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Steady-State Kinetics of Catecholamine Transport by Chromaffin-Granule 'Ghosts'

By JOHN H. PHILLIPS*
Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

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Resealed chromaffin-granule 'ghosts' were used to study the steady-state kinetics of catecholamine transport. The pump has a high affinity for (−)-noradrenaline, (−)-adrenaline, tyramine and 5-hydroxytryptamine (serotonin), but a lower affinity for (+)-noradrenaline. The measured rates of incorporation do not conform to Michaelis–Menten kinetics, but affinity constants for the former substrates are in the range 8–18 μM. Reserpine is a potent inhibitor. Incorporation as a function of ATP concentration also fails to show simple kinetics; the affinity constant for ATP is deduced to be about 3 mM at 1 mM-$MgCl_2$. Adenylyl ($\beta\gamma$-methylene)diphosphonate is a competitive inhibitor at low concentrations, but inhibits more strongly at high concentrations. The pump has a transition temperature at 29°C and does not seem to be identical with the Mg^{2+}-stimulated adenosine triphosphatase of chromaffin granules.

In the preceding paper (Phillips, 1974) a method for the preparation of resealed chromaffin-granule 'ghosts' from the bovine adrenal medulla was described. In the present paper I describe the use of such preparations to study the transport of catecholamines across the storage-granule membrane, against the concentration gradient. An ATPase†-driven pump is involved; this does not conform strictly to Michaelis–Menten kinetics but appears to show positive co-operativity for the catecholamine substrate, but negative co-operativity for ATP. It is unlikely that the Mg^{2+}-stimulated ATPase of the chromaffin granule (Banks, 1965; Kirshner *et al.*, 1966) is the enzyme involved in this transport; it must therefore have another function.

Experimental

Materials

The following radiochemicals (The Radiochemical Centre, Amersham, Bucks., U.K.) were used: (−)-[*methylene*-^{14}C]noradrenaline D-bitartrate (54 or 57 mCi/mmol); (+)-[*methylene*-^{14}C]noradrenaline D-bitartrate (21 mCi/mmol); [*side chain*-2-^{14}C]tyramine hydrochloride (42 mCi/mmol); and 5-hydroxy[*side chain*-2-^{14}C]tryptamine creatinine sulphate (57 or 58 mCi/mmol). ADP(CH_2)P was obtained from Miles-Seravac Ltd., Maidenhead, Berks., U.K. The sources of other materials were given in the preceding paper (Phillips, 1974).

* Present address: Department of Biochemistry, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

† Abbreviations: ATPase, adenosine triphosphatase; Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N*′-yl)ethanesulphonic acid; ADP(CH_2)P, adenylyl ($\beta\gamma$-methylene)-diphosphonate.

Methods

The methods used were described in the preceding paper (Phillips, 1974). Chromaffin-granule 'ghosts' were always used within 6h of preparation. Their ability to incorporate catecholamines decreases by 50% in about 24h at 4°C. Standard incubation conditions for transport experiments were as follows: chromaffin-granule 'ghosts' (usually 50–150 μg of protein/ml) were incubated for 10min at 37°C in 0.3M-sucrose buffered with 10mM-Hepes, pH7.0, in the presence of 6mM-ATP, 2mM-$MgCl_2$ and a radioactive substrate. (−)-[^{14}C]Noradrenaline was diluted with (−)-noradrenaline hydrochloride to a specific radioactivity of about 20×10^3 c.p.m./nmol except where indicated in the Figure legends. 5-Hydroxy[^{14}C]tryptamine was diluted to a similar psecific radioactivity. (+)-[^{14}C]Noradrenaline and [^{14}C]tyramine were used at the specific radioactivities at which they were supplied. In all cases except Fig. 1 the values of control incubations performed in the presence of 10 μM-reserpine and 30mM-Hepes were subtracted from the experimentals. When 5-hydroxytryptamine was used as a substrate, incubations were performed for 5min or 8min rather than 10min. Initial rates are expressed as uptake/10min, however.

Results

General features of incorporation

Chromaffin-granule 'ghosts' incorporate a variety of biogenic amines in an ATP-dependent reaction (Phillips, 1974). In the experiments described in the present paper (−)-noradrenaline or 5-hydroxytryptamine (serotonin) have generally been used as substrates, with similar results.

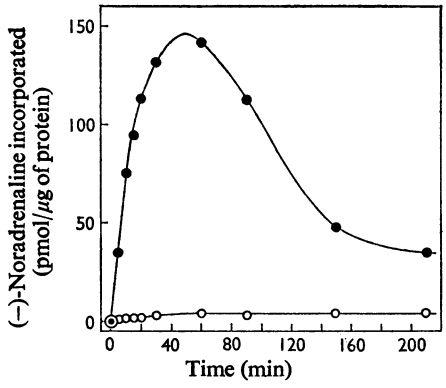

Fig. 1. *Incorporation of (−)-noradrenaline by 'ghosts'*

Chromaffin-granule 'ghosts' (1 ml; 69 μg of protein) were incubated under standard conditions at 37°C in the presence of 125 μM-(−)-[^{14}C]noradrenaline (●). A similar incubation contained 10 μM-reserpine (○). Samples (100 μl) were removed at intervals.

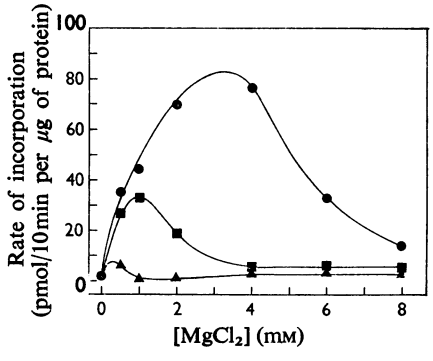

Fig. 2. *Mg^{2+} requirements for incorporation of (−)-noradrenaline*

Chomaffin-granule 'ghosts' (0.1 ml; 7.0 μg of protein) were incubated for 10 min at 37°C in the presence of 122 μM-(−)-[^{14}C]noradrenaline, the $MgCl_2$ concentration shown (or 2 mM-EDTA) and ATP at a concentration of 8 mM (●), 2 mM (■) or 0.5 mM (▲). Values for incorporation in the presence of 10 μM-reserpine have been subtracted.

Incorporation is directly proportional to the concentration of 'ghosts', is inhibited by reserpine, and the incorporated substrate can be released by osmotic shock (Phillips, 1974). Fig. 1 shows the time-course of incorporation of (−)-noradrenaline at 37°C. The uptake is linear with time for the first 10 min of incubation. The 'ghosts' leak after about 1 h, however; a similar phenomenon is found in transport experiments with bacterial membrane vesicles (Kaback, 1968). Incorporation in the presence of

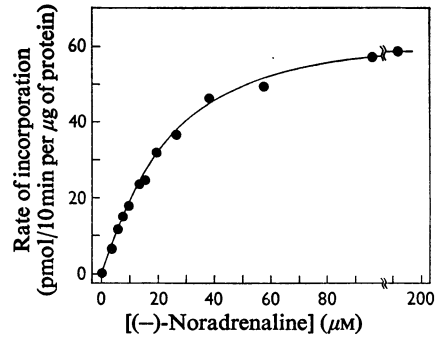

Fig. 3. *Dependence of incorporation rate on (−)-noradrenaline concentration*

Chromaffin-granule 'ghosts' (0.1 ml; 3 μg of protein) were incubated for 10 min at 37°C under standard conditions.

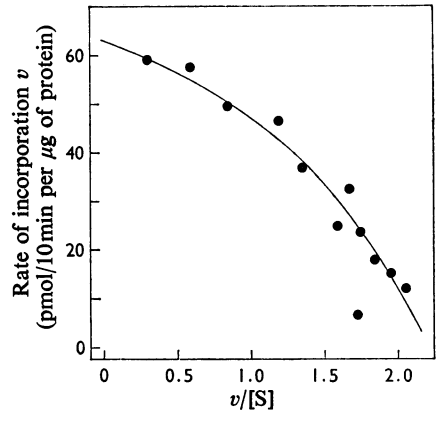

Fig. 4. *Plot of v against v/[S] for (−)-noradrenaline incorporation*

Data are taken from Fig. 3. [S] is in μM.

10 μM-reserpine is also shown in Fig. 1. These values represent binding of the substrate to membranes, as this material cannot be released by osmotic shock. Control incubations in the presence of 10 μM-reserpine were performed for all the experiments described in the present paper and these values have been subtracted from the experimental results. This procedure eliminates incorporation owing to artifacts such as sealing of membrane fragments during the incubation, and also eliminates any reserpine-independent incorporation (Slotkin *et al.*, 1971) from consideration. In general, these control values are low compared with the experimental values.

The dependence of incorporation on Mg^{2+} concentration varies with the concentration of ATP

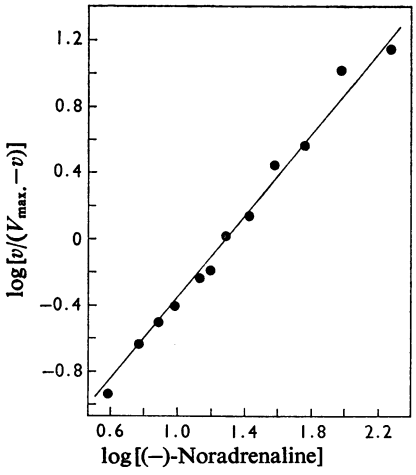

Fig. 5. *Hill plot for (−)-noradrenaline incorporation*

Data are taken from Fig. 3, by using a value for $V_{max.}$ of 63 pmol/10 min per μg of protein. [S] is in μM. The Hill coefficient n_H is 1.22, and the substrate concentration at $V_{max.}/2$ is 19 μM.

Table 1. *Kinetic parameters for substrates of the catecholamine pump*

Results are the mean of three determinations on different 'ghost' preparations. $V_{max.}$ was determined by extrapolation of Lineweaver–Burk plots. $s_{0.5}$, substrate concentration at $V_{max.}/2$; n_H, Hill coefficient.

	$s_{0.5}$ (μM)	n_H	$V_{max.}$ (pmol/10 min per μg of protein)
(−)-Noradrenaline	18	1.3	77
(+)-Noradrenaline	70	1.2	60
(−)-Adrenaline	16*	—	—
5-Hydroxytryptamine	9	1.2	70
Tyramine	8	1.2	11

* Obtained from inhibition of 5-hydroxytryptamine incorporation. The value given is the concentration of (−)-adrenaline which doubles the $s_{0.5}$ value for 5-hydroxytryptamine, as obtained from Hill plots for 5-hydroxytryptamine in the presence of (−)-adrenaline.

added (Fig. 2). In general, Mg^{2+} ions were added to a concentration equal to one-third that of ATP.

Dependence of transport on substrate concentration

On the basis of inhibition studies with intact chromaffin granules, Kirshner (1962) suggested that the transport process has very wide specificity. The influence of (−)-noradrenaline, an important substrate *in vivo*, on the initial rate of incorporation is shown in Fig. 3. These data give a concave Lineweaver–Burk plot (or convex Eadie plot, Fig. 4), and the same is true for other substrates. This form of dependence could result from positive co-operativity, and the data are shown in the form of a Hill plot in Fig. 5. Hill coefficients (n_H) and substrate concentrations at half saturation ($s_{0.5}$) for four substrates are presented in Table 1. Values found for maximum initial velocities (by extrapolation of Lineweaver–Burk plots) are also given, although these values vary somewhat from one preparation of 'ghosts' to another, and with the age of the preparation. An affinity constant for (−)-adrenaline has been deduced from inhibition studies, since this stereoisomer could not be obtained radioactive commercially.

Incorporation in the presence of 10 μM-reserpine is directly proportional to the substrate concentration over the range tested (up to about 200 μM). It is very small for substrates other than tyramine, being less than 5% of total incorporation at a substrate concentration which half-saturates the pump. With 8 μM-tyramine, however, about 20% of the incorporation was reserpine-insensitive. This incorporation has been discussed by Slotkin *et al.* (1971). However, even the reserpine-sensitive incorporation of tyramine had a low-affinity component, which complicated the interpretation of the data.

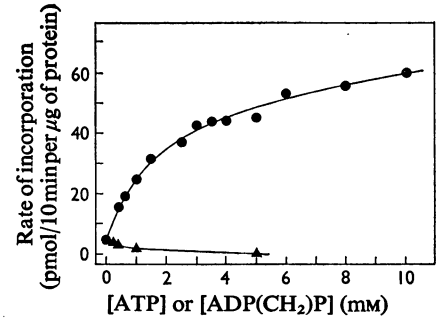

Fig. 6. *ATP requirement for incorporation of (−)-noradrenaline*

Chromaffin-granule 'ghosts' (0.1 ml; 15 μg of protein) were incubated for 10 min at 37°C in the presence of 135 μM-(−)-[^{14}C]noradrenaline and the ATP concentrations shown (●). Samples were also incubated in the absence of ATP in the presence of $ADP(CH_2)P$ (▲). $MgCl_2$ was present at one-third of the ATP and $ADP(CH_2)P$ concentration. Values obtained in the presence of 10 μM-reserpine have been subtracted.

Dependence of transport on ATP concentration

Dependence on the second substrate, ATP, is shown in Fig. 6. In this experiment Mg^{2+} is present at

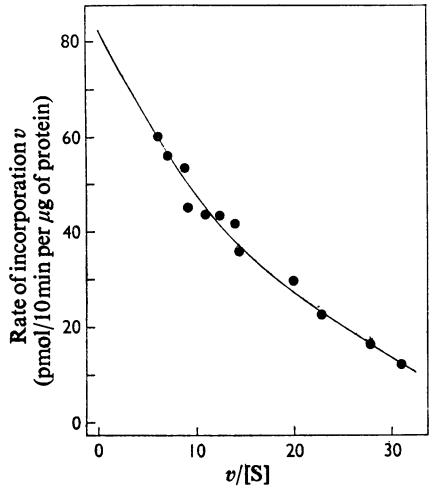

Fig. 7. *Plot of v against v/[S] for ATP dependence of (−)-noradrenaline incorporation*

Data are taken from Fig. 6. [S] is in mM.

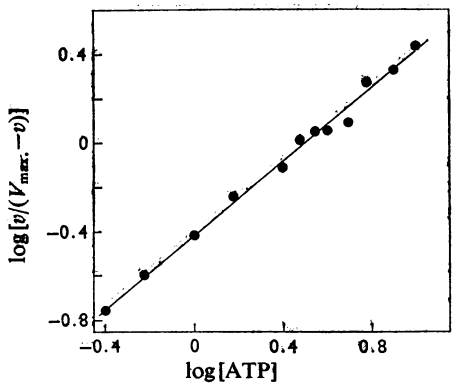

Fig. 8. *Hill plot for ATP dependence of (−)-noradrenaline incorporation*

Data are taken from Fig. 6, by using a value for $V_{max.}$ of 82pmol/10min per μg of protein. [S] is in mM. The Hill coefficient is 0.84, and the ATP concentration at $V_{max.}/2$ is 3mM.

a concentration equal to one-third of the ATP concentration, and all values shown have been decreased by the amount of a control value obtained in the presence of 10μM-reserpine.

One feature of this experiment is that there is a small residual reserpine-sensitive incorporation in the absence of added ATP. This is not attributable to ATP introduced into the incubation as a contaminant of the 'ghost' preparation; this ATP is approx. 1μM,

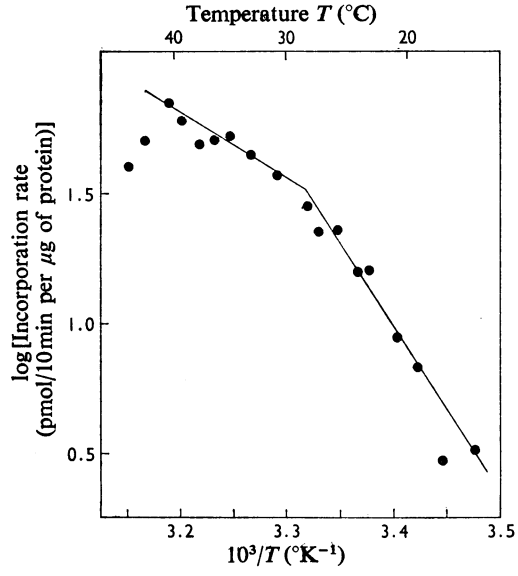

Fig. 9. *Arrhenius plot for incorporation of 5-hydroxytryptamine*

Chromaffin-granule 'ghosts' (0.1ml; 11μg of protein) were incubated under standard conditions in the presence of 104μM-5-hydroxy[^{14}C]tryptamine.

and the incorporation is insensitive to preincubation of the 'ghosts' with hexokinase and glucose. It is, however, inhibited by excess of free Mg^{2+} ions and by the ATP analogue, adenylyl (βγ-methylene)-diphosphonate [ADP(CH_2)P]. A likely explanation for this small incorporation (which is sensitive to osmotic shock) is that it represents an exchange reaction across the membrane by substrate molecules utilizing the carrier, in the absence of ATP hydrolysis, this exchange being blocked by addition of ADP-(CH_2)P (and, presumably, of ATP).

Although 10μM-reserpine fails to inhibit transport completely in the absence of ATP, complete inhibition is produced by a sufficient (e.g. 5mM) concentration of ADP(CH_2)P (Fig. 6). This suggests that all entry is through the pump; non-specific entry through the membrane is insignificant.

Investigation of the dependence of transport on ATP concentration requires a correction to be made to the data of Fig. 6 for the small ATP-independent uptake that is found at low ATP concentrations. The correction is difficult to make accurately, since this component of the transport presumably decreases as the concentration of ATP increases. A correction was based on inhibition experiments with ADP(CH_2)P (Fig. 6). The resultant data for ATP-dependence consistently yield convex Lineweaver–Burk plots or concave Eadie plots (Fig. 7); the data of Fig. 6 are

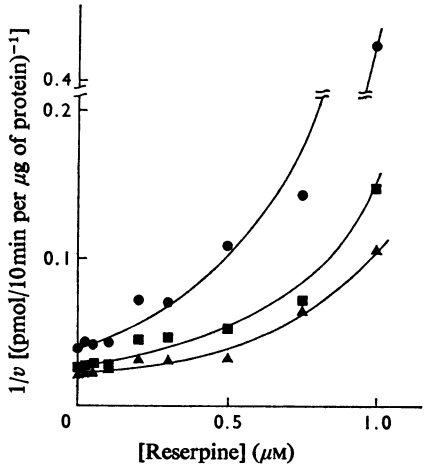

Fig. 10. *Reserpine inhibition of* (−)-*noradrenaline incorporation*

Chromaffin-granule 'ghosts' (0.1 ml; 11 μg of protein) were incubated under standard conditions in the presence of the following concentrations of (−)-noradrenaline: 26 μM (88×10^3 c.p.m./nmol) (●); 62 μM (37×10^3 c.p.m./nmol) (■); 114 μM (20×10^3 c.p.m./nmol (▲).

shown in the form of a Hill plot in Fig. 8, by using a value for $V_{max.}$ obtained by extrapolation of the Eadie plot. The Hill coefficient (n_H) is 0.84, with a substrate concentration at half-maximal velocity of 3 mM-ATP (1 mM-Mg^{2+}). Similar values have also been found with 5-hydroxytryptamine as substrate.

Effect of temperature

An Arrhenius plot for incorporation of 5-hydroxytryptamine is shown in Fig. 9. The activation energy for transport changes from 122 kJ·mol^{-1} to 50 kJ·mol^{-1} on raising the temperature above 29°C. Recent work on the Mg^{2+}-stimulated ATPase of chromaffin-granule membranes (H. B. Pollard, personal communication) has shown a transition temperature of 32°C; above this temperature the ATPase showed negative co-operativity with respect to ATP concentration ($n_H = 0.85$), but at lower temperatures the co-operativity was abolished. This was not found to be the case for 5-hydroxytryptamine transport, however. In one experiment, for example, n_H was found to be 0.88 at 37°C ($s_{0.5}$ for ATP, 2.8 mM) and 0.84 at 24°C ($s_{0.5}$, 0.9 mM).

Effect of inhibitors

The *Rauwolfia* alkaloid reserpine has long been recognized as a potent inhibitor of the transport of

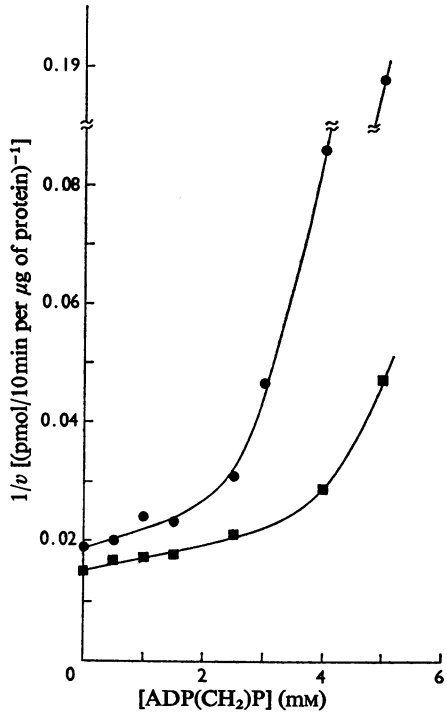

Fig. 11. *Inhibition of* (−)-*noradrenaline incorporation by ADP*(CH_2)*P*

Chromaffin-granule 'ghosts' (0.1 ml; 13 μg of protein) were incubated under standard conditions in the presence of 132 μM-(−)-noradrenaline and 3 mM-ATP (●) or 6 mM-ATP (■). In all incubations the concentration of $MgCl_2$ was one-third of the sum of the ATP and ADP(CH_2)P concentrations.

catecholamines into chromaffin granules (Kirshner, 1962). It proved very difficult, however, to obtain good results in studies of its inhibitory action, possibly because it may bind non-specifically to membranes and other surfaces. Inhibition at three concentrations of (−)-noradrenaline is shown in the form of a Dixon (1953) plot in Fig. 10. Experiments of this type consistently gave upward-curving inhibition plots, although the points were generally rather scattered. Expressing the data from several experiments in the form of Hill plots gave values for the Hill coefficient n_H of between 1.2 and 1.3, with inhibitor concentration at half-maximal velocity of about 0.3 μM.

Low concentrations of the ATP analogue ADP(CH_2)P appear to inhibit catecholamine transport competitively, with a K_i of about 3 mM (Fig. 11). There is a sharp increase in its inhibitory effect at

high concentrations, however, and this is discussed below.

Discussion

Criteria for active transport

The main difficulty in interpreting experiments on transport is the correction of experimental data to allow for two-way fluxes across the membrane. For intact chromaffin granules this problem is rendered almost insoluble because of the high concentrations of both substrates, catecholamines and ATP, inside the granules. Incorporation experiments are accompanied by both leakage of these components and lysis of the granules. In the present work this difficulty is overcome by the use of resealed 'ghosts'. The 'ghost' preparations do, however, contain low residual concentrations of catecholamines and ATP (Phillips, 1974), both inside the vesicles and free in solution. The latter may easily be allowed for in calculations, but the former presents two problems; firstly, included catecholamines may leak during incubations. Detailed measurements of such efflux have been made by Taugner (1971, 1972*a*), who showed that the rate of efflux is essentially constant at 31°C; in the present experiments short incubations coupled with the measurement of uptake of labelled catecholamines (rather than assays of total catecholamines) were used to obtain measurements of influx rates rather than of net uptake. The second problem is that, in the absence of a reliable value for the included volume of 'ghost' preparations, the internal concentration of catecholamines remains unknown.

Taugner (1971) has shown that the uptake of catecholamines appears to represent an uphill transport, rather than an exchange reaction. The results in the present paper confirm this. Taking the results of Fig. 1, for example, the concentration of (−)-[^{14}C]noradrenaline inside the 'ghosts' after 50min incubation is about 7mM, if the included volume of the preparation is 20μl/mg of protein (Phillips, 1974; if the included volume is less than this, the concentration is of course higher). Fluorimetric assay gave the initial concentration of catecholamines inside the 'ghosts' of this preparation as 1.4mM, by using the same volume assumption. The concentration of (−)-noradrenaline in the incubation medium was 0.13mM. This concentration is essentially saturating, and much greater concentration ratios are clearly achieved by using lower concentrations in the incubation medium.

There is no direct evidence for the source of energy for the transport, since no stoicheiometry can be demonstrated between ATP hydrolysis and catecholamine uptake (see below). However, the facts that ATP is required, that it cannot be replaced by ADP and that ADP(CH_2)P appears to be a competitive inhibitor strongly suggest that catecholamine transport is in fact driven by ATP hydrolysis.

Mechanism of transport

Taugner (1972*a*) showed that the pH optimum for the transport process was different from that of the Mg^{2+}-stimulated ATPase of the 'ghosts'. She also showed that, at pH7, the specific activity of the latter exceeded the transport rate by a factor of about 250. On the other hand, univalent anions have similar effects on both processes (Taugner, 1972*b*). The present study also suggests that transport is not linked to the bulk ATPase of the membranes. The activation energies for the two processes are quite different; that of the ATPase is found to increase from 15.4kJ·mol^{-1} to 36.4kJ·mol^{-1} above a transition temperature of 32°C (H. B. Pollard, personal communication). Dependence on ATP concentration as a function of temperature is also dissimilar, as noted above.

In calculating the results in Table 1, the contribution to the transport process of non-radioactive catecholamine, present as a contaminant of the 'ghost' preparation, has been ignored. The concentration of this extra substrate is, of course, low compared with that of the radioactive substrate in the medium, except at the lowest substrate concentrations. It can, however, be allowed for in the calculation, since (−)-adrenaline (which comprises about 75% of the endogenous catecholamines) and (−)-noradrenaline are transported with similar affinities. Only small differences are made to the deduced kinetic constants. In cases of severe contamination, values of $V_{max.}$ may be underestimated by about 10%; values for n_H and $s_{0.5}$ may also be slightly underestimated.

It is tempting to interpret the kinetic data in terms of positive co-operativity for the transported substrate and negative co-operativity for ATP. Positive co-operativity for cations is well known for the sodium pump (Squires, 1965; Hoffman & Tosteson, 1971). Several alternative interpretations are possible, however (see Levitzki & Koshland, 1969), especially when considering a subcellular fraction rather than a purified protein. In particular, the Hill coefficients found are not very far from unity. The most likely alternative is, of course, that there is more than one ATPase-catalysed catecholamine-transport system present. This could arise, for example, from different properties of the granules of the two cell types in the adrenal medulla (containing adrenaline and noradrenaline respectively).

If due to a co-operative interaction, the negative homotropic effect shown by ATP would seem to be both novel and interesting, in that the transport mechanism will tend to work effectively over rather a wide range of ATP concentrations. The inhibition by ADP(CH_2)P is noteworthy: it appears to be unusually

inhibitory at concentrations above its apparent K_i. This is not only found in the presence of ATP (Fig. 11), but also in the inhibition of catecholamine exchange by using the pump in the absence of ATP. This could be explained if there are two sites for ATP, with a 'half-of-the-sites' effect (Levitzki *et al.*, 1971; Stallcup & Koshland, 1973), so that occupation of the first site greatly decreases either the affinity of ATP or its rate constant at the second site. Curves such as those of Fig. 11 cannot be fitted by straight-line Hill plots. By contrast, reserpine inhibition can be fitted by Hill plots which are straight lines, and presumably reflects binding of the inhibitor to the catecholamine sites.

Specificity of the pump

It is well established that noradrenaline-containing granules of sympathetic neurons show specificity for the incorporation of (−)-noradrenaline from mixtures of both stereoisomers (Stjärne & von Euler, 1965; von Euler & Lishajko, 1967), and Taugner (1972*a*) showed that this also applied to chromaffin granules. A substantial difference in affinities is also revealed in the present work (Table 1).

The substrate concentrations for half-maximal velocity shown in Table 1 for (−)-noradrenaline and (−)-adrenaline are low; the mechanism of catecholamine storage in the adrenal medulla may present analogies with calcium storage in sarcoplasmic reticulum. In the latter case, the affinity of Ca^{2+} ions for the calcium pump may be as much as a thousand times greater than their affinity for the intravesicular storage protein (MacLennan & Wong, 1971).

Since the natural substrates of the pump in the adrenal medulla are dopamine, noradrenaline and adrenaline, it is perhaps surprising that 5-hydroxytryptamine is a substrate with high affinity (Table 1; Kirshner, 1962). An interesting possibility is that 5-hydroxytryptamine-storage granules share a common pump mechanism with catecholamine-containing granules. The similarity in structure between 5-hydroxytryptamine and part of the reserpine molecule has been pointed out by Green & Slotkin (1973) and reserpine has been found to be a potent inhibitor of 5-hydroxytryptamine uptake by the storage granules of blood platelets (Minter & Crawford, 1974).

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