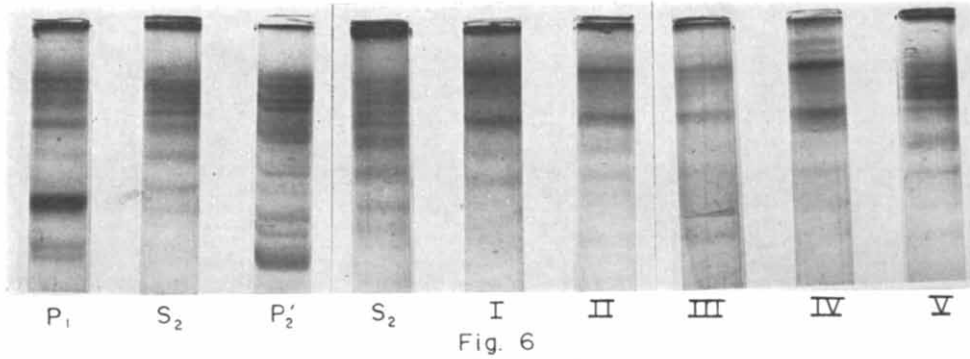

Fig. 6

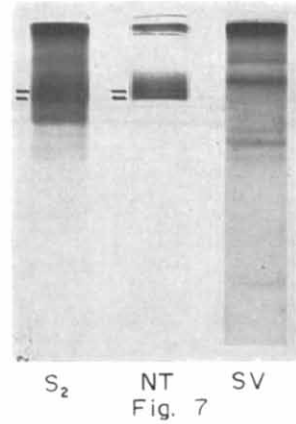

Fig. 7

FIG. 6. Acrylamide gel electrophoresis patterns of the fractions P_1, S_2, P_2', S_2', I, II, III, IV and V. Note the similarity of the patterns of the fractions I-IV.

FIG. 7. Acrylamide gel electrophoresis patterns of protein present in the supernatant (S_2), isolated neurotubular protein (NT) and synaptic vesicle protein (IV).

colchicine binding protein. The activities and RSA of MAO and ChE in fractions I and II show that the membrane fragments in these fractions are derived from mitochondria and nerve endings, whereas the synaptic vesicle fraction (IV) is hardly contaminated with these membranes. Some LDH activity, however, is present in this fraction which indicates a slight contamination with cytoplasmic protein. This may account for the colchicine binding found in this fraction (the same holds for fractions I–III). Acrylamide gel electrophoretic patterns (Fig. 6) show that there is some similarity among the proteins in fractions I–IV. This suggests that the protein compositions of synaptic vesicles and nerve ending membranes are not entirely different (cf. Breckenridge and Morgan[30]), which supports the view that exocytosis of synaptic vesicles may occur in the central nervous system. The proteins present in the soluble fractions (S_2 and V) are quite different from those in the particulate fractions. The prominent bands of the neurotubular protein are found in the S_2 fraction whereas they are absent in the synaptic vesicles (Fig. 7).

TABLE 3. RELATIVE SPECIFIC ACTIVITY OF MAO, ChE, LDH AND COLCHICINE BINDING. SPECIFIC ACTIVITY IS DEFINED AS ENZYME ACTIVITY AND COLCHICINE BINDING ACTIVITY PER MG PROTEIN. THE SPECIFIC ACTIVITY OF THE HOMOGENATE IS TAKEN TO BE 1·0. FIGURES IN BRACKETS DENOTE NUMBER OF EXPERIMENT. MEANS ± S.E.M.

Fraction	MAO	ChE	LDH	Colchicine binding
P_1	1·4 ± 0·1 (2)	0·5 ± 0·2 (2)	0·4 ± 0·1 (2)	0·3 ± 0·1 (2)
S_2	0·2 ± 0·0 (2)	0·9 ± 0·1 (2)	1·5 ± 0·0 (2)	1·2 ± 0·1 (2)
P_2'	2·2 ± 0·1 (2)	1·5 ± 0·1 (2)	0·4 ± 0·1 (2)	0·3 ± 0·0 (2)
S_2'	0·4 ± 0·1 (2)	1·3 ± 0·1 (2)	1·7 ± 0·1 (2)	0·5 ± 0·1 (2)
I	2·0 ± 0·2 (3)	5·8 ± 0·6 (3)	0·8 ± 0·2 (3)	0·4 ± 0·0 (3)
II	2·9 ± 0·1 (3)	5·9 ± 1·1 (3)	0·9 ± 0·1 (3)	0·3 ± 0·1 (3)
III	0·8 ± 0·1 (3)	2·4 ± 0·3 (3)	1·4 ± 0·1 (3)	0·3 ± 0·1 (3)
IV	0·2 ± 0·0 (3)	0·4 ± 0·1 (3)	1·1 ± 0·1 (3)	0·1 ± 0·1 (3)
V	0·0 ± 0·0 (3)	0·4 ± 0·0 (3)	1·6 ± 0·1 (3)	0·5 ± 0·0 (3)

DISCUSSION

The results presented here indicate that the colchicine binding protein of rat brain homogenates, made in 0·32 M sucrose, is cytoplasmic. Particulate fractions show a low colchicine binding activity which is probably due to contamination with cytoplasmic protein as is revealed by comparison with the activity of LDH in these fractions.

These observations do not agree with those of Feit and Barondes[11] and Lagnado[12] who found that fractions containing synaptosomal and microsomal membranes bound more colchicine per milligram of protein than the soluble fractions. The discrepancy between their findings and ours may be explained as follows. Lagnado *et al.* report that the colchicine binding activity of membrane fractions is much more stable than the activity of the soluble (free) protein. The colchicine binding activity in membrane fractions is therefore favoured above the colchicine binding in the soluble fractions in which the protein is not stabilized at all. This implies that the specific activity (colchicine bound per milligram of protein) found in particulate fractions is too high in proportion to the specific activity found in the soluble fractions.

If GTP, which is known to stabilize soluble colchicine binding protein[26,29] is present during the determination of colchicine binding, as in our experiments, particulate as well as soluble fractions are measured under stabilizing conditions. In this case only the specific activity (S.A.) of the supernatant fraction appears to be higher than the S.A. of the homogenate, whereas all other fractions, including the nerve ending cytoplasm, show lower values. Feit *et al.*[13] found that synaptosomes of young (3–7 days old) mouse brain contain relatively high amounts of colchicine binding (neurotubular) protein. With the technique they used to extract protein from nerve ending membranes, however, only a part of the total membrane-bound protein is solubilized (cf. Lim and Tadayyon[27]) whereas adsorbed protein, if present, preferentially will be extracted.

The presence of neurotubular protein in nerve endings of young animals suggests that this protein plays some role in the development of the nervous system (cf. ref. 31).

As our experiments show that nerve endings of adult animals contain only a low amount of neurotubular protein speculations about the role of this protein in synaptic transmission have to be regarded cautiously.

Acknowledgement—The authors thank Mrs. Marjolijn Hannaart-Toen for her assistance with the electron microscopic procedures.

REFERENCES

1. R. M. BERGLAND and R. M. TORACK, *Exp. cell Res.* **54,** 132 (1969).
2. G. BUSSOLATI and G. MONGA, *Experientia* **26,** 881 (1970).
3. D. FEWER and H. SHELDON, *J. cell Biol.* **47,** 62a (1970).
4. P. NÈVE, C. WILLEMS and J. E. DUMONT, *Exp. cell Res.* **63,** 457 (1970).
5. J. A. WILLIAMS and J. WOLFF, *Proc. natl. Acad. Sci., U.S.* **67,** 1901 (1970).
6. F. MALAISSE-LAGAE, M. H. GREIDER, W. J. MALAISSE and P. E. LACY, *J. cell Biol.* **49,** 530 (1971).
7. M. PERÍSIC and M. CUÉNOD, *Science* **175,** 1140 (1972).
8. B. ROSSIGNOL, G. HERMAN and G. KERYER, *FEBS Lett.* **21,** 189 (1972).
9. R. TEMPLE J. A. WILLIAMS J. F. WILBER and J. WOLFF, *Biochem. biophys. Res. Commun.* **46,** 1454 (1972).
10. N. B. THOA, G. F. WOOTEN, J. AXELROD and I. J. KOPIN, *Proc. natn. Acad. Sci., U.S.* **69,** 520 (1972).
11. H. FEIT and S. H. BARONDES, *J. Neurochem.* **17,** 1355 (1970).
12. J. R. LAGNADO, C. LYONS and G. WICKREMANSINGHE, *FEBS Lett.* **15,** 254 (1971).
13. H. FEIT, G. R. DUTTON, S. H. BARONDES and M. L. SHELANSKI, *J. cell Biol.* **51,** 138 (1971).
14. S. L. TWOMEY and F. E. SAMSON, *Brain Res.* **37,** 101 (1972).
15. G. R. DE LORES ARNAIZ and E. DE ROBERTIS, *J. Neurochem.* **9,** 503 (1962).
16. E. DE ROBERTIS, *J. Pharm. Pharmac.* **20,** 146 (1968).
17. O. Z. SELLINGER and F. DE BALBIAN-VERSTER, *J. biol. Chem.* **237,** 2886 (1962).
18. R. M. MARCHBANKS, *Biochem. J.* **100,** 65P (1966).
19. V. P. WHITTAKER and M. N. SHERIDAN, *J. Neurochem.* **12,** 363 (1965).
20. E. G. GRAY and V. P. WHITTAKER, *J. Anatomy* **96,** 79 (1962).
21. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
22. D. J. REED, K. GOTO and C. H. WANG, *Anal. Biochem.* **16,** 59 (1966).
23. M. KRAML, *Biochem. Pharmac.* **14,** 1684 (1965).
24. A. KORNBERG, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN) Vol. I, p. 441. Academic Press, New York (1955).
25. V. J. NICKOLSON and H. VELDSTRA, *FEBS Lett.* **23,** 309 (1972).
26. R. C. WEISENBERG, G. G. BORISY and E. W. TAYLOR, *Biochemistry* **7,** 4466 (1968).
27. R. LIM and E. TADAYYON, *Anal. Biochem.* **34,** 9 (1970).
28. J. B. KIRKPATRICK, *Science* **163,** 187 (1969).
29. V. J. NICKOLSON, Thesis, Leiden (1972).
30. W. C. BRECKENRIDGE and I. G. MORGAN, *FEBS Lett.* **22,** 253 (1972).
31. D. A. REDBURN and D. R. DAHL, *J. Neurochem.* **18,** 1689 (1971).