COMPARATIVE BIOCHEMICAL AND BIOPHYSICAL STUDIES ON RAT BRAIN SYNAPTOSOMES

P. HARGITTAI, D. ÁGOSTON+ and Á. NAGY*

Central Research Institute for Physics, 1525 Budapest, +Department of Anatomy, University Medical School, 6701 Szeged and **Institute of Biochemistry, Biological Research Center, 6701 Szeged, Hungary*

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1. Introduction

Synaptosome isolation methods should meet two principal but contradicting requirements: The fraction should be enriched as much as possible in synaptosomes, while the amount of time used during the isolation process should be as short as possible. The conditions of synaptosomal preparation and the duration of each step of purification greatly affect the physiological state of the subcellular fractions. In some biochemical and biophysical examinations of synaptic activities the surveyor needs information about the state of the electrogenic mechanism. Several procedures are available to measure the membrane potential in suspension as a physiological parameter of the synaptosomes. Some of these methods are based on the distribution of radioactive isotopes [1,2] while others utilize specific, potential-sensitive dyes [3,4].

In our experiments, we have, for the first time, compared synaptosomes which were prepared by 3 established techniques, on the basis of biochemical criteria, and also by a biophysical measurement which indicates the physiological state of the plasma membrane.

2. Methods

2.1. *Preparation of synaptosomes and reference fractions*

Rat cortical synaptosomes were prepared by using 3 different methods: Two slightly hyperosmotic sucrose media were used according to [5] and [6]; and the method in [7] involving an isoosmotic Ficoll/sucrose solution. The final synaptosomal fractions were kept at 4°C before use. The purity of the synaptosomal fractions was also checked by electron microscopy.

Purified mitochondrial fractions were prepared according to [8] and purified microsomal fractions were prepared by centrifuging the supernatant of crude mitochondrial fraction (100 000 × g for 1 h) and washing the pellet twice.

2.2. *Enzyme assays*

The following marker enzymes activities were determined by spectrophotometric techniques on a Cary 15 recording spectrophotometer: Lactate dehydrogenase (EC 1.1.1.27) [9]; NADPH–cytochrome c reductase (EC 1.6.2.3) [10]; and fumarate hydratase (EC 4.2.1.2) [11]. Protein content was determined as in [12].

2.3. *Fluorescent dye studies*

All synaptosomal fractions were carefully resuspended in Krebs medium (145 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 2.0 mM NaH_2PO_4, 10 mM glucose, 20 mM Tris–base; pH adjusted to 7.4 with maleic acid) prior to optical registration. In all additions, the summed concentration of NaCl and KCl was 150 mM. The solutions which contained different KCl concentrations are noted as 'X K' media.

The fluorescence measurements were done using the 3,3'-dipentyl-2,2'-oxocarbocyanine (diO-C_5(3)) potential-sensitive fluorescent dye as in [3], except that the conventional fluorescence detecting system was constructed in our laboratory.

3. Results

3.1. *Enzyme assays*

Table 1 outlines the specific activities of the

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Table 1
Contamination of various synaptosomal preparations by other subcellular constituents

Synaptosomal fractions prepared as in [ref.]:		No. expt	NADPH cytochrome *c* reductase (NCR)	Fumarate hydratase (FH)	Lactate dehydrogenase (LDH)	
					−Detergent	+1% Triton-X-100
[5]	(a_1)	3	2.5 ± 0.5	32.9 ± 3.9	n.d.	0.90 ± 0.05
[6]	(a_2)	6	2.4 ± 0.3	49.2 ± 7.3	0.09 ± 0.01	1.30 ± 0.12
[7]	(a_3)	4	1.5 ± 0.2	46.5 ± 6.8	n.d.	0.95 ± 0.11
Purified microsomes	(b)	4	9.6 ± 1.3	30.7 ± 8.8	n.m.	n.m.
Purified mitochondria	(c)	4	n.d.	404.5 ± 75.6	n.m.	n.m.
100 000 × *g* Supernatant	(d)	4	n.m.	n.m.	1.98 ± 0.16	2.05 ± 0.19

Synaptosomal preparations	% Contamination by subcellular constituents based on assays above		
	Microsomes (NCR) $(a_i/b) \times 100$	Mitochondria (FH) $(a_i/c) \times 100$	Soluble protein (LDH) $(a_i/d) \times 100$
[5]	26.0 ± 6.3	8.1 ± 1.8	n.d.
[6]	25.0 ± 4.6	12.2 ± 3.0	5 ± 1
[7]	15.6 ± 3.0	11.5 ± 2.8	n.d.

n.d., non-detected; n.m., non-measured

Specific activities expressed in nmol . min^{-1} . mg $protein^{-1}$ are the means ± SD except for LDH where units are μmol . min^{-1} . mg^{-1}

marker enzymes in the 3 different synaptosomal preparations. The data were similar to those reported for synaptosomes by the originators of the preparation techniques [5,7] except for the method in [6] where no enzyme activities had been reported. This table also contains data related to the known contaminations of synaptosomal fractions. The integrity of synaptosomes was shown by the negligible lactate dehydrogenase activity measured in the absence of detergents. Percentage contaminations were calculated by comparing the activities of synaptosomal fractions and the contaminants, purified microsomes, mitochondria and soluble proteins. On the basis of the mean specific activities determined from several experiments we found that the synaptosomal fractions contained ~12% 'free' mitochondrial contamination. The estimate for microsomal contamination in these fractions was $\lesssim$25%, on a protein basis.

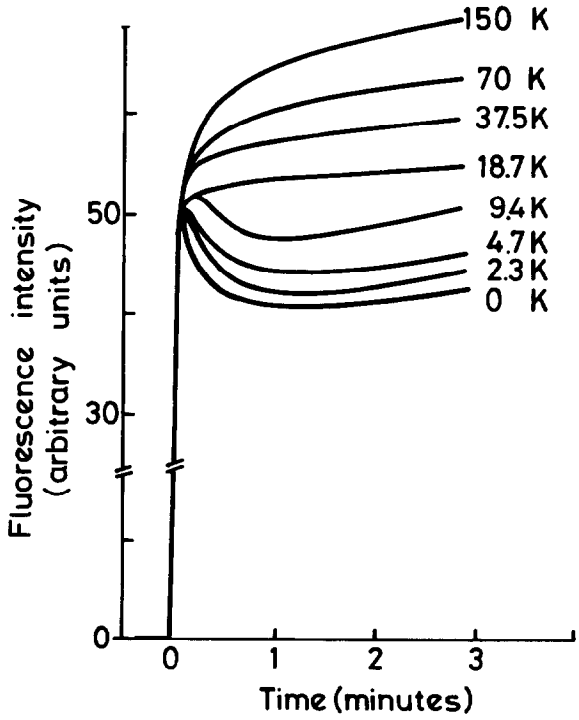

Fig.1. The variation of the fluorescence light intensity *vs* time at various potassium concentrations: 100 μl synaptosome suspension (6 mg protein/ml) prepared as in [7] was diluted in 3 ml of dye containing Krebs solution at different [K^+]. The final dye concentration was 4.44 μM at 37°C.

3.2. *Fluorescent dye studies*

Carbocyanine dye, an optical indicator for membrane potential in suspension, was added to Krebs media. When synaptosomes were mixed into this solution, the fluorescent light intensity increased. The size and time course of the changes in light intensity depended on the potassium concentration of the solution (fig.1). If 100 μl '150 K' medium was added to 3 ml control system, the fluorescence intensity showed a sudden increase. Addition of the same volume of '0 K' medium had no effect beside diluting the mixture.

The control synaptosomal fluorescence, i.e., synaptosomes in '5 K' medium, at constant protein concentration (4 mg/ml) varied ~50% with the type of the synaptosomal preparations, due to the different composition of the contaminants (table 1). The $[K^+]$-dependent synaptosome fluorescence [3], which is a measure of the membrane potential, can be characterized by the difference of the fluorescent light intensity observed in '5 K' and '150 K' media. These differences were similar in all 3 preparations. The changes in fluorescence intensities of synaptosomes as a function of extrasynaptosomal potassium concentrations are shown in fig.2. At 15–150 mM KCl the synaptosome fluorescence is proportional to the logarithm of

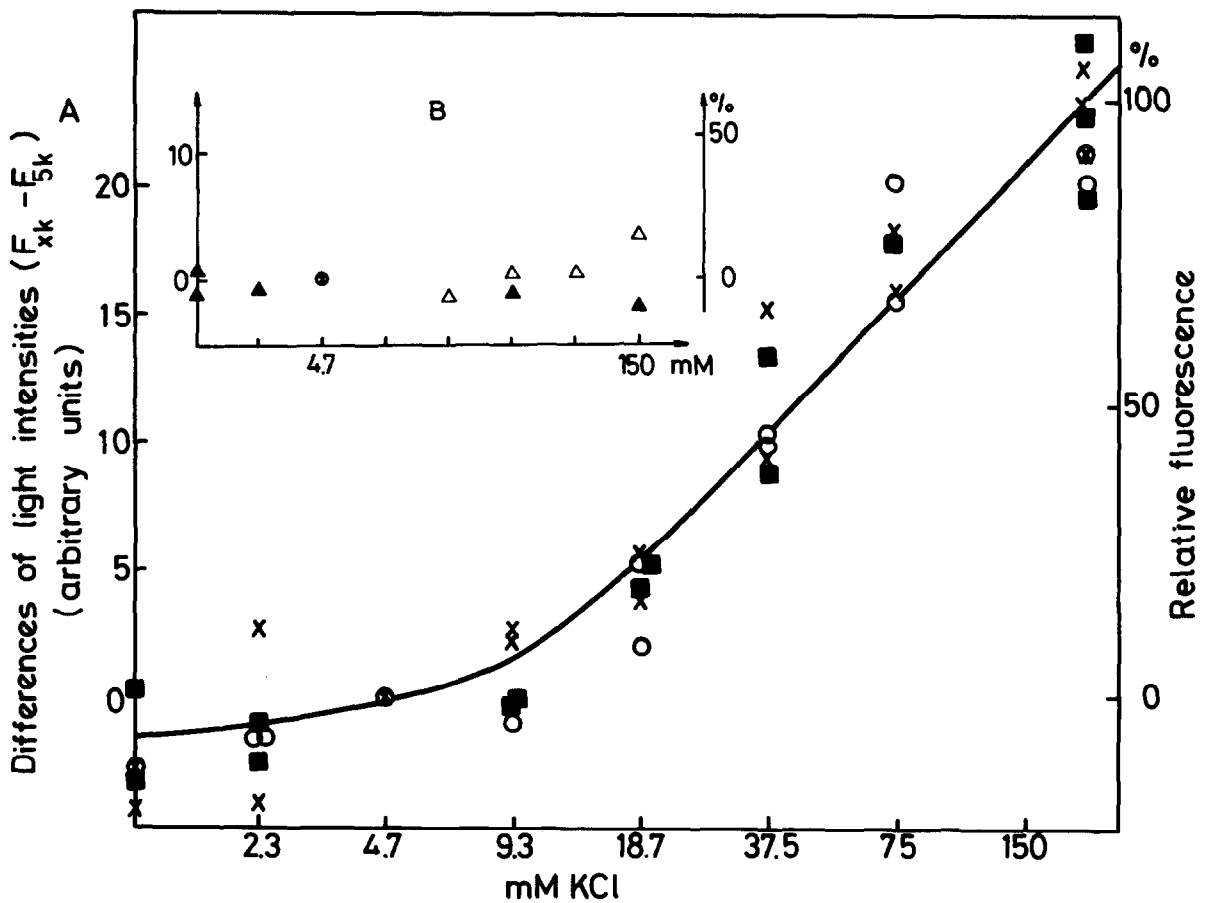

Fig.2. (A) Potassium concentration-dependent fluorescence intensity changes in various synaptosomal preparations. Differences of fluorescence light intensities were calculated from the test value by subtracting the intensity value measured in '5 K' media ($F_{XK} - F_{5K}$). The relative fluorescence is 100% for the average value of $F_{150K} - F_{5K}$. X-Synaptosomes prepared as in: (X–X) [5]; (■——■) [6]; (○——○) [7]. The final protein concentrations were 0.13 mg/ml and dye was 4.44 μM. The temperature was maintained at 37°C.

Fig.2. (B) Potassium dependence of the fluorescence light intensity of reference fractions. The calibration of relative fluorescence is taken from the synaptosome suspension: (△) Microsomal fraction; (▲) mitochondrial fraction. Concentrations were as in fig.2(A). Temperature was 37°C and protein was 0.13 mg/ml.

the $[K^+]$ gradient across the membrane.

Purified microsomal and mitochondrial suspensions served as reference fractions. The light intensities of these fractions were virtually independent of the concentration of potassium (fig.2B).

4. Discussion

It has been a subject of debate in the literature for some time whether or not synaptosomes prepared according to different purification methods are equivalent for various functional, biochemical and physiological investigations. Although there is a great difference in osmolarity of the separating media, our 3 synaptosomal fractions do not differ significantly from each other by the marker enzyme criteria. More information about the 'condition' of the synaptosomes can be obtained by detecting the membrane potentials of the synaptosomal fractions.

The changes of membrane potential produced by increased [KCl] and registered as fluorescence light intensity differences [13], were similar for all preparations. Synaptosomal suspensions in physiological saline, independent of preparation technique, preserved most of their resting potentials and polarisabilities for ~24 h after separation (at 0°C).

Using the slightly hyperosmotic preparation techniques [5,6], synaptosomes can be damaged by osmotic shock. This is caused by a sudden dilution of the separating sucrose solution to isoosmolarity. The optical signal in such cases decreases and may disappear altogether. This type of damage can be avoided by addition of the necessary amount of distilled water gradually over 30 min at 0°C while the suspension was gently stirred. This problem was not encountered using the isoosmotic separation technique [7].

To summarise, the diO-C_5(3) fluorescent dye method gave useful results regarding the conditions of synaptic membranes by making possible the continuous monitoring of the membrane potentials. We could not show a preference for any of the synaptosomal preparations we tried.

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References

[1] Deutsch, C. and Rafalowska, U. (1979) FEBS Lett. 108, 274–278.
[2] Hanson, E., Jacobson, I., Venema, R. and Sellström, A. (1980) J. Neurochem. 34, 569–573.
[3] Blaustein, M. P. and Goldring, J. M. (1975) J. Physiol. 247, 589–615.
[4] Kamino, K. and Inouye, A. (1978) Jap. J. Physiol. 28, 225–237.
[5] Gray, E. G. and Whittaker, V. P. (1962) J. Anat. London 96, 79–87.
[6] Hajós, F. (1975) Brain Res. 93, 485–489.
[7] Booth, R. F. G. and Clark, J. B. (1978) Biochem. J. 176, 365–370.
[8] Clark, J. B. and Nicklas, W. J. (1970) J. Biol. Chem. 245, 4724–4731.
[9] Johnson, M. K. (1960) Biochem. J. 77, 610–618.
[10] Omura, T. and Takesue, S. (1970) J. Biochem. Tokyo 67, 249–257.
[11] Racker, E. (1950) Biochim. Biophys. Acta 4, 211–214.
[12] Peterson, G. L. (1977) Anal. Biochem. 83, 346–356.
[13] Waggoner, A. S., Wang, C. H. and Tolles, R. L. (1977) J. Membrane Biol. 33, 109–140.