METHODS

STIMULATION RECORDING OF UPTAKE AND SECRETION OF ^{14}C-NORADRENALIN BY RAT BRAIN SYNAPTOSOMES

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To determine the efficiency of synaptic transmission simultaneous recording of the uptake and subsequent evoked secretion of mediator by the nerve endings is important. The procedures used at the level of the whole organism may at the same time influence the intensity of secretion and uptake of mediator. In most cases when uptake and secretion of neurotransmitters are studied the tissue (sections) or isolated nerve endings (synaptosomes) are incubated with labeled mediator in isotonic Krebs–Ringer medium containing Ca^{++} ions (0.5-2.0 mM). Numerous investigations have demonstrated the strict Ca-dependent secretion of mediators [3]. At the same time, there is evidence to the contrary [4, 6-11] on the role of Ca^{++} ions in active uptake of mediators by corresponding nerve endings.

The object of this investigation was to compare the intensity of uptake of labeled ^{14}C-noradrenalin (NA) by synaptosomes isolated from the rat brain, determined by the usual method, with results obtained by simultaneous recording of uptake and secretion of NA, and also to study the effect of Ca^{++} ions on the active uptake of NA by synaptosomes and the subsequent secretion of this mediator.

EXPERIMENTAL METHOD

Experiments were carried out on 56 noninbred male albino rats weighing 180-200 g. Synaptosomes were isolated from the brain (without the cerebellum) as described by Hajôs [5] and suspended in Krebs–Ringer medium (pH 7.4) of different composition (in mM): No. 1) NaCl 104, KCl 5, $MgCl_2$ 1.3, Na_2HPO_4 1.2, glucose 10, $CaCl_2$ 1.2, Tris-HCl 20; No. 2) NaCl 74, KCl 35, $MgCl_2$ 1.3, Na_2HPO_4 1.2, glucose 10, $CaCl_2$ 1.2, Tris-HCl 20; No. 3) NaCl 74, KCl 35, $MgCl_2$ 1.3, Na_2HPO_4 1.2, glucose 10, $CaCl_2$ 2.4, Tris-HCl 20; No. 4) NaCl 104, KCl 5, $MgCl_2$ 1.3, Na_2HPO_4 1.2, glucose 10, Tris-HCl 20; No. 5) NaCl 104, KCl 35, $MgCl_2$ 1.3, Na_2HPO_4 1.2, glucose 10, EGTA 2.0, Tris-HCl 20; No. 6) NaCl 74, KCl 35, $MgCl_2$ 1.3, Na_2HPO_4 1.2, glucose 10, EGTA 2.0, Tris-HCl 20.

Protein was determined by Lowry's method. The suspension of synaptosomes were diluted with incubation medium to a concentration of 250 µg protein/mg. Samples (1 ml) were preincubated during mixing for 15 min (37°C) and ^{14}C-NA was added up to $6 \cdot 10^{-6}$ M (from the Radiochemical Centre, Amersham, England; 35 mCi/mmole). After incubation (10 min, 37°C) the synaptosomes were sedimented at 22,000g for 2 min (K-24 centrifuge, East Germany). The residues were washed five times with incubation medium (20°C). Some samples were taken for determination of the quantity of NA taken up (usual method), other samples were suspended in various Krebs–Ringer media and incubated for 5-30 min (37°C); after incubation the samples were centrifuged and the supernatant was collected and 0.1 ml of it transferred to a scintillation flask. The residue was dissolved in 1 ml 1 N NaOH (45°C, 1.5 h); 0.1 ml of the solution was transferred to the scintillation flask containing a mixture of scintillator (1 liter toluene, 4 g PPO, 0.1 g POPOP) with ethanol in the ratio of 7:3. Radioactivity was measured on an Intertechnique SL-30 scintillation counter (France) and expressed in cpm/mg protein. Specific uptake activity (U) was calculated by the equation: $U = 40 (S + R)$, where S and R represent the specific activity of the supernatant and residue, respectively, and the intensity of NA secretion was calculated by the formula $I = S/(S + R) \cdot 100\%$. The results were subjected to

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TABLE 1. Effect of Ca^{++} on Uptake of ^{14}C-NA by Synaptosomes Isolated from Rat Brain, Determined by Method [1] and by Method [2] of Simultaneous Recording of Uptake and Secretion (M ± m)

Medium No.	Specific radioactivity, cpm/mg protein			
1	56936,7 ± 1427,3	(100 %)	56636,8 ± 1897,4	(100 %)
4	34348,1 ± 995,6	(60,3 %)*	30587,1 ± 971,3	(54,0 %)*
5	37635,2 ± 1124,1	(66,1 %)*	38179,6 ± 1075,8	(67,4 %)*

*$P \leq 0.01$ compared with corresponding control.

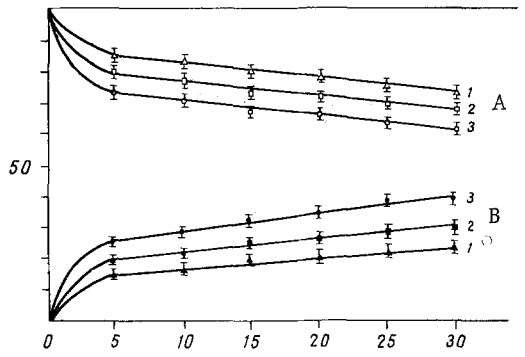

Fig. 1. Changes in content (A) and dynamics of spontaneous and evoked secretion (B) of labeled NA in synaptosomes. Abscissa, incubation time (in min); ordinate, percent of total uptake of labeled NA, taken as 100%. 1) Spontaneous secretion (medium No. 1); 2 and 3) potassium depolarization in the presence of 1.2 and 2.4 mM Ca^{++} (media Nos. 2 and 3). Results of five or six experiments shown.

statistical analysis using the Wilcoxon–Mann–Whitney criterion.

As the adsorption control the synaptosomes were subjected to preliminary osmotic shock and freezing and thawing, after which the material was centrifuged for 2 min at 22,000g. The residue was then suspended in incubation medium and tested by the method described above. Radioactivity due to adsorbed NA accounted for 15% of the total specific radioactivity.

EXPERIMENTAL RESULTS

The results obtained by simulteneous recording of uptake and secretion of labeled NA showed that the method adopted is adequate for the study of these processes (Table 1).

As Table 1 shows, removal of Ca^{++} from the incubation medium led to marked inhibition of NA uptake by synaptosomes. Similar results were obtained previously with respect to uptake of NA [11], choline [6], and GABA [10] by rat brain synaptosomes. However, experiments indicating that removal of Ca^{++} from the incubation medium intensifies neuronal uptake of NA in the rat heart have been described [4, 7, 9]. The physiological role of NA uptake in the central and peripheral nervous system differs in essential respects. In the peripheral nervous system much of the secreted NA can be removed from the synaptic space by diffusion into the blood stream [8, 9]. The main function of NA uptake in the peripheral nervous system can be reduced to making good the labile reserves of vesicular NA [2]. In the central nervous system much of the NA from the synaptic space is taken up by nerve endings and is the principal mechanism of its inactivation [1]. It may perhaps be this fact which determines the opposite effect of Ca^{++} on NA uptake in the central and peripheral nervous system.

On incubation of synaptosomes for 5-30 min (Fig. 1) an increase in the spontaneous and evoked secretion of labeled NA was observed. The spontaneous liberation of NA was due to two causes: leakage of NA from synaptosomes "damaged" during isolation and (principally) the existence of dynamic equilibrium between extracellular, cytoplasmic, and vesicular NA. During prolonged incubation of the synaptosomes this equilibrium could be shifted toward accumulation of NA in the medium through the action of factors such as oxidation of NA and its destruction by monoamine oxidase of the synaptic mitochondria of the nerve endings.

It will be clear from Fig. 1 and Table 2 that secretion of labeled NA during depolar-

TABLE 2. Intensity of Spontaneous and Evoked Secretion of ^{14}C-NA under Different Conditions of Incubation (M ± m)

Conditions of incubation (No. of medium)	5 min			15 min			30 min		
	%			%			%		
	S_1	S_2	S_1/S_2	S_1	S_2	S_1/S_2	S_1	S_2	S_1/S_2
1,2 / 1	14,2±0,9	20,0±1,2†	1,42	19,3±1,1	26,2±1,3†	1,36	25,6±1,3	32,5±1,5‡	1,25
4 / 1	14,8±0,9	—	—	20,1±1,2	—	—	24,7±1,2	—	—
1,2 / 4	15,2±1,0	21,5±1,4†	1,41	18,9±1,1	29,1±1,7*	1,53	25,2±1,3	35,3±1,6*	1,40
4,6 / 4	14,9±1,0	15,4±1,1	1,03	19,5±1,2	20,8±1,3	1,06	24,9±1,3	24,2±1,4	0,97
5,7 / 5	14,4±0,9	15,9±1,2	1,10	19,1±1,3	18,8±1,4	0,98	25,5±1,2	25,8±1,5	1,01

*P < 0.05.
†P < 0.01.
‡P < 0.001.
Legend. Numerator — secretion, denominator — uptake; S_1) spontaneous secretion, S_2) evoked secretion (35 mM (KCl).

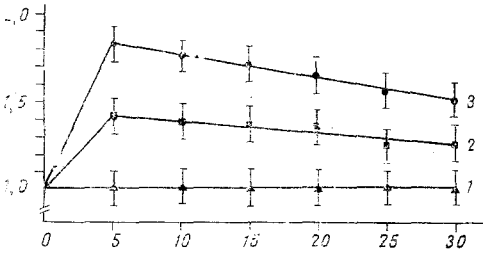

Fig. 2. Intensity of evoked NA secretions as a function of time of incubation of synaptosomes during potassium depolarization. Ordinate, ratio S_1/S_2 (Table 2). Remainder of legend as in Fig. 1.

ization of the synaptosomal membranes with 35 mM KCl was Ca-dependent in character. In a calcium-free incubation medium practically no evoked NA secretion was observed, but an increase in the Ca^{++} concentration in the incubation medium led to a corresponding increase in secretion.

Analysis of the results showed (Fig. 2) that the intensity of evoked secretion was maximal under these experimental conditions during the first 5 min. This rule was most clearly expressed during an increase in the Ca^{++} concentration in the incubation medium. The characteristics of evoked NA secretion noted above likewise were independent of the procedure of saturation of the synaptosomes with labeled NA, whether conducted in the presence or absence of Ca^{++} ions.

These investigations thus showed that the technique of simultaneous recording of uptake and secretion of ^{14}C-NA by rat brain synaptosomes is perfectly adequate and can be used in experiments involving procedures aimed at the whole organism, The results also indicate that the process of NA uptake by synaptosomes is dependent on Ca^{++} ions. These results can be explained by an increase in the affinity of NA for its carrier (in the active uptake system) in the Ca-containing medium, It was also noted that spontaneous liberation of NA, unlike evoked secretion, is independent of the presence of Ca^{++} in the incubation medium.

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MODIFICATION OF THE "HANGING DROP" METHOD FOR CULTURING REAGGREGATES OF DISSOCIATED CELLS AND EXPLANTS OF EMBRYONIC LUNG

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Of the few methods of obtaining reaggregates of dissociated embryonic tissue cells the "hanging drop" method, in the modern form suggested by Steinberg [6], is attractive because it reproduces the natural quality of the reaggregation process, which takes place spontaneously. Other methods are based on artificial approximation of cells by agitating the suspension in special shakers [5] or by centrifugation [7]. Another advantage of the "hanging drop" method is that the process of cell aggregation can be kept under constant observation without being disturbed. A disadvantage of the method is the comparatively small volume of nutrient medium (1 drop = 0.05 ml), as a result of which the nutrients in the system are quickly exhausted. As the writers showed previously [2] the process of organotypical aggregation consists of several successive stages, so that the suspension of chicken embryonic lung cells must be cultured for at least 3-4 days without a change of nutrient medium.

The object of this investigation was to modify the "hanging drop" method by increasing the volume of nutrient medium and to compare the results of aggregation of dissociated mouse and chicken embryonic lung cells when the classical method and the suggested modification were used. The possibility of applying the modified "hanging drop" method to culture pieces of embryonic tissue also was investigated.

EXPERIMENTAL METHOD

Lungs of 10-day Brown Leghorn chick enbryos and 17-day A and C57BL mouse embryos were used. Embryonic lungs were dissociated into cells by Moscona's method [4]. The finely shredded pieces of embryonic lung, measuring 1-2 mm in diameter, were incubated at 37°C in calcium-free Tyrode solution and in a 0.25% solution of trypsin, after which the dissociating solutions were replaced by "growth" nutrient medium consisting of the following components: Eagle's medium (100%), glutamine (1%), inactivated bovine serum (10%), and chick embryonic extract prepared from 10-day embryos in Eagle's medium in the ratio of 1:1 and added to the medium after centrifugation. The chick embryonic lungs were kept in the dissociating solutions for 15-20 min and the mouse embryonic lungs for 25-30 min, for the latter are much harder to dissociate into cells. Undissociated cell complexes were removed by centrifugation and the completeness of their sedimentation was verified under the microscope. The resulting cell suspension was adjusted to a concentration of $2 \cdot 10^6$-$6 \cdot 10^6$ cells/ml medium and 1 drop was applied to siliconized coverslips. These were inverted with the drop beneath and placed on glass rings glued with epoxide glue to the bottom of a petri dish. Besides the classical "hanging drop" method as described above, cells also were cultured by a modified method in specially designed vessels, in which the conditions for cell culture on the surface of tension of the drop were similar to those in the "hanging drop" method. The vessels were

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