between them, in agreement with the very considerable difference in the anticoagulation activity of HP-3 and HP-4 [3, 4]. The relative content of organic components is greater in the HP-3 anion than in the HP-4 anion, and the bands at 1615 and 1550 cm^{-1} are weaker in the spectra of HP-3. The maximum at 1250 cm^{-1} is clearer in the spectrum of HP-3 than in that of HP-4. The presence of the "amide I" and "amide II" bands in the spectra of the HP-3 and HP-4 preparations not contaminated by other proteoglycans is probably due to the presence of a certain number of N-acetyl groups in heparin, as is confirmed by other physical methods [10]. However, this is a problem for further investigation, which is particularly necessary in view of evidence that heparin contains mannose derivatives as structural components [17].

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FUNCTIONAL CHARACTERISTICS OF NERVE ENDINGS ISOLATED FROM THE BRAIN BY HAJOS' METHOD

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KEY WORDS: synaptosomes of the brain and spinal cord; Hajos' method; respiration; secretion of GABA.

Isolated nerve endings (synaptosomes) provide a unique opportunity for the direct study of mechanisms of the fundamental processes lying at the basis of synaptic function: biosynthesis and secretion of mediators, accumulation of mediators in vesicles, uptake of the extracellular mediator by the terminal.

The solution of these problems requires the possession of synaptosome preparations free from impurities and preserving the initial level of functional activity as far as possible. The most widely used method of isolation of synaptosomes [6] satisfies these demands partially, but is time-consuming. In many cases, the long duration of fractionation required is unacceptable, and for that reason investigators are content with the initial stages of fractionation and work with the P_2 fraction of unpurified synaptosomes, in which synaptosomes proper account for up to 55% of the total (as protein). To allow for interference caused by the impurities, an additional series of experiments is set up.

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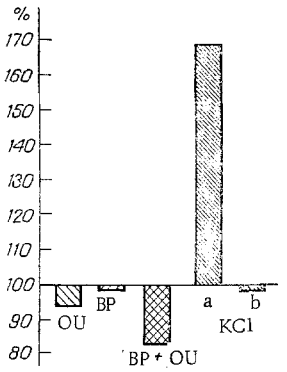

Fig. 1

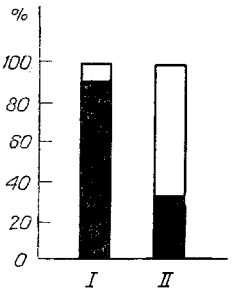

Fig. 2

Fig. 1. Effect of ouabain, benzylpenicillin, and high concentrations of K^+ ions on oxygen consumption (in % of control) by synaptosomes. BP) In benzylpenicillin medium compared with respiration in Krebs' medium; OU, BP+OU) 5 min after addition of ouabain to suspensions of synaptosomes in Kreb's medium and in benzylpenicillin medium compared with respiration in these same media before addition of ouabain. KCl: a) 5 min after addition of KCl, b) during next 15 min.

Fig. 2. Distribution of GABA (in %) between synaptosomes (shaded parts of columns) and incubation medium (unshaded part of columns) at rest (I) and after inhibition of Na,K-ATPase by 0.3 mM ouabain (II).

Recently, Hajos [3] suggested a rapid method of isolation of a highly purified fraction of synaptosomes and showed that, during incubation in physiological media, morphology of synaptosomes isolated by his method is almost identical with that of nerve endings in situ. The object of the present investigation was to undertake the functional evaluation of this highly purified preparation of synaptosomes with respect to a number of biochemical criteria: the oxygen consumption and maintenance of transmembrane mediator gradients at rest and under the influence of some depolarizing agents.

EXPERIMENTAL METHOD

Synaptosomes were isolated on the K-24 centrifuge (East Germany) by Hajos' method [3]. In the experiments of series I, to study oxygen consumption, synaptosomes from rat cerebral cortex were suspended (3-4 mg protein/ml) in Krebs' medium of the following composition (in mM): NaCl 124, KCl 5, KH_2PO_4 1.2, $CaCl_2$ 0.75, $MgCl_2$ 1.3, glucose 10, $Na_2H_2PO_4$ 20, pH 7.5. In the penicillin medium, the NaCl was replaced by the equivalent quantity of the sodium salt of benzylpenicillin. Oxygen absorption was measured manometrically in a Warburg apparatus for 40 min at 37°C. To study the action of depolarizing agents (0.3 mM ouabain or 56 mM K^+ – final concentrations) the appropriate solutions were added 20 min after the beginning of incubation and changes in respiration were studied during the next 20 min. The synaptosomes were then separated from the incubation medium by centrifugation (10,000g, 10 min) and the content of amino acids in the medium and extract of synaptosomes was determined on the AAA-881 (Czechoslovakia) automatic amino acid analyzer.

In the experiments of series II synaptosomes were isolated from the lumbar enlargement of the rat spinal cord. Synaptosomes (100 μl of a suspension in 0.32M sucrose) were added to 0.9 ml of incubation medium containing 0.5 μCi [^{3}H] GABA (New England Nuclear, Boston, Mass.). The composition of the incubation medium was as follows (in mM): NaCl 104, KCl 5, $CaCl_2$ 1.2, $MgCl_2$ 1.3, KH_2PO_4 1.2, Tric-HCl 20; pH 7.4. Synaptosomes (0.25-0.30 mg protein/ml) were incubated at 37°C for 8 min, after which they were separated from the incubation medium on Synpore (Czechoslovakia) filters with a pore size of 0.8 μ and diameter of the filter itself of 24 mm. The residues were washed with 10 ml of 0.32M sucrose at 22°C. To study the effect of potassium depolarization (final concentration of K^+ 56 mM), KCl was added with a micropipet 8 min after the beginning of incubation, and the synaptosomes were separated on the filter 5 min later as described above. After drying, the filtrates containing synaptosomes were mixed in flasks with a mixture of 3 ml 2-methoxyethanol and 7 ml toluene scintillator. Radioactivity was measured after 24 h in an SL-30 (Intertechnique, France) scintillation spectrometer.

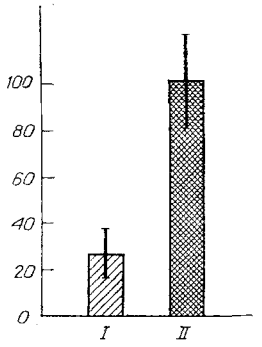

Fig. 3

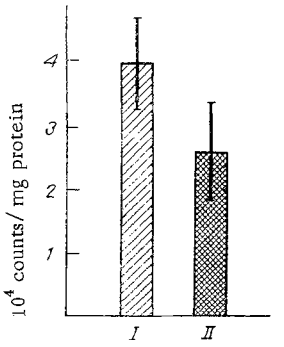

Fig. 4

Fig. 3. Effect of ouabain on GABA content (in nmoles/100 mg protein) in incubation medium of synaptosomes. I) Control, II) in presence of ouabain.

Fig. 4. Accumulation of [^{3}H]GABA by synaptosomes from spinal cord at rest (I) and during potassium depolarization (II). Mean results of eight experiments. Ordinate, radioactivity of synaptosomes.

EXPERIMENTAL RESULTS

The yield of synaptosomes when isolated from the cerebral cortex was the same as that reported by Hajos [3]: about 10 mg protein from 1 g wet weight of tissue. The preparation contained mainly intact synaptosomes*. The yield of synaptosomes from spinal cord tissue was only about 30% of their yield from brain, and besides synaptosomes the fraction also contained many fragments of myelin. These differences can evidently be explained by the fact that the spinal cord, with its powerful conducting system, contains far fewer nerve cells and synapses than the cerebral cortex.

The oxygen consumption of the suspension of brain synaptosomes was linear for 40 min. The absolute values of oxygen consumption, namely 49 ± 3 μ moles O_2/100 mg protein/h, were close to values obtained for synaptosomes isolated by the standard method [6].

It was decided to study changes in the oxygen consumption under the influence of factors changing active or passive ion transport through the plasma membrane. The results are given in Fig. 1. To reveal the action of these agents more clearly, variations in the rate of respiration are expressed in percentages of the rate of oxygen consumption by the same synaptosomes before exposure to the corresponding agent. Ouabain, a specific inhibitor of transport Na,K-ATPase, caused inhibition of respiration, probably on account of inhibition of respiratory control through a decrease in ADP formation.

In media in which the principal anion was benzylpenicillin, inhibition of respiration of the synaptosomes was well reproducible, but small in magnitude. It might be supposed that the effect of inhibition was due to interference with the passive entry of Na^+ into the nerve endings: The massive anion did not penetrate into the endings and Na^+ transport was restricted through the formation of a Donnan equilibrium potential. This explanation of the inhibition of respiration is in agreement with known facts indicating that respiration of synaptosomes depends essentially on the degree of stimulation of Na,K-ATPase by Na^+ ions penetrating into the nerve ending [5]. Since the permeability of synaptosomal membranes for Na^+ is low at rest, the weak effect of benzylpenicillin ought not to cause surprise. Against the background of benzylpenicillin, the action of ouabain was stronger (Fig. 1).

The action of 56 mM K^+ on respiration of the synaptosomes was biphasic: Initially the rate of oxygen consumption rose sharply, but later it fell (Fig. 1). The increase in oxygen consumption by the synaptosomes under the influence of potassium depolarization of the membrane is in agreement with data in the literature [2], and the subsequent inhibition could perhaps reflect the special sensitivity of energy processes in synaptic mitochondria to the increased osmolarity of the incubation medium. On the whole, the results described above indicate a connection between the mechanism of active ion transport and the intensity of energy processes in

*The authors are grateful to Dr. Med. Sci. O. M. Pozdnyakov for electron-microscopic verification of the purity of the fractions and for valuable advice.

the synaptic mitochondria, making good the expenditure of energy on cation transport. Preliminary experiments showed that cationic regulation of the processes of synaptosomal respiration may be substantially improved by the use of 0.30-0.32 M sucrose, buffered with Tric-HCl and containing chelating agents for Ca^{++}, as the brain tissue homogenization medium.

Data on the GABA content in the synaptosomes and incubation medium under normal conditions and after inhibition of Na,K-ATPase activity by ouabain are given in Figs. 2 and 3. Under normal conditions (Fig. 2), almost 90% of the GABA is retained in the cytoplasm of the synaptosomes, evidence of the normal functioning of the mechanism of mediator reassimilation and the provision of energy for the functioning of this mechanism. Under the influence of ouabain, GABA is released into the incubation medium (Fig. 3); its concentration in the incubation medium thereupon increases fourfold ($P < 0.001$). The GABA content in the synaptosomes fell sharply under these circumstances (Fig. 2A).

Data on the uptake of [^{3}H]GABA from the external medium by synaptosomes isolated from spinal cord tissue are given in Fig. 4. Synaptosomes accumulated GABA and released part of the accumulated mediator during potassium depolarization of the membranes. An increase in the incubation time to 2.5 h was accompanied by a decrease in the ability of the synaptosomes to take up labeled GABA (by 50%, compared with accumulation of the mediator by synaptosomes immediately after their isolation).

The data described above confirm the good functional characteristics of synaptosomes obtained by Hajos' method. Considering that the method does not require the use of expensive centrifuges and that it gives fractions of higher purity yet requires a shorter time, it can be concluded that it has important advantages when used to isolate synaptosomes from rat cerebral cortex.

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