A METHOD FOR THE RAPID SEPARATION OF SOLUBLE AND PARTICULATE COMPONENTS OF RAT BRAIN SYNAPTOSOMES

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1. Introduction

The preparation of synaptosomes or 'pinched off' nerve endings was first described 17 years ago [1,2]. Since that time much information about these preparations has become available (see [3–5]) and it has become apparent that in many respects the synaptosome may act as an in vitro tool for studying biochemical events at the nerve ending. For example, on depolarization a number of transmitters are released [6,7] and high affinity uptake systems for choline [8] and catecholamines [9] have been described. All the enzymes of transmitter synthesis are present [10] within the synaptosome and this may allow its use as a model for the regulation of transmitter metabolism.

An understanding of cellular metabolism has until recently been limited by difficulties in measuring metabolic concentrations in the cytosolic and mitochondrial compartments. Major factors which may influence the direction and flow of metabolic processes in the cell are the redox state ($[NAD]^+$ / [NADH]) and the phosphate potential ([ATP] / [ADP] + $[P_i]$) in each of the cell compartments [11]. Two main experimental approaches have been used to determine these parameters:

(i) The metabolite indicator method [12–14] where the redox state and the phosphorylation state are estimated by measurement of the appropriate enzyme couple [11,15];

(ii) The direct method where both the nicotinamide and adenine nucleotides, and inorganic phosphate are measured after rapid physical separation of the two compartments. The primary problem with the latter technique has been to separate the two compartments sufficiently rapidly to prevent dismutation of the metabolites between the two compartments.

The digitonin technique was introduced in [16] for the fractionation of hepatocytes into particulate and cytosolic compartments. This has been subsequently modified [17] and also applied to adipocytes [18]. Here we report the application of this technique to the synaptosome and its use in measuring adenine nucleotide levels in the cytosolic and particulate compartments of synaptosomes prepared from the rat brain. We also report measurements of the levels of guanine nucleotides in each of these compartments which have been unavailable due to methodological difficulties. A preliminary communication of some of this work has been presented [19].

2. Materials and methods

ADP, ATP, NADH and oxaloacetate were obtained from the Boehringer Corp. (London) Ltd., Lewes, E. Sussex BN7 1LG. Acetyl CoA, MOPS (3-(*N*-morpholino)propanesulphonic acid), digitonin and sodium pyruvate were obtained from the Sigma Chemical Co., Kingston, Surrey. Glucose, 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's Reagent), bovine plasma albumin (fraction V) and Aristar KH_2PO_4 for high performance liquid chromatography (HPLC) were obtained from British Drug Houses, Poole, Dorset BH12 4NN. [U-^{14}C]Sucrose (381 mCi/mmol) and [8-^{14}C]inosine (55 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks.

The p-trifluoromethoxyphenylhydrazone of carbonyl cyanide (FCCP) was the gift of Dr P. Heytler of E. I. Du Pont de Nemours and Co. Inc., Wilmington, DE. The silicone oil Versilube F.50 was the gift of Jacobson, Van den Berg and Co., London. The silicone oil AR.20 was the gift of Wacker-Chemie, Burghansen.

All other reagents used were of the highest purity commercially available and were purchased from one of the aforementioned suppliers. Double glass-distilled water was used in the preparation of all solutions.

Male rats of the Wistar strain (200–250 g body wt) were used throughout. They were fed ad libitum on laboratory diet no. 1 (Spratts, Reading, Berks.) and drinking water and food were always available.

Synaptosomes were prepared from rat cerebral cortex essentially as in [20]. The synaptosomes were resuspended after the final centrifugation step in an incubation medium containing: 136 mM NaCl; 5.6 mM KCl; 16.2 mM $NaHCO_3$; 2.2 mM $CaCl_2$; 1.2 mM $MgCl_2$; 1.2 mM NaH_2PO_4; 10 mM Tris–Hepes (pH 7.4). The medium was gassed with 95% O_2/5% CO_2 for 30 min before use.

Enzyme assays were carried out at 25°C in a Unicam SP1800 recording spectrophotometer. Enzyme activities in synaptosomes or lysed synaptosome preparations were measured in the presence (where necessary) of sufficient Triton X-100 to release maximal activity. Samples for enzyme assays were always measured on the same day that synaptosomes were prepared. Citrate synthase (EC 4.1.3.7.) was assayed essentially by the method in [21]. Lactate dehydrogenase (EC 1.1.1.27.) was assayed as in [22].

2.1. *Incubation of synaptosomes and separation of cytosolic (extramitochondrial) and particulate (mitochondrial) constituents*

Synaptosomes (6 mg/ml) were incubated in the incubation medium described earlier at 37°C in a shaking water bath with 95% O_2–5% CO_2 as the gas phase. Fractionation of the synaptosomes was achieved as follows: 0.2–0.4 ml of the incubation mixture was injected into 1 ml of an ice cold solution of 125 mM KCl, 20 mM MOPS buffer (pH 7.2), 3 mM EDTA and 1 mg digitonin (= digitonin medium). The mixture was whirlimixed for 10 s and then 1.0 ml was transferred to an Eppendorf cup which had been maintained at 0°C containing 0.1 ml 10% (w/v) trichloroacetic acid (TCA) plus 15% (v/v) methanol plus 0.1 μCi [8-^{14}C]inosine beneath a layer of a silicone oil mixture (AR20: Versilube F50 = 1:1) which was then placed in an Eppendorf centrifuge (type 3200) rotor. Centrifugation for 30 s was started 30 s after mixing with digitonin. Enzyme activities were subsequently measured in the top layer and compared with the total activity in synaptosomes.

2.2. *Nucleotide measurements*

For nucleotide measurements of the 'particulate' compartments the oil phase was carefully aspirated and the pellet homogenized in the TCA/methanol mixture. The pellet was centrifuged at 14 000 × *g* for 2 min in an Eppendorf microcentrifuge and 90 μl of the supernatant removed. The supernatant was washed 5 times with 1 ml additions of 'wet' ether (diethyl ether : H_2O, 1:1, equilibrated for 24 h) to remove the TCA. Finally the sample was neutralized to pH 7–8 by addition of crystals of Trizma base.

The nucleotides were measured using high performance liquid chromatography (HPLC). The apparatus consisted of a Partisil PXS/10/25 SAX column no. 10 (Whatman Ltd., Springfield Mill, Kent, ME14 2LE), fitted with a valve injector. Two Altex Model 10A pumps were used in conjunction with an Altex Model 410 Solvent Programmer (Anachem Ltd., Luton, Beds.). The column eluant was monitored at 260 nm by a Cecil CE2012 Reference Channel Variable Wavelength UV Detector (Cecil Instruments Ltd., Cambridge, CB4 4AZ) equipped with a 10 μl flow cell. The output from the monitor was recorded and integrated with a Hewlett Packard Model 3380 A Integrator.

For the elution of the nucleotides the buffers used were 0.005 M KH_2PO_4 (pH 4.5) and 0.9 M KH_2PO_4 (pH 4.5). ATP was eluted within 25 min. The volume of sample injected onto the column was calculated by collection of the eluant from the column and using the [8-^{14}C]inosine in the TCA/methanol mix as an internal standard.

2.3. *Protein determination*

Proteins were determined [23] using bovine plasma albumin for the construction of the standard curves. Sodium deoxycholate was added to 2% (w/v) prior to the addition of the Biuret reagent.

3. Results and discussion

3.1. *Enzyme marker studies*

The efficiency of the digitonin fractionation of the synaptosomes was determined by the release of the enzymes lactate dehydrogenase and citrate synthase, the enzymes being used as markers for the cytosolic and mitochondrial compartments, respectively. Table 1 shows the release of the enzymes to be a function of both the digitonin concentration and their time of exposure to the detergent. At 2.0 mg digitonin/ml considerable leakage of citrate synthase occurs, whilst below this figure the leakage is $< 10\%$ suggesting that under these conditions the integrity of the mitochondrial membrane is only marginally affected. For the remaining experiments where fractionation of synaptosomes into cytosolic and mitochondrial compartments was carried out, incubation for 30 s together with 1.0 mg digitonin/ml was employed. Under these conditions, although the release of lactate dehydrogenase is only ~80–85% the actual release of the cytosolic nucleotides was probably in excess of this figure. This is supported by the observation [24] that digitonin treatment of hepatocytes causing a leakage of only ~45% of the lactate dehydrogenase results in the sucrose permeable space increasing to 95% from 50% in untreated hepatocytes. Thus, in hepatocytes the plasma membrane becomes permeable to small molecules such as sucrose at a time when large enzyme molecules still do not readily leak out.

Table 1
Release of marker enzymes from synaptosomes exposed for different times to varying concentrations of digitonin

Digitonin (mg/ml)	Incubation time					
	30 s		30 s		40 s	
	CS	LDH	CS	LDH	CS	LDH
0	10.6	12.6	11.6	13.7	11.3	14.1
0.5	8.9	78.7	9.4	82.4	8.7	81.2
1.0	10.9	90.3	10.0	83.9	15.9	86.0
2.0	18.5	86.7	21.3	89.6	26.5	81.0

Citrate synthase (CS) and lactate dehydrogenase (LDH) were measured in the supernatant fraction after treatment of synaptosomes with digitonin as in section 2. The values given indicate the enzyme activities and are expressed as a % of the total synaptosomal enzyme activities prior to fractionation. The figures are the means of 2 experiments. Lactate dehydrogenase and citrate synthase were measured as in section 2

Furthermore, examination by electron microscopy of thin sections of digitonin-treated synaptosomes revealed a loss of their previously intact plasma membrane, the disappearance of all neurotransmitter containing vesicles and the appearance of the mitochondria as inner-membrane matrix vesicles (cf. [25]). This would suggest that the experimental conditions employed were suitable for the disruption of the synaptosomal membrane with minimal damage to the intrasynaptosomal mitochondria.

3.2. *Latency of citrate synthase and lactate dehydrogenase in digitonin-treated synaptosomes*

The latency of the lactate dehydrogenase of intact synaptosomes has been reported to be ~30 when compared in the presence and absence of 0.1% Triton X-100 [20]. To determine the latency of lactate dehydrogenase after treatment with 1.0 mg digitonin/ml for 30 s (as in section 2), the Eppendorf tube containing the lysed synaptosomes was spun at 15 000 × g for 60 s to pellet the synaptosomes. The supernatant was decanted and replaced with fresh digitonin-free medium. The synaptosomes were resuspended and measurement of the lactate dehydrogenase in the presence and absence of Tritox X-100 revealed a latency of 1. These results thus correlated well with the evidence from electron microscopy indicating lysis of all the synaptosomal membranes.

When the latency of the inner mitochondrial matrix enzyme, citrate synthase, was measured in the pellet fraction after digitonin treatment, a latency of between 11 and 12 was observed thus indicating that the inner mitochondrial membrane was intact. This result is in accord with the data shown in table 1, indicating minimal leakage of citrate synthase under the conditions employed.

3.3. *Carry-over contamination*

Table 2 shows the carry-over contamination of [U-^{14}C]sucrose from the supernatant to the oil phase and the pellet fraction. The synaptosomes were treated in the usual fashion except that [8-^{14}C]inosine was omitted from the TCA/methanol mix and [U-^{14}C]-sucrose was included in the digitonin medium.

Table 2
[U-^{14}C]Sucrose carry-over contamination during fractionation of synaptosomes

	pmol	%
A. Before centrifugation	72.4	100
B. After centrifugation		
1. Supernatant	71.5 ± 2.9	98.9 ± 0.4
2. Oil phase	0.14	0.02
3. TCA/methanol plus solubilized pellet	0.8 ± 0.3	1.1 ± 0.4

[U-^{14}C]Sucrose (~0.05 μCi) was added to the digitonin medium and synaptosomes were added from an incubation and centrifuged as in section 2. The counts present in the supernatant, oil and TCA/methanol phase plus the solubilized pellet were then measured. The results for each phase are expressed as a % of the total counts present. The number of pmoles in each phase has also been given and was calculated from original specific activity of the sucrose

Where standard deviations are indicated $n = 4$ and these results are the means of 4 different experiments

The results show that a negligible amount of sucrose was recovered in the oil fraction and < 1.5% was found in the TCA/methanol phase plus the solubilized synaptosomal pellet. The results suggest that in terms of carry-over contamination the methodology is comparable with that reported for the fractionation of hepatocytes [25].

3.4. *Adenine and guanine nucleotide compartmentation*

Lysis of the synaptosomal membrane with digitonin results in a rapid dilution of the extramitochondrial metabolites which could result in a rapid efflux of mitochondrial metabolites. Thus, where measurements of adenine nucleotides were to be made, atractylate (0.5 mM) was included in the digitonin medium to reduce any possible exchange of mitochondrial adenine nucleotides.

Table 3 shows the distribution of the adenine and guanine nucleotides between the extramitochondrial (cytosolic) and particulate (mitochondrial) compartments. Cytosolic values have been calculated from the difference between the whole synaptosome nucleotide values and the digitonin treated synaptosome (particulate) values. This method of estimation was considered to be the most accurate as the time required for the quenching of the adenine nucleotides was the same in both cases, whereas if the cytosolic values had been measured directly the time required for the quenching of the nucleotides in this compartment would have been somewhat longer.

Evidence that no alteration in the adenine nucleotide values occurs throughout the fractionation procedure is given by a comparison of the values for whole synaptosomes treated in a digitonin free medium and then spun through silicone oil (see table 3) with the values for synaptosomes directly quenched with TCA/methanol. When the synaptosomes were directly quenched, the values are 1.55 ± 0.17 nmol/mg synaptosomal protein ($n = 3$) and 1.13 ± 0.21 nmol/mg synaptosomal protein ($n = 3$) for ATP and ADP, respectively. These values correspond well with those observed for synaptosomes spun through silicone oil (see table 3). It would therefore appear that no major changes occur throughout the course of the fractionation.

The results in table 3 show that ~75% of the ATP is located in the extramitochondrial compartment and 25% in the mitochondrial compartment. In contrast, there is nearly twice as much ADP in the mitochondrial compartment as in the cytosol. This produces ATP/ADP ratios of 3.1 and 0.44 for the cytosol and mitochondria, respectively. The effect of FCCP, an uncoupler of oxidative phosphorylation, is to severely reduce the ATP content of both the mitochondrial and cytosolic compartments, whilst the ADP content increases in the cytosol but decreases by ~25% in the mitochondrial compartment. The overall loss of ADP plus ATP is probably due to adenylate kinase activity converting ADP into ATP plus AMP while phosphorylation of ADP is completely inhibited by the action of the uncoupler, thereby resulting in an overall loss of ADP and ATP to AMP.

The distribution of the guanine nucleotides differs from those of the adenine nucleotides, there being nearly twice as much GDP as GTP in the synaptosome. Most of the synaptosomal guanine nucleotide is located in the cytosolic compartment with only 28% of the total GDP and 18% of the total GTP being present in the mitochondrial compartment. The GTP/GDP ratios in both the mitochondrial and cytosolic compartments are rather

Table 3
Compartmentation of adenine and guanine nucleotides in synaptosomes in the presence and absence of FCCP

Incubation Addition	Whole Synaptosome Pellet			Digitonin Treated Synaptosome Pellet			Cytosol		
	ATP	ADP	ATP/ADP	ATP	ADP	ATP/ADP	ATP	ADP	ATP/ADP
	(nmol/mg)			(nmol/mg)			(nmol/mg)		
10 mM Glucose	1.31 ± 0.19 (n = 7)	0.99 ± 0.16 (n = 8)	1.32	0.29 ± 0.06 (n = 7)	0.66 ± 0.16 (n = 7)	0.44	1.01 ± 0.18 (n = 7)	0.32 ± 0.08 (n = 7)	3.1
10 mM Glucose + 20 μM FCCP	0.37 ± 0.07 (n = 6)	1.03 ± 0.14 (n = 6)	0.36	< 0.04 (n = 4)	0.49 ± 0.12 (n = 4)	< 0.08	0.23 ± 0.14 (n = 5)	0.44 ± 0.14 (n = 4)	0.52
	GTP	GDP	GTP/GDP	GTP	GDP	GTP/GDP	GTP	GDP	GTP/GDP
	(nmol/mg)			(nmol/mg)			(nmol/mg)		
10 mM Glucose	0.52 ± 0.11 (n = 7)	0.92 ± 0.20 (n = 6)	0.56	0.12 ± 0.06 (n = 6)	0.37 ± 0.06 (n = 7)	0.32	0.37 ± 0.15 (n = 7)	0.53 ± 0.29 (n = 5)	0.70
10 mM Glucose + 20 μM FCCP	0.31 ± 0.07 (n = 6)	1.0 ± 0.16 (n = 6)	0.31	< 0.08 (n = 5)	0.55 ± 0.17 (n = 5)	< 0.02	0.27 ± 0.06 (n = 5)	0.56 ± 0.22 (n = 5)	0.48

Synaptosomes (~5 mg/ml) were incubated at 37°C as in section 2. After 10 min the synaptosomes were fractionated and analysed as in section 2. When present, FCCP was at 20 μM and was added at the beginning of the incubation. Cytosolic values of nucleotides were calculated by subtraction of the digitonin treated synaptosome values (particulate) from the whole synaptosome values

lower than those seen for the adenine nucleotides being 0.32 and 0.70, respectively. The effect of FCCP is similar to that seen with the adenine nucleotides with the triphosphate/diphosphate ratio being reduced. In the case of the guanine nucleotides however, no loss of total nucleotide is observed.

The application of the digitonin fractionation technique to synaptosomes for the separation of the cytosolic and mitochondrial compartments will allow investigation into the role of the adenine and guanine nucleotide systems in different cellular compartments and their influence on metabolism at the nerve terminal. Hitherto, technical difficulties have precluded investigation of the relevant problems.

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