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A Rapid Method for the Preparation of Relatively Pure Metabolically Competent Synaptosomes from Rat Brain

By ROBERT F. G. BOOTH and JOHN B. CLARK
Department of Biochemistry and Chemistry, The Medical College of St. Bartholomew's Hospital, Charterhouse Square, London EC1M 6BQ, U.K.

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A rapid (less than 2h) method is described for the preparation of synaptosomes from rat brain by using a discontinuous Ficoll/sucrose gradient by a flotation technique. These synaptosomes are metabolically active and minimally (less than 5%) contaminated with 'free' mitochondria as judged by marker-enzyme assays and electron microscopy.

Since the pioneering work of Gray & Whittaker (1962) and De Robertis *et al.* (1962), which led to the isolation of the synaptosome or nerve-ending particle, a considerable amount of effort has been put into the study of the components and biochemical activities of these structures (see Whittaker, 1969; Rodriguez de Lores Arnaiz & De Robertis, 1972; Barondes, 1974).

The original techniques of preparation involved long high-speed centrifugations on discontinuous sucrose gradients, which led to preparations that at best were often almost 50% contaminated with structural elements other than synaptosomes (see Whittaker, 1969). The purity, but not the yield, was improved slightly by the use of continuous sucrose gradients (Whittaker, 1968), but in either case the high concentration of the sucrose required and hence the hyperosmolality was such that the final state of the synaptosomes so prepared was less than satisfactory (Whittaker, 1969).

The introduction of Ficoll/sucrose discontinuous gradients (Kurokawa *et al.*, 1965; Abdel-Latif, 1966; Autilio *et al.*, 1968; Cotman & Matthews, 1971) provided two advantages over the sucrose gradients: (*a*) it was possible to prepare suitable gradients while retaining iso-osmolality; (*b*) the time of centrifugation was shortened. Nevertheless, the synaptosome fractions prepared by various Ficoll/sucrose procedures, although metabolically more active (Verity, 1972), were not significantly less contaminated (25–40% contamination; Cotman, 1974) than those prepared by the original sucrose techniques.

As a preliminary step therefore to the study of the compartmentation of metabolism of synaptosomes, a method has been developed for the preparation of synaptosomes that is based on flotation in a Ficoll/sucrose gradient. The method allows the preparation to be carried out, in less than 2h of synaptosomes that are minimally contaminated with free mitochondria and metabolically active.

Experimental

Materials

ADP, NAD^+, $NADP^+$, 2-oxoglutarate and succinate were purchased from Boehringer Corp. (London), Bell Lane, Lewes, E. Sussex BN7 1LG, U.K. Glutamate, malate, Trizma base and antimycin A were obtained from Sigma (London) Chemical Co., Norbiton Station Yard, Kingston upon Thames, Surrey KT2 7BH, U.K. Ficoll was obtained from Pharmacia, Uppsala, Sweden, and purified by dialysis against double-glass-distilled water for at least 5h before use. Glutaraldehyde and pyruvate were purchased from Koch–Light Laboratories, Colnbrook, Bucks, U.K., and the pyruvate was twice distilled under vacuum and stored at −20°C before use. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was kindly given by Dr. P. Heylter of E. J. Dupont de Nemours and Co., Wilmington, DE, U.S.A. Glucose, rotenone, sodium cacodylate and all other laboratory chemicals were AnalaR grade and were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K. All solutions were made up in double-glass-distilled water.

Animals

Adult male rates (150–190g) of the Wistar strain were used in all the experiments.

Synaptosome preparation

Rats were killed by decapitation and the forebrain of the animal was rapidly removed. The forebrains were dropped into ice-cold isolation medium (0.32M-sucrose/1mM-potassium EDTA/10mM-Tris/HCl, pH7.4) and chopped into small pieces with scissors. The blood and other debris were washed off the brain tissue by adding more isolation medium

and decanting the supernatant from the top of the minced tissue.

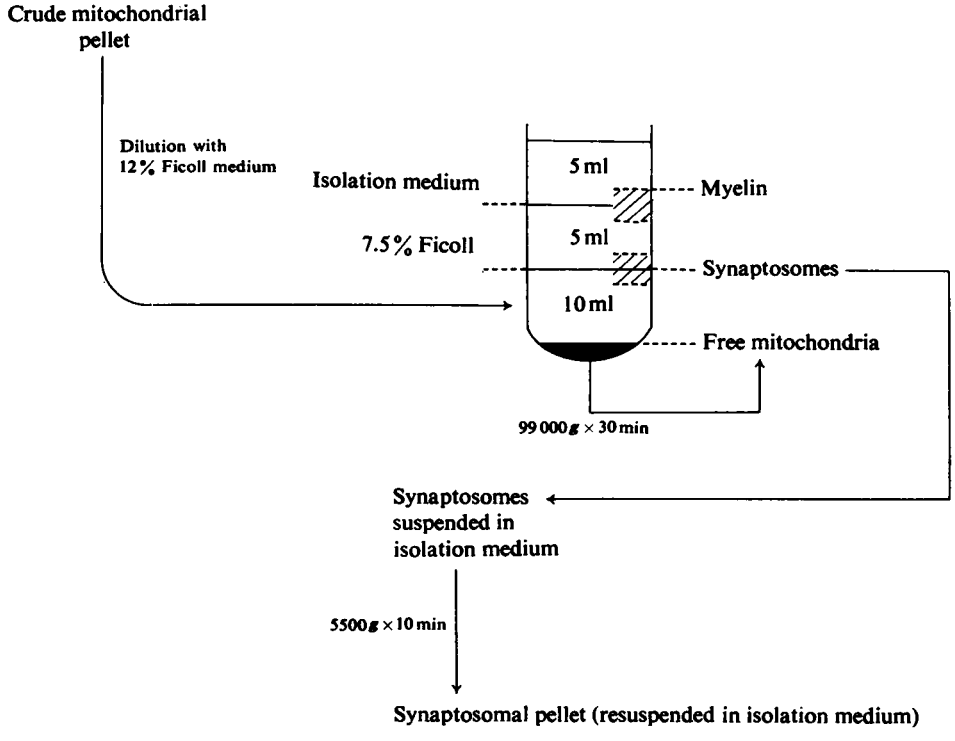
This washing procedure was repeated. The chopped tissue was then homogenized in a Dounce-type glass homogenizer by 12 up-and-down strokes with a glass pestle (total clearance 0.1 mm). This homogenate was diluted to 60ml with isolation medium and spun at 1300*g* for 3min in a MSE 18 high-speed centrifuge at 4°C. The supernatant from this spin was centrifuged at 17000*g* for 10min, producing the crude mitochondrial/synaptosomal pellet. This pellet (see Scheme 1) was resuspended in 5ml of isolation medium, diluted to 30ml with 12% Ficoll/sucrose medium [12% (w/w) Ficoll, 0.32M-sucrose, 50μM-potassium EDTA, pH7.4] and gently homogenized by hand in a Potter-type homogenizer (clearance 0.375mm). The crude mitochondrial suspension was introduced into a centrifuge tube and above this 5ml of 7.5% Ficoll/sucrose medium [7.5% (w/w) Ficoll, 0.32M-sucrose, 50μM-potassium EDTA, pH7.4] was carefully layered. Finally, on top of this 5ml of isolation medium was layered. The tubes were centrifuged at 99000*g* for 30min in a 3 × 23ml swing-out rotor in a MSE 50 centrifuge. Myelin and synaptosomes banded at the first and second interphases respectively, with the free mitochondria being pelleted at the bottom. The myelin layer was carefully removed and the synaptosomes were gently sucked off from the interphase. The synaptosomes were diluted to 30ml with isolation medium and gently homogenized as above in a Potter homogenizer. The synaptosomes were finally diluted to 60ml and spun at 5500*g* for 10min. The final synaptosomal pellet from four rats was taken up in isolation medium in a final volume of 1–2ml.

Synaptosomal respiration studies

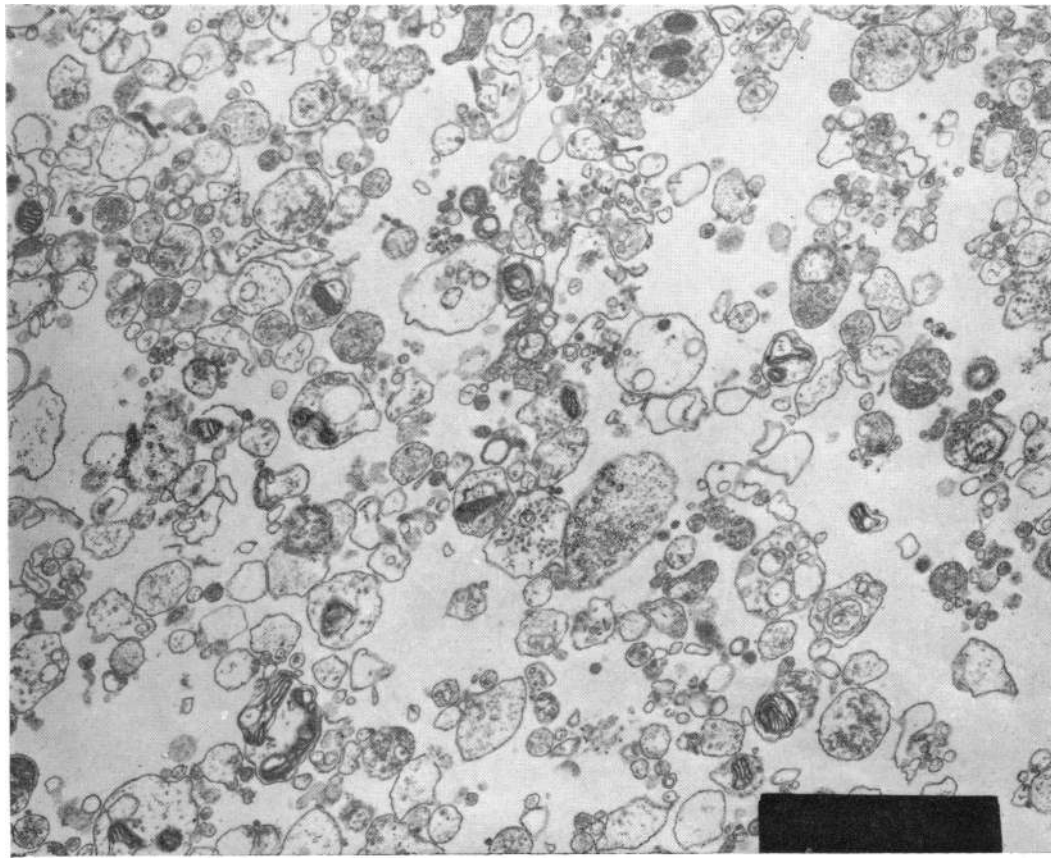
Synaptosomal respiration was measured polarographically at 25°C in an incubation medium containing either 100mM-K^+ (100mM-KCl, 75mM-mannitol, 25mM-sucrose, 10mM-phosphate/Tris, 10mM-Tris/HCl, 0.05mM-EDTA, pH7.4) or 100mM-Na^+, where 100mM-NaCl replaced 100mM-KCl. To the incubation medium was added 2–5mg of synaptosomal protein together with the appropriate substrates. Respiration was stimulated by the addition of 250μM-ADP (State 3; Chance & Williams, 1956).

Enzyme assays

These were carried out at 25°C by using an SP.1800 recording spectrophotometer. NADH- and



Scheme 1. *Fractionation of crude mitochondrial pellet*



EXPLANATION OF PLATE 1

Electron micrograph of a representative section through the synaptosomal pellet
Magnification × 24000. The specimen was prepared as outlined in the Experimental section.

NADPH–cytochrome *c* reductase (rotenone-insensitive) (EC 1.6.99.3) were measured essentially as described by Duncan & Mackler (1966). The reaction mixture contained 0.2M-potassium phosphate buffer, pH7.4, 1mM-KCN, 0.25mM-NAD(P)H, 0.1% (w/v) cytochrome *c*, 5μM-rotenone plus sample. The rate of increase in A_{550} was measured.

Acetylcholinesterase (EC 3.1.1.7) and lactate dehydrogenase (EC 1.1.1.27) were determined as previously described (Lai & Clark, 1976).

Protein concentration was measured by the biuret method (Gornall *et al.*, 1949).

Cytochrome measurements

Low-temperature spectra were carried out at 77K in an Aminco–Chance dual-wavelength spectrophotometer with a low-temperature attachment. Synaptosomal preparations were suspended to a concentration of 1–2.5mg/ml in (final concns.) 200mM-mannitol, 50mM-sucrose, 0.1mM-potassium EDTA, 20mM-Tris/HCl, pH7.4, and 10mM-potassium phosphate, pH7.4, as recommended by Wilson & Epel (1968). Complete oxidation of cytochromes was achieved by the addition of 10μM-rotenone, 2μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and bubbling with O_2. Reduced cytochromes were obtained by the addition of dithionite and allowing the suspension to become anaerobic. Reduced cytochrome *b* was determined in samples from incubations containing 10μg of antimycin A/ml together with 5mM-succinate and 10μM-rotenone. Samples were taken from each incubation and injected into microcuvettes with a 2mm light-path, maintained at liquid-N_2 temperature.

Electron microscopy

This was kindly carried out by Dr. D. Landon of the Institute of Neurology, Queen Square, London W.C.1. The synaptosomes were pelleted in 1% glutaraldehyde buffered in 0.1M-sodium cacodylate (pH7.3) and post-fixed in 1% OsO_4 in the same buffer. After embedding in epoxy resin, these sections were stained with ethanolic 2% uranyl acetate and aq. 0.1% lead citrate and examined with a Philips EM/30/G electron microscope at 80kV.

Results and Discussion

Electron microscopy of fractions

The synaptosomes as prepared by this technique are shown in Plate 1, which is a representative field of the final pellet (magnification ×24000). Several well-formed synaptosomes containing mitochondria and vesicles can be observed. Cutting random sections of the entire pellet indicated that the population was relatively homogeneous throughout. Semi-quantitative analysis of all of the sections when only well defined synaptosomes were counted indicates a 'free' mitochondrial contamination of the order of 3.8%.

Enzyme-contamination studies

Table 1 outlines the results of experiments in which the 'free' mitochondrial and microsomal contamination of the synaptosomes has been assessed by marker enzyme studies. For this purpose the activities of the rotenone-insensitive NAD(P)H–cytochrome *c* reductase have been measured. The NADH–cytochrome *c* reductase is located in both the outer mitochondrial membrane and the endoplasmic reticulum (Beattie, 1968; Gurd *et al.*, 1974), but the NADPH–cytochrome *c* reductase is specific to the endoplasmic reticulum in origin (Gurd *et al.*, 1974). Thus the difference between the two allows an estimate to be made of the 'free' mitochondrial contamination of the synaptosomes. These values are at best overestimates, since they rely on the accuracy of the specific activities of these two enzymes in a 'purified' brain mitochondrial and microsomal preparation. Any protein contamination in these preparations will decrease the specific activities of these marker enzymes and hence increase the apparent percentage contamination of the synaptosomal fraction. On this basis, therefore, by using mean specific activities derived from several experiments, these synaptosomes are contaminated by 'free' brain mitochondria on a protein basis by approx. 4% or less. Further support for this may be derived from the work of Gurd *et al.* (1974), who have also used the NADH/NADPH–cytochrome *c* reductase systems as a means of assessing synaptosomal contamination. If their specific activities for these enzymes in their purified mitochondrial/microsomal preparations are used instead of those in Table 1, the estimate for synaptosomal contamination by 'free' brain mitochondria is approx. 1% on a protein basis. Measurement of the acetylcholinesterase activity of this synaptosomal preparation, which may be taken as a marker of membrane contamination (Cotman & Matthews, 1971), indicates an activity of 51.9 (S.D. $\pm$ 7.7) ($n = 6$) nmol/min per mg of protein (S. Harvey, unpublished work). This represents a marked decrease in synaptosomal acetylcholinesterase activity as compared with previously published methods for synaptosome preparations, e.g. 79.1nmol/min per mg of protein (Lai *et al.*, 1977), which may be interpreted as a significant decrease in free membrane contamination of the synaptosomes. The integrity of the synaptosomal membrane may be judged by the measurement of lactate dehydrogenase activity in the presence and absence of 0.1% Triton. The mean specific activity of the lactate dehydrogenase in the presence of 0.1%

Table 1. *Free mitochondrial contamination of synpatosomal fraction*

Synaptosomes were prepared as in the Experimental section and the purified mitochondrial preparations by the method of Clark & Nicklas (1970). The purified microsomal fraction was prepared by centrifuging the post crude mitochondrial supernatant (see the Experimental section) for 1 h at 100000*g* and resuspending the pellet. Enzymes were assayed as outlined in the Experimental section and the results in nmol/min per mg of protein are the means ± S.D., where *n* is the number of separate experiments.

	Enzyme activity (nmol/min per mg of protein)	
	Rotenone-insensitive NADH–cytochrome *c* reductase	Rotenone-insensitive NADPH–cytochrome *c* reductase
Synaptosomal fraction (*a*)	3.8 ± 0.4 (*n* = 4)	0.9 ± 0.1 (*n* = 4)
Purified mitochondrial fraction (*b*)	31.3 ± 3.8 (*n* = 4)	Not detectable
Purified microsomal fraction (*c*)	19.3*	11.7*
Synaptosomal contamination		
(I) Total	$(a/b) \times 100 = 12\%$	$(a/c) \times 100 = 7.8\%$
(II) Free mitochondria	$12 - 7.8 = 4.2\%$	

* The values for the purified microsomal preparation are the means for at least two experiments and are taken from the work of Lai (1975).

Triton was 650.1 (S.D. ± 73.4) nmol/min per mg of synaptosomal protein ($n = 3$) and in its absence 22.3 (S.D. ± 0.9) nmol/min per mg of synaptosomal protein ($n = 3$), from which a value for the latency of this enzyme of 29.2 (S.D. ± 3.0; $n = 3$) may be obtained. Further data concerning the purity of the synaptosomes are shown in Fig. 1, which is a spectrum of the cytochrome content of this preparation taken at 77K. It is clear that the synaptosomes contain a complete range of cytochromes and that the concentrations on a protein basis are comparable and in some cases slightly higher than those reported previously by Clark & Nicklas (1970) for synaptosomes prepared by the classical Gray & Whittaker (1962) technique (cytochrome values in nmol/mg of protein: *b*, 0.034; c_1, 0.06; *c*, 0.137; *a*, 0.077; a_3, 0.035; see Fig. 1). The ratios of the different cytochromes are, however, very similar in the two synaptosome preparations and are comparable with those found in purified synaptic mitochondria (Lai & Clark, 1978).

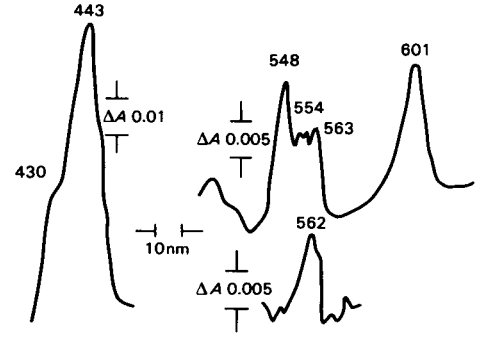


Fig. 1. *Difference spectra (oxidized against reduced) of cytochrome content of synaptosomal preparation at temperature of liquid N_2 (77 K)*

Synaptosomes were incubated as indicated in the Experimental section and spectra were taken by using the trapped steady-state technique (Wilson & Epel, 1968). Cytochrome concentrations were calculated by using the following wavelength pairs and extinction coefficients (mM): cytochrome *b* (563–575nm), 22; cytochrome *c* (550–540nm), 19; cytochrome *a* (605–630nm), 24; cytochrome a_3 (445–460nm), 164; cytochrome c_1 (556nm) was assumed to have the same extinction coefficient to that of cytochrome *c* (Wilson & Epel, 1968). The absorption of cytochromes in sucrose is known to intensify at 77 K and an intensification factor of 7 has been assumed (Wilson, 1967).

Respiration studies

Table 2 indicates the rates of respiration of these synaptosomes in media containing either 100mM-K^+ or 100mM-Na^+ with a variety of substrates. In both cases succinate was oxidized at almost twice the rate of other tricarboxylic acid-cycle intermediates (glutamate, pyruvate and 2-oxoglutarate). Glucose was also oxidized, but at rates that were approx. 4–5 times as slow as for succinate. The rates of oxidation were generally higher in the Na^+ medium (except for pyruvate and 2-oxoglutarate), the extent of the increase being dependent on the substrate. Verity (1972) also observed a Na^+ stimulation of synaptosomal respiration with glutamate as substrate, which

Table 2. *O_2 uptake by synaptosomes*

O_2-uptake values were measured by an oxygen electrode in either the 100mM-K^+ or 100mM-Na^+ medium (see the Experimental section) at 25°C either in the presence of 250μM-ADP (State 3) or in the absence of added ADP (State 4) together with the indicated substrate. The results are expressed in ng-atoms of O/min per mg of mitochondrial protein and are the means ± S.D. for several separate synaptosomal preparations.

		Rate of respiration (ng-atoms of O/min per mg of protein)	
Medium	Substrate	State 3 (+ADP)	State 4 (−ADP)
100mM-K^+	5mM-Glutamate ($n = 6$)	7.8 ± 1.9	2.7 ± 1.0
	5mM-Pyruvate ($n = 5$)	8.0 ± 1.7	5.0 ± 1.0
	10mM-Succinate + 5μM-rotenone ($n = 3$)	16.7 ± 3.3	7.8 ± 1.4
	7.5mM-2-Oxoglutarate ($n = 3$)	9.0 ± 0.7	4.3 ± 1.6
	10mM-Glucose ($n = 5$)	2.8 ± 0.7	2.8 ± 0.7
100mM-Na^+	5mM-Glutamate ($n = 4$)	11.7 ± 1.9	11.7 ± 1.9
	5mM-Pyruvate ($n = 3$)	7.7 ± 0.9	7.7 ± 0.9
	10mM-Succinate ($n = 3$) + 5μM-rotenone	21.3 ± 4.4	21.3 ± 4.4
	7.5mM-2-Oxoglutarate ($n = 4$)	8.7 ± 2.7	8.7 ± 2.7
	10mM-Glucose ($n = 4$)	4.5 ± 1.6	4.5 ± 1.6

was, however, greater than 2-fold. Table 2 also indicates that in the 100mM-Na^+ medium there was no stimulation of respiration on the addition of ADP, whereas in the 100mM-K^+ medium there were stimulations (1.6–2.9-fold) in the presence of ADP when glutamate, pyruvate, succinate and 2-oxoglutarate were being oxidized. These stimulations were, however, considerably less than those observed by Verity (1972), and the presence of bovine plasma albumin and/or EGTA made no difference in the experiments reported in the present paper. This is distinct from the situation reported by Verity (1972), where both albumin and 1mM-EGTA in the incubation were necessary to observe 'adequate respiratory control'. In this context it is important to recognize that, because of the much higher specific activities of brain mitochondria to oxidize all the substrates utilized in Table 3 (except for glucose) as compared with synaptosomes (Clark & Nicklas, 1970; Lai & Clark, 1976), even a few per cent contamination of the synaptosomes by free mitochondria will contribute a disproportionate increase in O_2 uptake on the addition of ADP. This might account for the relatively high absolute rates of O_2 consumption observed by Verity (1972) as compared with the rates reported here [i.e. 49ng-atoms of O/min per mg of protein with glutamate and ADP at 30°C (Verity, 1972); 7.8ng-atoms of O/min per mg of protein at 25°C (Table 2)] and also for the relatively high State 3/State 4 ratios (Chance & Williams, 1956) observed. Further comparison of the data of Table 2 with other observations reported in the literature is difficult because of differences in the incubation conditions used. However, when synaptosomes were incubated under the conditions used by Bradford (1969), i.e. in Krebs–Ringer medium at 37°C in the presence of 10mM-glucose, they showed an O_2 uptake very comparable with that reported by Bradford (1969), approx. 20ng-atoms of O/min per mg of protein (R. F. G. Booth, unpublished work). It appears therefore that this preparative procedure produces rat brain synaptosomes that are at least as metabolically competent as those produced by alternative existing methods. This method, however, has two distinct advantages over existing methods: (*a*) the relatively short time required for preparation (less than 2h), and (*b*) the marked decrease in contamination by non-synaptosomal material, particularly 'free' brain mitochondria (less than 4%), associated with this preparation. The minimization of the time that this synaptosomal preparation has to spend in the density-gradient procedure, and the fact that in this procedure this involves iso-osmotic Ficoll media rather than hyperosmotic sucrose media, may have important implications for the final metabolic and membrane integrity of the preparation and hence on its suitability as a model of the nerve ending.

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