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The Membrane of Catecholamine Storage Vesicles of Adrenal Medulla

Catecholamine Fluxes and ATPase Activity

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Summary. 1. A method of preparing isolated membranes of the catecholamine storing vesicles of the adrenal medulla is described. The membrane protein amounts to 23% of the total vesicular protein and still contains 0.1% of the catecholamine. 2. At 31°C in the presence of ATP the membrane preparation accumulates catecholamine; in the absence of ATP, however, catecholamine is released into the medium. At 0°C the catecholamine content of the membrane preparation remains unchanged, whether ATP is present or not, although a low catecholamine turnover is observed. 3. From the kinetics of the catecholamine turnover in the absence or presence of ATP a low ATP-independent catecholamine influx can be distinguished from a considerably higher ATP-dependent influx of catecholamine. 4. The catecholamine influx as well as the ATPase activity of the membrane preparation decreases with time; in contrast, the catecholamine efflux remains constant and is higher in the presence of ATP than in its absence. 5. Catecholamine is to a great extent accumulated within a membrane-enclosed space into newly formed particles; only a minor amount of the catecholamines is actually bound within the membrane.

Key-Words: Membrane Function — Active Transport of Catecholamines — Storage Mechanism of Catecholamine — Net Uptake of Catecholamine — Reorganization of Membrane Fragments.

Introduction

The storage vesicles of the adrenal medulla contain two different proteins, which are easy to separate from isolated vesicular preparations by hypo-osmotic shock. More than 70% (Hillarp, 1958) of vesicular protein consists of the water-soluble protein, chromogranin (Blaschko *et al.*, 1967a), which is characterized by a high content of acidic amino acids, especially glutaminic acid, and a very low content of cysteine (Smith and Winkler, 1967; Helle and Serck-Hanssen, 1969). This protein is secreted by the adrenal gland, together with the catecholamines and ATP (Banks and Helle, 1965; Blaschko *et al.*, 1967a). The vesicular membrane remains within the cell after secretion of the soluble components (Schneider *et al.*, 1967; Malamed *et al.*, 1968; Diner, 1967;

Viveros *et al.*, 1969). These biochemical and electron-microscopic results led to the conception of exocytosis as the mechanism of secretion for catecholamines (Banks and Helle, 1965; Schneider *et al.*, 1967; Douglas, 1968). The vesicular membrane, which is characterized by its high content of lysophosphatidylcholine (lysolecithine), was assigned the function of fusion with the cell membrane (Blaschko *et al.*, 1967b; Winkler *et al.*, 1967). On the other hand, the problem of accumulation and preservation of the stored catecholamines within the vesicular particles until their liberation from the medullary cell has not been considered in the discussion of the secretion mechanism for catecholamines.

In previous papers (Taugner and Hasselbach, 1966a, 1966b, 1968; Hasselbach and Taugner, 1970) it was demonstrated, from a study of the kinetics of catecholamine fluxes, that storage of catecholamines is an active energy-consuming process which takes place at the vesicular membrane: the vesicular ATPase is located exclusively within the membrane, while the water-soluble protein fraction does not contain any ATPase activity. Blocking of the thiol groups on the vesicular membrane leads to inhibition of ATPase activity as well as inhibition of the ATP-dependent catecholamine influx. Conversely, ATPase activity as well as catecholamine influx remains unaffected if some of the thiol groups are protected by ATP against thiol reagents. Furthermore, the water-soluble protein contains fewer thiol groups accessible to thiol reagents than can be protected by ATP under these experimental conditions (Taugner and Hasselbach, 1968). Therefore, these results are incompatible with the assumption that storage of catecholamines is brought about by binding to a storage complex across a membrane freely permeable for catecholamines (compare Kirshner *et al.*, 1966).

This storage complex, consisting of ATP, catecholamine, Mg^{2+}, Ca^{2+} and chromogranin, ought rather to bind the catecholamines which are previously transported into the interior of the vesicle. By this mechanism the concentration of "free" and exchangeable catecholamine in the vesicular interior can be kept constant and low in order to diminish the concentration gradient for the transport system (Taugner and Hasselbach, 1966a).

Various attempts to prove extravesicular formation of this storage complex (Blaschko and Helle, 1963; Weiner and Jardetzky, 1964) did not produce convincing evidence for a mechanism able to store catecholamine against a concentration gradient. More recent studies, however, (Berneis *et al*, 1969) demonstrated the formation of a complex consisting of catecholamine and ATP with molecular weight between 4,000—8,000 on the addition of suitable concentrations of earth alkali ions. To form this complex, however, concentrations of complex partners were neces-

sary, which are hardly likely within the cell plasma. For this reason it seems plausible that the formation of the complex within the vesicles is preceeded by a process producing suitable conditions for its formation. In order to differentiate the active transport events from storage by the complex, the kinetics of the catecholamine fluxes across the vesicular membrane were studied on isolated, highly purified membrane preparations.

Methods

The Preparation of Vesicular Membranes was carried out at 2—4°C. Homogenized bovine adrenal medullae from 10—12 animals were centrifuged in 0.3 M sucrose at 900×g for 6 min in order to remove coarser particles, then sedimented at 12,000×g for 15 min. The upper brownish layer of the sediment was discarded. The more tightly packed pinkish layer was resuspended in 0.3 M sucrose and centrifuged for 90 min through a discontinuous density gradient (1.0, 1.2, 1.4, 1.6 M sucrose) at 60,000×g. Vesicles, sedimented in 1.6 M sucrose, with a catecholamine/protein ratio of 2.96 μmoles catecholamine/mg of protein, were osmotically lysed in 0.015 M KCl. After sedimentation at 45,000×g (30 min) they were washed again in 0.015 M KCl and resuspended in 0.16 M KCl. The catecholamine/protein ratio of this preparation still amounted to 95.6 nmoles (112.5—87.8) catecholamine/mg of protein. Therefore, the membrane preparation was dialysed overnight against 200 times its volume of 0.16 M KCl at 2°C, and was subsequently washed twice in 0.16 M KCl. By this means the catecholamine content of the vesicular membranes could be reduced to 12.6 nmoles/mg of protein. Even after dialysis for 24 h, and five consecutive washings in 0.16 M KCl, the catecholamine of the membrane preparation was not completely removed.

Measurements of the Kinetics of Catecholamine Turnover. The membrane preparation was incubated at 31°C under constant stirring in a mixture of the following composition: membrane protein 0.3 (0.112—0.63) mg/ml KCl 0.16 M, NaCl 5 mM. In some experiments sodium glutarate, 0.1 M, or sucrose, 0.3 M was also used. The mixtures were buffered at pH 7.4 with sodium glycerophosphate, 50 mM, containing Mg^{2+} 5 mM, and ascorbic acid, 0.1 mM. Catecholamine was added as (–)-noradrenaline-bitartrate, 0.5—0.03 mM, with the respective ^{14}C-isotope. In the experiments with ATP, the concentration of added ATP was 5 mM.

At intervals aliquots of 1 ml were taken, cooled by dilution with 5 ml ice-cold 0.16 M KCl, and centrifuged at 18,000×g for 15 min. The pellet was washed again with 5 ml cold 0.16 M KCl and was then extracted at 0°C with cold 0.1 n HCl, which was buffered with Tris to pH 2. The catecholamine was measured fluorimetrically, according to von Euler and Lishajko (1961), with a Hitachi Perkin Elmer fluorescence spectrophotometer. The ^{14}C-radioactivity was measured in a scintillator mixture consisting of toluene/Triton X 100, 1100:400, Omnifluor 4 g/1000 in a Packard Tri-Carb spectrometer.

As a control in some experiments aliquots were taken before and after incubation with noradrenaline, either in the presence or absence of ATP, cooled to 0°C, sonicated for 15 sec (Branson Sonic Power), and sedimented at 105,000×g for 30 min.

Determinations of the Efflux of Catecholamine. The membrane suspension, which had been dialysed overnight and washed, was incubated at 31°C in the presence of ATP with ^{14}C-(–)-noradrenaline of high specific activity for 15 min. This preparation, after being cooled to 0°C, was washed twice in order to remove excess of

^{14}C-activity so that only that ^{14}C-(–)-noradrenaline remained which was incorporated during the preincubation period. This suspension was incubated in either the presence or absence of ATP at 31°C in a medium containing 0.5 mM non-radioactive (–)-noradrenaline. Then the ^{14}C-radioactivity as well as total catecholamine in the membrane protein was determined as described above.

The ATPase Activity of the Membrane Protein was measured at 31°C in incubation mixtures with the same composition as above, except that catecholamine was omitted, if not mentioned otherwise. The splitting reaction was terminated by the addition of 10% (w/v) trichloroacetic acid after certain time intervals, and the inorganic phosphate determined according to Rockstein and Herron (1951).

The splitting rates were calculated from three successive values derived from the first 15 min of the experiment. The protein was determined according to the Kjeldahl method.

Materials

ATP was obtained from P-L Biochemicals Inc., Milwaukee, Wisc., U.S.A., (–)-noradrenaline bitartrate from Farbwerke Hoechst[1], Frankfurt a.M., Germany, ^{14}C-(–)-noradrenaline bitartrate, with a specific activity of 57 mCi/mmol from the Radiochemical Centre, Amersham, Bucks., U.K., Omnifluor [98% 2,5-diphenyloxazol + 2% p-bis(o-methylstyryl)-benzene] was from Merck, Darmstadt, Germany. All other substances used were of analytical purity.

Results

Protein and Catecholamine Content of the Membrane Preparation

Table 1 demonstrates the decrease of catecholamine and water-soluble protein during the course of preparation. The main part of the water-soluble protein is removed by the first hypo-osmotic shock. The second shock reduces the amount of protein by only a further 10% of the total vesicular protein. After the two washings in iso-osmotic KCl solution after dialysis, the amount of sedimentable membrane protein remains practically constant. However, the catecholamine content of the membrane preparation is considerably reduced by the second shock. During the 15-h dialysis the catecholamine content is reduced to 1/5 of its previous value, so that the preparation finally contains only 0,1% of the original vesicular catecholamine. Subsequent washings at 2—4°C in 0.16 M KCl yield only negligable decreases in membrane-bound catecholamine.

Uptake and Release of Catecholamine in Media Containing (–)-Noradrenaline

Warming to 31°C causes an initial decrease of 40—50% in the catecholamine content of the membrane protein (Fig. 1). This initial loss is independent of either the presence or absence of catecholamine in the medium. In the absence of ATP, in spite of the high concentration of

1 (–)-noradrenaline-bitartrat was a generous gift of Farbwerke Hoechst, Frankfurt a.M.

Table 1. *Protein and catecholamine content of membrane preparation obtained from adrenal medullary vesicles during the preparation. The preparation was carried out at 2—4° C. Semipurified vesicles were centrifuged through a density gradient for 90 min. The vesicles sedimented in 1.6 M sucrose were used for further purification. The results are shown as mean ± S.E.M. of numbers of experiments given in parentheses*

Steps	Vesicular suspension: 2.956 μmoles catecholamine/mg of vesicular protein		
	percent of vesicular protein	nmoles catecholamine/mg of membrane protein	percent of vesicular catecholamine
Shock I KCl 15 mM sedimentation at 45,000 × g 30 min	36.2 ± 3.89 (5)	1162 ± 109 (7)	14.3
Shock II KCl 15 mM sedimentation at 45,000 × g 30 min	26.4 ± 2.97 (6)	95.6 ± 9.75 (8)	0.85
Dialysis KCl 160 mM, 2—4° C 200 fold volume, 15 h sedimentation at 45,000 × g 30 min	25.8 ± 3.16 (4)	16.8 ± 1.8 (10)	0.15
Final washing KCl 160 mM sedimentation at 45,000 × g 30 min	23.2 ± 2.10 (7)	12.6 ± 0.84 (25)	0.10

noradrenaline, amounting to 500 nmoles/ml, membrane-bound catecholamine is slowly released into the medium. As the increase of ^{14}C-labelled (–)-noradrenaline in the membrane protein indicates, the catecholamine release is opposed by an ATP-independent catecholamine uptake. After about 15 min the specific activity in the medium equals that in the membrane protein.

In the presence of ATP the equilibrium between the specific activity in the membrane protein and that in the medium is achieved after 5 min. The amount of membrane-bound catecholamine increases linearly with time during the first 15 min, reaching a value three times the original; after a further 15 min the amount of catecholamine is 4.5 times the initial. Therefore, ATP not only accelerates the turnover of catecholamine but also induces a net uptake of catecholamine. A reduction of the catecholamine concentration in the medium to either 250 or 125 nmoles/ml does not alter either the uptake or release of catechol-

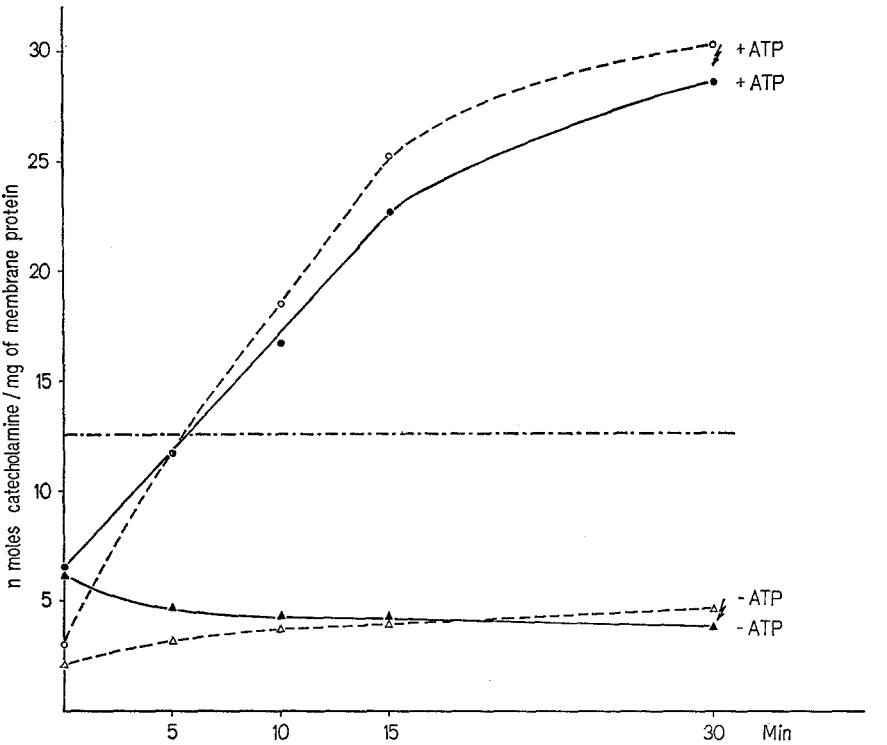

Fig. 1. Uptake and release, of (–)-noradrenaline by membrane preparations obtained from adrenal medullary vesicles. Ordinate: nmoles of catecholamine/mg of protein; Abscissa: time in minutes. (–)-noradrenaline concentration in the medium: 0.5 mM, temperature: 31° C. - — - — - nmoles catecholamine/mg of protein before incubation at 31° C; the value at zero time, is lower due to the initial loss of catecholamine on warming. Closed symbols: nmoles catecholamine/mg of protein measured fluorimetrically; open symbols: nmoles catecholamine/mg of protein, calculated from specific activity in the medium. •,○ in the presence of ATP; ▲,△ in the absence of ATP (average of 9 experiments)

amine. At noradrenaline concentrations in the medium of 65 or 32 nmoles/ml, however, catecholamine uptake is reduced to $^1/_2$ or $^1/_3$, respectively, while the catecholamine release remains unaffected.

At 0° C the catecholamine content of the membrane protein remains nearly constant for 90 min, irrespective of the presence or absence of ATP. Although no net uptake or release was observed, a catecholamine turnover obviously takes place, because the specific activity—about 0.4 at zero-time—approaches the value of 1 after 90 min. Control experiments in KCl-free media (0.3 M sucrose, 0.1 M sodium glutarate) at 31° C revealed no differences in the uptake or release of catecholamine from the membrane protein. This is in agreement with the results for ATPase

activity, which also does not change in KCl-free media. Freezing and thawing of the membrane preparation in liquid nitrogen decreases ATPase activity only insignificantly in comparison with the control. However, it abolishes completely the ability to accumulate catecholamine in ATP-containing media.

Determination of Catecholamine Efflux

From the experiments on the uptake of (–)-noradrenaline by vesicular membranes in ATP-containing media (see above) it was not possible

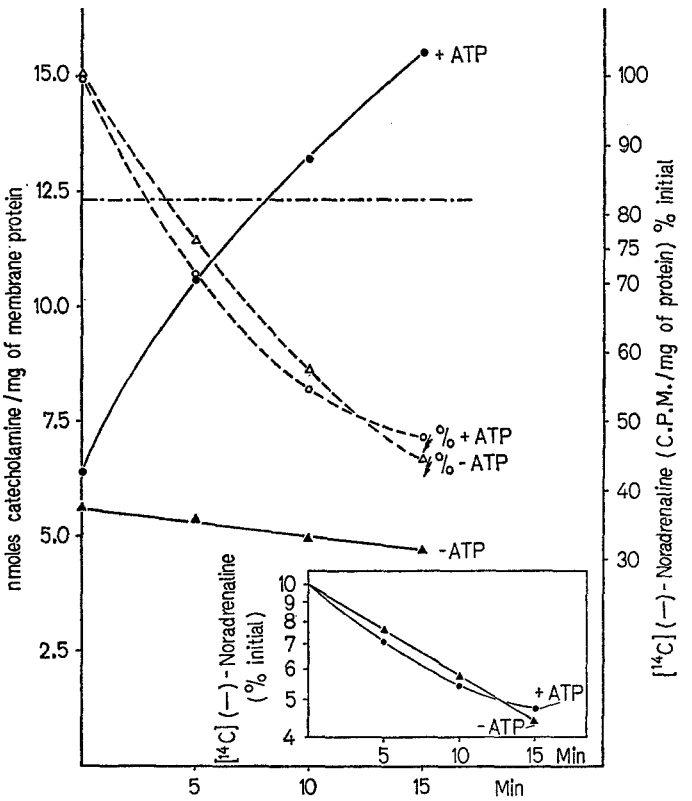

Fig. 2. Catecholamine turnover of vesicular membranes previously incubated with ^{14}C-(–)-noradrenaline. - — - — - nmoles catecholamine/mg of membrane protein after incubation with ^{14}C-(–)-noradrenaline. Closed symbols: nmoles catecholamine/mg of protein measured fluorimetrically, in the presence (•) or absence (▲) of ATP (left ordinate). Open symbols: Decrease of ^{14}C-radioactivity of membrane protein as a percentage of the initial value; in the presence (○) or absence (△) of ATP (right ordinate). Abscissa: time in minutes, Temperature 31°C, concentration of (–)-noradrenaline in the medium 0.5 mM. Inset: semilogarithmic plot of the percentage decrease in ^{14}C-(–)-noradrenaline in the membrane protein (average of 6 experiments)

Table 2. *Catecholamine fluxes and hydrolysis of ATP of membrane preparations in the presence or absence of ATP calculated for time intervals of 5 min. Experimental conditions were as described in the legend of Fig. 2 and in Methods. The results are given as mean ± S.E.M. of 6 experiments*

Time interval	nmoles catecholamine/mg of protein/time interval in presence of ATP			nmoles P_i/mg of membrane protein/time interval splitting rate
	net uptake	efflux	influx	
0— 5	4.07 ± 0.75	2.11 ± 0.68	6.18 ± 1.31	840
5—10	2.40 ± 0.65	2.90 ± 0.93	5.29 ± 0.98	730
10—15	1.70 ± 0.75	2.87 ± 0.91	4.57 ± 0.69	435
	in absence of ATP			
0— 5	− 0.52 ± 0.20	1.72 ± 0.58	1.21 ± 0.16	—
5—10	− 0.79 ± 0.25	1.73 ± 0.55	0.94 ± 0.29	—
10—15	− 0.34 ± 0.13	1.52 ± 0.28	1.17 ± 0.18	—

to determine whether and to which extent a catecholamine release opposes the analytically measurable catecholamine uptake. Therefore, the highly purified vesicular membranes were incubated in the presence of ATP with ^{14}C-(−)-noradrenaline of high specific activity. Thereafter, the exchange of membrane-bound ^{14}C-catecholamine for inactive (−)-noradrenaline in the medium was measured.

As Fig. 2 shows, except for small quantitative differences, membranes preincubated in ATP-containing media react in the same way as the untreated membranes: upon heating to 31° C about 50% of catecholamine is instantly released into the medium. In the presence of ATP the catecholamine content of the vesicular membranes rises during the first 15 min, while in the absence of ATP it gradually decreases. ^{14}C-radioactivity in the membrane protein decreases at the same rate in the presence or absence of ATP at 31° C. The inset of Fig. 2 shows that the ^{14}C-radioactivity decreases according to a first order function in the absence of ATP, while the time course of release in the presence of ATP is modified, apparently by reincorporation of ^{14}C-(−)-norardenaline from the medium. The calculation of the efflux rates from the respective mean specific activities at certain time intervals shows, however, that the efflux of catecholamine is considerably higher in the presence of ATP than in its absence (Table 2). While the efflux rates in both cases remain nearly constant during the observation period of 15 min, the catecholamine influx gradually slows down in the presence of ATP. In the absence of ATP the incorporation of catecholamine is only $^1/_5$ of that observed in its presence, hence, the efflux is not compensated. This is the cause of the net release of catecholamine into the medium in the absence of

ATP. At 0°C, as expected, the catecholamine content of the vesicular membranes remains almost constant over 60 min. A small decrease of ^{14}C-activity within the membrane protein indicates, however, that even in the cold a certain, slow, catecholamine exchange occurs which is independent of the presence or absence of ATP.

ATPase Activity of Vesicular Membranes

Fig. 3 shows the time course of ATP cleavage by isolated vesicular membranes. The mean cleavage rate amounts to 155 nmoles P_i/mg of protein/min. The splitting rate, however, is not constant but decreases with time, as can be seen from Table 2 and Fig. 3. Addition of (−)-noradrenaline or (±)-adrenaline in concentrations of 65—500 nmoles/ml of medium does not influence ATPase activity. Since the membrane protein, even after 15 h dialysis at 2—4°C against isotonic KCl solution, still contains on average 16 nmoles catecholamine/mg of protein, it was tried to oxidize the residual, apparently membrane-bound catecholamine, at 31°C and pH 8.5 (Tris buffer). After incubation for 1 h at pH 8.5 and subsequent washing the membrane preparation still contained 2.3 nmoles catecholamine/mg of protein. No enhancement of ATPase activity was achieved by the addition of (−)-noradrenaline to the medium.

In control experiments in which isotonic solutions of sucrose or sodium glutarate were used, no differences in ATPase activity were detected as compared with those found in KCl medium. This contrasts with results obtained with intact vesicles (Taugner, 1969, 1971).

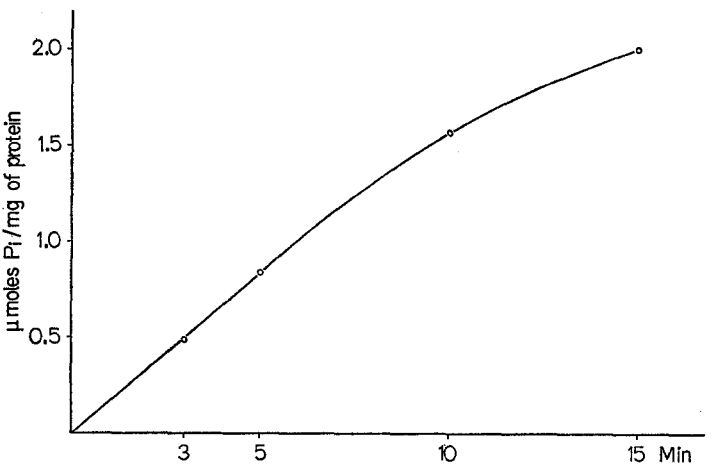

Fig. 3. Kinetics of hydrolysis of ATP by vesicular membranes. Ordinate: µmoles P_i/mg of membrane protein. Abscissa: time in minutes. ATP concentration: 5 mM: temperature 31°C

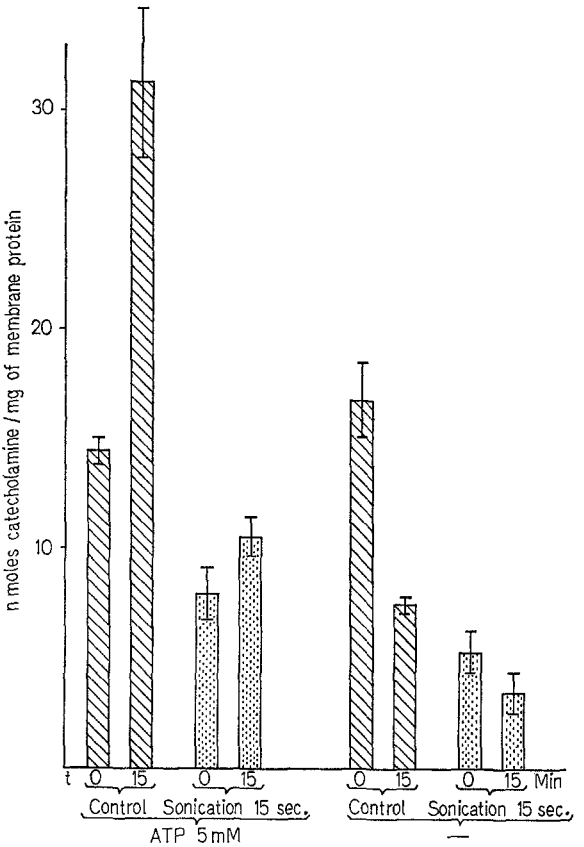

Fig. 4. Effect of sonication on vesicular membranes as compared to controls, at zero time and 15 min after the beginning of the experiment. Temperature 31° C, concentration of (–)-noradrenaline in the medium 0.5 mM, duration of sonication 15 sec, in ice-bath, sedimentation at 105,000 × g, 30 min. (Average of 4 experiments ± S.E.M.)

Effect of Sonication on Vesicular Membranes

In order to decide whether binding of catecholamine to protein structures, or genuine catecholamine storage in probably newly-formed vesicular particles is responsible for the observed catecholamine accumulation, the experimental mixture was sonicated for 15 sec in an ice-bath subsequent to the incubation at 31° C, and then sedimented as for the control.

The results are shown in Fig. 4. At the beginning of the experiment the catecholamine content of the membrane preparation was reduced by sonication to approximately $^1/_2$ the control value. Particularly striking

is the effect of sonication after an incubation period of 15 min in the presence of ATP in catecholamine-containing medium. As the control shows, the membrane preparation accumulated an average of 16 nmoles catecholamine/mg of protein within 15 min. The preparation sonicated subsequently, however, contains only 2.6 nmoles catecholamine/mg of protein more than the initial value. In the absence of ATP the effect of sonication after incubation is smaller. While spontaneous catecholamine release of the control in ATP-free medium amounts to 9 nmoles/mg of protein during 15 min, the catecholamine content of the preparation sonicated after incubation is only 2 nmoles less than that observed for vesicular membranes sonicated at the beginning of the experiment.

Discussion

The membrane preparation from bovine adrenal medulla vesicles, as described above, still contains 23% of the total vesicular protein. This value agrees well with findings of Winkler *et al* (1970), who separated both protein fractions by repeated freezing and thawing in hypo-osmotic media, and also with the results of Hillarp (1958). In contrast to this Helle and Serck-Hanssen (1969) found that 45% of the total protein was still sedimentable after isolation of the vesicular membranes in iso-osmotic NaCl solution, and showed immunologically that 75% of this preparation was chromogranin. Apparently the hypo-osmotic shock is necessary for the complete separation of both vesicular protein fractions.

According to electronmicroscopic results (Agostini and Taugner, in preparation) the membrane preparation consists chiefly of membrane-enclosed, empty vesicles of considerably smaller size than that of intact vesicles; apparently they are newly formed from membrane fragments. Electrondense intact, or partly intact, particles, which could possibly be responsible for the measured fluxes of catecholamines, were not detected. Therefore, it can be assumed that the residual 0.1% of the total catecholamine, which was not removed by repeated washings and dialysis, must be bound to membrane structures.

On warming the membrane preparation to 31°C, half of the bound catecholamine is released into the medium immediately independently of the presence or absence of ATP. It cannot be decided with certainty, however, whether, through warming, some of the presumbably newly formed vesicular particles are opened and emptied of their contents, or whether catecholamine binding within the membrane decreases (e.g. by a temperature-induced alteration of the structure of membrane lipids).

In the presence of ATP this initial loss of catecholamine is compensated for within a few minutes. Moreover a net accumulation of added catecholamine occurs resulting in an increase to three or four times the initial value. Such net uptakes have hitherto not been observed for

"full" adrenal medullary vesicles (Taugner and Hasselbach, 1966a), although they were observed for smaller transmitter vesicles of sympathetic nerve endings (v. Euler and Lishajko, 1963, 1964; Burger *et al.*, 1969).

The stimulation by Mg-ATP, as well as the temperature dependence of the process, indicates that catecholamine uptake is caused by an enzymatic process. This process could either catalyse the binding of catecholamine to the membrane, or alternatively could supply energy for a pumping mechanism for catecholamine. It is not possible, however, to stimulate the ATPase activity any further by addition of catecholamine to the medium; stimulation of ATPase activity by the transported material (K-Na-pump, Skou, 1957; Ca-pump, Hasselbach and Makinose, 1961) would indeed prove an active transport system. However, from quantitative comparison of ATPase activity and catecholamine influx one can calculate that, on the basis of a transport quotient of 0.7—0.8 (as found by two independent methods, Hasselbach and Taugner, 1970; Taugner, 1971), the ATPase activity stimulated by addition of catecholamine can amount to only 1% the total activity. However, such a small amount is below the limits of accuracy of the measurements. The missing proof of an extra-ATPase activity therefore does not contradict the existence of an active transport mechanism.

The above mentioned alternative assumption (i.e. catecholamine binding to membrane structures on the basis of an enzymatic process) could also explain the net accumulation of catecholamine within the vesicular membranes. In this case the geometry (i.e., whether vesicular particles or open fragments are involved) should not make any difference. It is obvious from experiments with sonicated membrane preparations, that at least 50—60% of the catecholamine is stored in the interior of vesicular particles, while a smaller part ought to be bound within the membrane. It was impossible to determine this portion exactly, since one cannot exclude the possibility that some of the particles remained intact after sonication. Prolongation of time of sonication resulted in incomplete protein sedimentation.

As experiments with membranes previously treated with radioactive (–)-noradrenaline demonstrated the observed net uptake of catecholamine is the result of catecholamine influx and efflux. In the presence of ATP the catecholamine efflux is about $1^1/_2$ times higher than in the absence of ATP. With an ATP-induced binding of catecholamine to a complex an inverse relation should be obtained.

The catecholamine efflux (either in the presence or absence of ATP) remained constant throughout the experimental period. As described for intact adrenal medullary vesicles (Taugner and Hasselbach, 1966b, 1968; Hasselbach and Taugner, 1970) it is independent of catechol-

amine influx, and only indirectly coupled with the influx by the respective permeability state of the membrane.

Even in the absence of ATP, catecholamine from the medium is incorporated into the membrane. Therefore a low, apparently ATP-independent turnover, of catecholamine is to be distinguished from a considerably higher ATP-dependent one: While the low catecholamine influx in the absence of ATP remains almost constant over the observed period, the ATP-dependent catecholamine influx decreases with time.

The decrease in the influx of catecholamine in the presence of ATP is paralleled by a decrease in the ATPase activity. Since the ATP concentration in the medium is diminished at most by 15—20% during the experimental period of 15 min, deficiency of ATP cannot be the cause for the simultaneous decrease of catecholamine influx and ATPase activity. Alternatively, the increasing loading of the vesicular membranes with catecholamine could inhibit ATPase activity and therefore further transport. Such a mechanism, for instance, is known from the Ca-vesicles of sarcoplasmatic reticulum (Makinose and Hasselbach, 1965). In the case of the membrane preparation presented here those components which might be responsible for complex formation with catecholamine (such as chromogranin, ATP, Ca^{2+}) were removed.

From this might also result the very low catecholamine influx and likewise the low efficiency of transport mechanism of isolated membranes compared with those obtained from intact vesicles: in the presence of ATP in media containing KCl the catecholamine influx of intact vesicles amounts to 8 nmoles/mg of vesicular protein (Taugner, 1971). Related to the membrane protein (which makes up approximately $^1/_4$ of the total protein) the influx rate ought to be 32 nmoles catecholamine/mg of protein, if it is assumed that the accumulation of catecholamine is exclusively brought about by membrane transport. The influx rate obtained from isolated membranes, however, amounts to only 1.2 nmoles catecholamine/mg of membrane protein at a splitting rate of 155 nmoles P_i/mg of protein. This difference between the two preparations indicates that the complex inside the vesicles seems to be necessary for the storage of large amounts of catecholamine. Obviously, by raising the concentration of "free" catecholamine inside the vesicles to a high level, active transport creates the conditions giving rise to complex formation. For the preservation of the complex the high free catecholamine concentration which is inside the vesicles and in equilibrium with the complex, must be maintained by the catecholamine transport system of the membrane.

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References

Banks, P., Helle, K. B.: The release of protein from the stimulated adrenal medulla. Biochem. J. **97**, 40c (1965).

Berneis, K. H., Pletscher, A., Da Prada, M.: Metal-dependent aggregation of biogenic amines: A hypothesis for their storage and release. Nature (Lond.) **224**, 281 (1969).

Blaschko, H., Comline, R. S., Schneider, F. H., Silver, M., Smith, A. D.: Secretion of a chromaffine granule protein, chromogranin, from the adrenal gland after splanchnic stimulation. Nature (Lond.) **215**, 58 (1967a).

— Firemark, H., Smith, A. D., Winkler, H.: Lipids of the adrenal medulla. Lysolecithin, a characteristic constituent of chromaffin granules. Biochem. J. **104**, 545 (1967b).

— Helle, K. B.: Interaction of soluble protein fractions from bovine adrenal medullary granules with adrenaline and adenosine triphosphate. J. Physiol. (Lond.) **169**, 120p (1963).

Burger, A., Philippu, A., Schümann, H. J.: ATP-Spaltung und Aminaufnahme durch Milznervengranula. Naunyn-Schmiedebergs Arch. Pharmak. exp. Path. **262**, 208 (1969).

Diner, O.: L'expulsion des granules de la médullo-surrénale chez le Hamster. C. R. Acad. Sci. (Paris) **265**, 616 (1967).

Douglas, W. W.: Stimulus-secretion coupling: The concept and clues from chromaffine and other cells. The first Gaddum memorial lecture. Brit. J. Pharmacol. **34**, 451 (1968).

Euler, U. S. v., Lishajko, F.: Improved technique for the fluorimetric estimation of catecholamines. Acta physiol. scand. **51**, 348 (1961).

— — Effect of adenine nucleotides on catecholamine release and uptake in isolated nerve granules. Acta physiol. scand. **59**, 454 (1963).

— — Uptake of L- and D-isomers of catecholamines in adrenergic nerve granules. Acta physiol. scand. **60**, 217 (1964).

Hasselbach, W., Makinose, M.: Die Calciumpumpe der „Erschlaffungsgrana" des Muskels und ihre Abhängigkeit von der ATP-Spaltung. Biochem. Z. **333**, 518 (1961).

— Taugner, G.: The effect of a cross-bridging thiol reagent on the catecholamine fluxes of adrenal medulla vesicles. Biochem. J. **119**, 265 (1970).

Helle, K. B., Serck-Hanssen, G.: Chromogranin: The soluble and membrane bound lipoprotein of the chromaffine granule. Pharmacol. Res. Commun. **1**, 25 (1969).

Hillarp, N. Å.: Adenosinephosphates and inorganic phosphate in the adrenaline and noradrenaline containing granules of the adrenal medulla. Acta physiol. scand. **42**, 321 (1958).

Kirshner, N., Holloway, C., Kamin, D.: Permeability of catecholamine granules. Biochem. biophys. Acta (Amst.) **112**, 532 (1966).

Makinose, M., Hasselbach, W.: Der Einfluß von Oxalat auf den Calcium-Transport isolierter Vesikel des sarkoplastischen Reticulum. Biochem. Z. **343**, 360 (1965).

Malamed, S., Poisner, A. M., Trifaro, J. M., Douglas, W. W.: The fate of chromaffine granule during catecholamine release from the adrenal medulla. III. Recovery of a purified fraction of electron-translucent structures. Biochem. Pharmacol. **17**, 241 (1968).

Rockstein, M., Herron, P. W.: Colorimetric determination of inorganic phosphate in microgram quantities. Analyt. Chem. **23**, 1500 (1951).

Schneider, F. H., Smith, A. D., Winkler, H.: Secretion from the adrenal medulla: biochemical evidence for exocytosis. Brit. J. Pharmacol. **31**, 94 (1967).

Skou, J. Ch.: The influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochim. biophys. Acta (Amst.) **23**, 394 (1957).

Smith, A. D., Winkler, H.: Purification and properties of an acid protein from chromaffine granules of bovine adrenal medulla. Biochem. J. **103**, 483 (1967).

Taugner, G., ATP als Katecholamin-Freisetzer in isotonischen Salzlösungen? Naunyn-Schmiedebergs Arch. Pharmak. **264**, 315 (1969).

— The effect of salts on catecholamine fluxes and adenosine triphosphatase activity from storage vesicles of the adrenal medulla. Biochem. J. **123**, 219 (1971).

— Hasselbach, W.: Über den Mechanismus der Catecholamin-Speicherung in den „chromaffinen Granula" des Nebennierenmarks. Naunyn-Schmiedebergs Arch. Pharmak. exp. Path. **255**, 266 (1966a).

— — Kinetic aspects of catecholamine storage. Biochem. J. **102**, 23p (1966b).

— — Die Bedeutung der Sulhydryl-Gruppen für den Catecholamin-Transport der Vesikel des Nebennierenmarkes. Naunyn-Schmiedebergs Arch. Pharmak. exp. Path. **260**, 58 (1968).

Viveros, O. H., Arqueros, L., Kirshner, N.: Mechanism of secretion from the adrenal medulla. Retention of storage vesicle membranes following release of adrenaline. Molec. Pharmacol. **5**, 342 (1969).

Weiner, N., Jardetzky, O.: A study of catecholamine nucleotide complexes by nuclear magnetic resonance spectroscopy. Naunyn-Schmiedebergs Arch. Pharmak. exp. Path. **248**, 308 (1964).

Winkler, H., Hörtnagl, H., Hörtnagl, H., Smith, A. D.: Membranes of the adrenal medulla. Behaviour of insoluble proteins of chromaffin granules on gel electrophoresis. Biochem. J. **118**, 303 (1970).

— Strieder, N., Ziegler, E.: Über Lipide, insbesondere Lysolecithin, in den chromaffinen Granula verschiedener Species. Naunyn-Schmiedebergs Arch. Pharmak. exp. Path. **256**, 407 (1967).

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