

## Kinetic Mechanism of the Exchanges Catalysed by the Adenine-Nucleotide Carrier

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Initial rates of the exchange  $\text{ADP}_{\text{in}}/\text{ADP}_{\text{out}}$  catalysed by the adenine-nucleotide carrier of rat-heart mitochondria have been studied under conditions where internal and external ADP may be varied. The initial rate was measured within 1 s by the carboxyatractyloside-stop method, using a rapid-mixing technique.

The double-reciprocal plots  $v_0^{-1}$  versus  $[\text{ADP}]_{\text{out}}^{-1}$  at different internal-ADP concentrations and  $v_0^{-1}$  versus  $[\text{ADP}]_{\text{in}}^{-1}$  at different external-ADP concentrations exhibit straight-line relationships having a common point of intersection on the axis of ordinates.

These results demonstrate the essential role of a ternary complex and thus exclude the ping-pong mechanism generally accepted. The kinetic equation implies a strong positive cooperativity in the binding of the two substrates.

Two models are proposed: (a) the ternary complex performs the exchange and the transport of the substrates in a single step; (b) the carrier is mobile and transports the substrates one by one, the formation of a ternary complex being needed to release the first product.

An intriguing situation encountered in the field of the adenine-nucleotide carrier (modeling) is the lack of extensive kinetic studies, although this kind of study provides information that may often be interpreted simply and surely. In fact, the limited kinetic data so far reported have been obtained under conditions which do not permit one to discriminate the usually accepted ping-pong mechanism from other possible mechanisms. It has been proposed [1-3] that the adenine-nucleotide carrier possesses a single accessible binding site for its substrates and exists in two forms; the first can be loaded by the external substrate, the second can be loaded by the internal substrate inside the mitochondrial matrix. Since the transformation of one form into the other can only occur if the carrier is loaded with one substrate, the carrier performs an obligatory one-to-one exchange. The models described in [1-3] in which the translocator binds alternately both its substrates correspond to the so-called 'ping-pong' mechanism [4]. Another possibility would be a sequential mechanism [4] in which the external substrate and the internal substrate must

*Abbreviation.* Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

bind to the translocator before the exchange occurs. In this case the active complex is the ternary complex external-substrate-translocator-internal-substrate while in the 'ping-pong' mechanism only binary complexes are formed: carrier-internal-substrate and carrier-external-substrate.

An initial-rate analysis permits one to distinguish between these two types of mechanisms [4]. In the case of a 'ping-pong' mechanism, and only in this case, the ratio between the apparent Michaelis constant and the apparent maximal velocity for a substrate is constant at different fixed concentrations of the second substrate. Thus the Lineweaver-Burk plot ( $v_0^{-1}$  versus  $[\text{S}]^{-1}$ ) for one substrate consists of parallel straight lines each of which corresponds to a fixed concentration of the second substrate.

In order to determine the kinetic mechanism of the translocation it is thus necessary to measure initial rates at various concentrations of both internal and external substrates. The theoretical interpretation is clear and would thus permit the non-equivocal demonstration or rejection of a mechanism which is widely accepted. This method has not been applied yet and is the subject of this paper. Its correct applica-



tion is of course subject to real initial-rate measurements and to the accurate determination of varied internal-substrate concentrations.

Preliminary results have been present elsewhere [5].

## MATERIALS AND METHODS

Rats were 200–250-g fed males from inbred strains of Wistar R/A Pfd f. Mitochondria from rat-heart ventricles were prepared according to Tyler and Gonze [6]; the isolation medium contained: 225 mM mannitol, 75 mM sucrose and 0.05-mM EDTA neutralized with 1 M Tris. The mitochondrial pellet was resuspended in isolation medium without EDTA but supplemented with 2 mM Hepes, pH 7.4.

The following pretreatments were found to provide mitochondrial preparations in which (a) the internal adenine-nucleotide concentration may be varied, (b)  $[ADP]_{in}$  exceeds  $[ATP]_{in}$ , (c) the ratio  $[ADP]_{in}/[ATP]_{in}$  remains essentially constant.

a) Mitochondria were incubated for 30 min at 0°C in the presence of 2 µg rotenone and 3 µg antimycin/mg mitochondrial protein, in a medium containing 20 mM Tris/Cl pH 7.4, 1 mM EDTA, 10 mM ADP, as well as 165.4 mM mannitol, 55 mM sucrose and 1.5 mM Hepes derived from the isolation medium. In these conditions the ATP/ADP ratio is low (0.11–0.25).

b) Mitochondria were also incubated for 10 min at 25°C in the presence of 1 µg rotenone/mg mitochondrial protein, plus 2 mM succinate and 1 mM or 2 mM inorganic phosphate in a medium containing 20 mM Tris/Cl pH 7.4, 45 mM mannitol, 15 mM sucrose, and 0.8 mM Hepes (the last three were derived from the isolation medium), 0, 2.5 mM or 20 mM  $MgCl_2$ ; the solution was supplemented by sucrose to obtain the same osmolarity (0.3 osM). In these conditions the  $Mg^{2+}$  content was lowered [7] as well as the total adenine-nucleotide content. If the mitochondria were then incubated as in (a), the same ATP/ADP ratio was obtained.

The two mitochondrial preparations were washed twice afterwards in the presence of rotenone and antimycin in order to eliminate external added ADP.

Initial rate of  $[^{14}C]ADP$  uptake at 2°C was measured by the rapid technique described in [8] with 10 µM carboxyatractyloside as stop inhibitor in the following incubation medium: 15 mM KCl, 57.5 mM Tris/Cl pH 7.4, 2 mM EDTA, 22.5 mM mannitol, 7.5 mM sucrose, 0.2 mM Hepes and 1 µg rotenone, 1.5 µg antimycin, and 3 µg oligomycin for each mg mitochondrial protein. At 2°C and in the presence of 2 mM EDTA, adenylate kinase is not active [9] and thus does not affect the concentration of the external ADP.

The internal ADP and ATP content were determined enzymatically [10, 11]. The magnesium content was determined with a Perkin-Elmer atomic-absorption spectrophotometer (model 303).

Special reagents were obtained as follows:  $[U-^{14}C]$ -sucrose, tritiated water,  $[8-^{14}C]$ adenosine 5'-diphosphate and  $[8-^{14}C]$ adenosine 5'-triphosphate, ammonium salts (The Radiochemical Center, Amersham, Great Britain); rotenone (Sigma Chemical Company, St Louis, Missouri, U.S.A.); antimycin A, oligomycin, carboxyatractyloside (Boehringer, Mannheim, Federal Republic of Germany).

## RESULTS

Fig. 1 shows the time course of ATP uptake at 2°C and demonstrates the shortness of the initial phase during which one can measure initial rates: this is not more than 2 s.

Fig. 2 illustrates the time resolution of our method leading to accurate slope determinations (initial rates) at different external-ADP concentrations with incubations of less than 1 s (0.26–0.85 s) and with six points for each determination of the initial rate.

Fig. 3 represents the double-reciprocal plots ( $v_0^{-1}$  versus  $[ADP]_{out}^{-1}$ ) at different internal-ADP concentrations (2.01–6.66 mM). The straight lines are certainly not parallel and seem to have a common point of intersection. If we assume the existence of such a common point, its coordinates may be calculated in order to minimize the sum of the squared deviations for the whole set of lines (see Appendix in [12]). The values obtained are: abscissa ( $-K_m^{-1}/\mu M^{-1}$ ) = -0.01, ordinate ( $V^{-1}/\text{pmol}^{-1} \cdot \text{s} \cdot \mu\text{l mito}$ ) =  $10^{-3} \times (3.67 \pm 0.66)$ . The probable common point is thus very near the axis of ordinates, if not on it.

Fig. 4 shows the double-reciprocal plots ( $v_0^{-1}$  versus  $[ADP]_{in}^{-1}$ ) at different external-ADP concentrations. Again the straight lines are not parallel and also appear to have a common point of intersection of which the coordinates may be calculated in the same way. The values obtained are: abscissa ( $-K_m^{-1}/\mu M^{-1}$ ) = 0.015, and ordinate ( $V^{-1}/\text{pmol}^{-1} \cdot \text{s} \cdot \mu\text{l mito}$ ) =  $10^{-3} \times (4.4 \pm 0.6)$  the last one being very close to the value obtained in  $v_0^{-1}$  versus  $[ADP]_{out}^{-1}$  (Fig. 3). The slightly positive value of  $-K_m^{-1}$  suggests that the common point is probably on the axis of ordinates. If we assume so, the ordinate of the common point becomes  $10^{-3} \times (3.64 \pm 0.63)$ . The maximal rate at infinite internal-substrate and external-substrate concentrations is then  $275 \text{ pmol} \cdot \text{s}^{-1} \cdot (\mu\text{l mito})^{-1}$  or  $1375 \text{ pmol} \cdot \text{s}^{-1} \cdot (\text{mg protein})^{-1}$ . As the total amount of adenine-nucleotide binding sites in rat-heart mitochondria is of the order of 1200 pmol/mg protein [13], the catalytic rate constant is  $1.15 \text{ s}^{-1}$ . In comparison, the maximal rate of the oxoglutarate carrier is 834 pmol



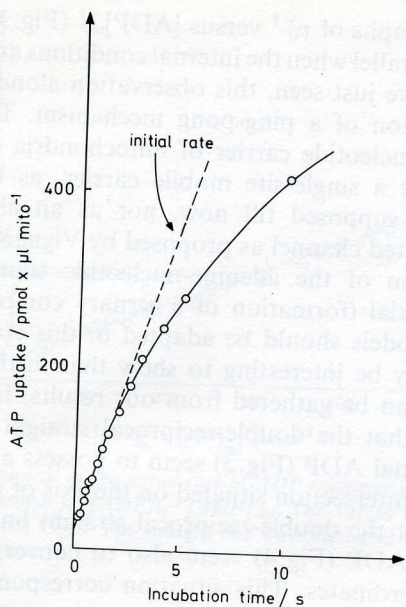


Fig. 1. Time course of the adenine-nucleotide translocation. ADP-loaded mitochondria (0.47 mg protein) contained 7.4 mM ADP and 0.89 mM ATP. The exchange reaction was initiated by addition of 10 μM <sup>14</sup>C-labelled ATP and stopped by addition of 10 μM carboxyatractyloside. The ATP uptake was not corrected for the sucrose-space contamination; it is expressed per μl of mitochondrial water-accessible space (mito)

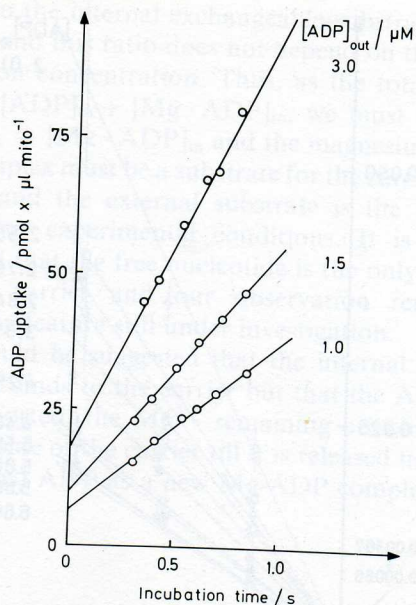
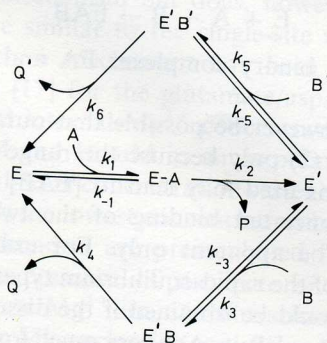


Fig. 2. Initial-rate determination of ADP uptake by ADP-loaded mitochondria. Mitochondria (0.47 mg protein) contained 5.5 mM ADP and 0.98 mM ATP. The incubation time represents the interval between injections of <sup>14</sup>C-labelled ADP and carboxyatractyloside. The ADP uptake was not corrected for sucrose-space contamination; it is expressed per μl of mitochondrial water-accessible space (mito). The initial rate is the slope of the straight line calculated from each set of six incubation times

$\cdot s^{-1} \cdot (\text{mg protein})^{-1}$ , the total binding to the active site is  $50 \text{ pmol} \cdot (\text{mg protein})^{-1}$  and the catalytic rate constant is  $16.7 \text{ s}^{-1}$ .

DISCUSSION

Taking into account the simultaneous presence of two internal substrates B (ADP) and B' (ATP), the reaction scheme of the ping-pong type and the corresponding kinetic equation are as follows [14]:



where E = translocator presenting its binding site to the outside, E' = translocator presenting its binding site to the inside, A = external (labelled) ADP, B = internal ADP, B' = internal ATP, P = internal transported (labelled) ADP, Q = external transported ADP, and Q' = external transported ATP.

This reaction scheme can be simplified (it does not consider the complexes of the translocator with the products of the translocation, i.e. EQ, EQ' and E'P) in order to lighten the kinetic equation:

$$\frac{E_0}{v_0} = \frac{k_3(k_2 + k_4)(k_{-5} + k_6) + k_5(k_2 + k_6)(k_{-3} + k_4)\beta}{k_2k_3k_4(k_{-5} + k_6) + k_2k_5k_6(k_{-3} + k_4)\beta} + \frac{k_{-1} + k_2}{k_1k_2[A]} + \frac{(k_{-3} + k_4)(k_{-5} + k_6)}{\{k_3k_4(k_{-5} + k_6) + k_5k_6(k_{-3} + k_4)\beta\} [B]} \quad (1)$$

where  $\beta = [B']/[B] = [\text{ATP}]_{\text{in}}/[\text{ADP}]_{\text{in}}$ ,  $v_0$  = the initial rate of external-ADP entrance into the mitochondria and  $E_0$  = the total concentration of the translocator.

This simplification does not modify the implications of the kinetic equation, i.e. for a ping-pong mechanism, the slope of the straight lines in the plot of  $v_0^{-1}$  versus  $[A]^{-1}$  does not depend on  $[B]$  nor on  $\beta$  and is exclusively characteristic of the external-substrate concentration  $[A]$ . The graph of  $v_0^{-1}$  versus  $[B]^{-1}$  will show parallel straight lines if the ratio between the internal-substrate concentrations ( $\beta$ ) remains constant. In our conditions  $\beta$  is rather low (0.11–0.25) so we have drawn the double-reciprocal plots using internal ADP concentrations (despite the fact that  $\beta$  is not exactly constant, its variation does not introduce a detectable dispersion into Fig. 4 and 5).



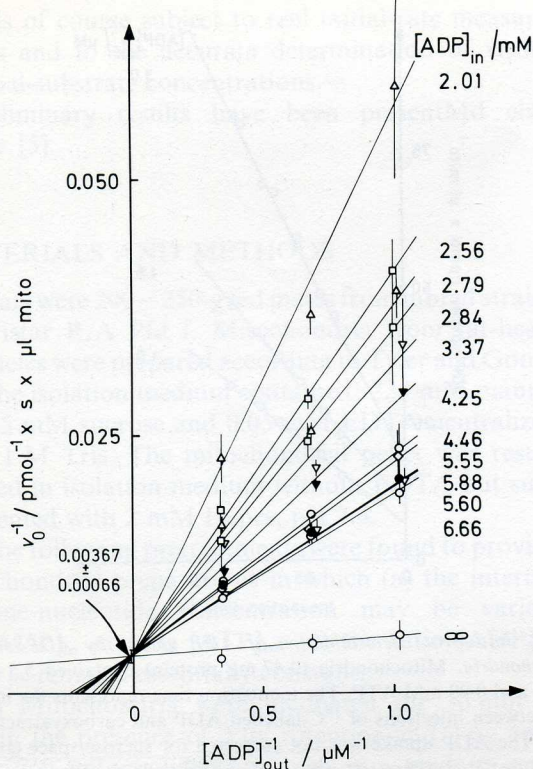


Fig. 3. Reciprocal of the initial rate of ADP exchange as a function of the reciprocal of the external-ADP concentration. ADP-loaded mitochondria (0.24–0.46 mg protein) were prepared to obtain an extended range of internal ADP concentrations, as described in Materials and Methods. The initial rates ( $\pm$  standard deviation) were measured for three external- $^{14}\text{C}$ ADP concentrations (0.97, 1.45 and 2.89  $\mu\text{M}$ ) at 11 internal-ADP concentrations and expressed per  $\mu\text{l}$  of water-accessible space in the mitochondrial pellet (mito). The initial rates for  $[\text{ADP}]_{\text{in}} = \infty$  were calculated by linear extrapolation of  $v_0^{-1}$  as a function of  $[\text{ADP}]_{\text{in}}^{-1}$  (Fig. 4). The straight lines were calculated by the method of least squares, imposing a common intersection point, unknown *a priori*, the coordinates of which were calculated in order to minimize the sum of the squared deviations for the whole set of lines

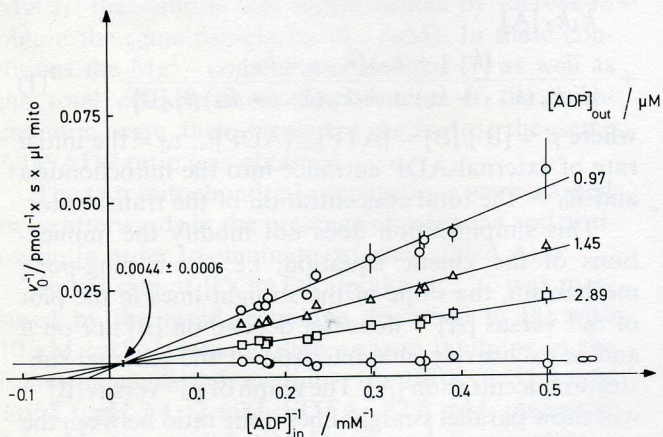


Fig. 4. Reciprocal of the initial rate of ADP exchange as a function of the reciprocal of the internal-ADP concentration. The initial rates of ADP uptake for three external-ADP concentrations at 11 internal-ADP concentrations are taken from Fig. 3; the initial rates for  $[\text{ADP}]_{\text{out}} = \infty$  were calculated by linear extrapolation of  $v_0^{-1}$  as a function of  $[\text{ADP}]_{\text{in}}^{-1}$ . The straight lines were calculated as in Fig. 3

The graphs of  $v_0^{-1}$  versus  $[\text{ADP}]_{\text{out}}^{-1}$  (Fig. 3) do not appear parallel when the internal conditions are varied. As we have just seen, this observation alone permits the rejection of a ping-pong mechanism. Thus, the adenine-nucleotide carrier of mitochondria does not behave as a single-site mobile carrier, as has been generally supposed till now, nor as an alternately loaded gated channel as proposed by Vignais [3]. The mechanism of the adenine-nucleotide translocation is sequential (formation of a ternary complex), and future models should be adapted to this conclusion.

It may be interesting to show that further information can be gathered from our results. Indeed we observe that the double-reciprocal straight lines for the external ADP (Fig. 3) seem to possess a common point of intersection situated on the axis of ordinates. Moreover the double-reciprocal straight lines for the internal ADP (Fig. 4) seem also to converge on the axis of ordinates. This situation corresponds to the following rate equation:

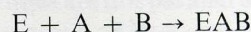
$$v = \frac{V \frac{[\text{A}][\text{B}]}{K_{\text{AB}}}}{1 + \frac{[\text{A}][\text{B}]}{K_{\text{AB}}}} \quad (2)$$

or

$$v_0^{-1} = \frac{K_{\text{AB}}}{V} \cdot \frac{1}{[\text{A}][\text{B}]} + \frac{1}{V} \quad (2')$$

the validity of which can be checked by drawing  $v_0^{-1}$  versus  $([\text{A}][\text{B}])^{-1}$ ; this must give a straight line as is satisfactorily observed in Fig. 5, where  $V^{-1} = 10^{-3} \times (3.64 \pm 0.55) \text{ pmol}^{-1} \cdot \text{s} \cdot \mu\text{l mito}$ , the slope =  $10^{-5} \times (9.66 \pm 0.23)$ ,  $K_{\text{AB}} = 10^{-3} \times (26.5 \pm 4.6) \text{ mM}^2$ ,  $r^2 = 0.98$ .

The mechanism related to Eqn (2) implies the formation of the active ternary complex EAB via a ternolecular reaction



in which the binary complexes EA and EB do not exist [15].

It may however be possible that our results conform to Eqn (2) only because the range of substrate concentrations used may lead to  $[\text{EAB}] \gg [\text{EA}]$  and  $[\text{EB}]$ ; the concerted binding of the two substrates would thus be apparent only. For example, if the kinetics are of the rapid-equilibrium type, an equation of type (2) could be obtained if the dissociation constant of  $\text{EAB} \rightarrow \text{EB} + \text{A}$  is very much lower than  $[\text{A}]$ , the latter being very much lower than the dissociation constant of  $\text{EA}$ , and if the dissociation constant of  $\text{EAB} \rightarrow \text{EA} + \text{B}$  is very much lower than  $[\text{B}]$ , the latter being very much lower than the dissociation constant of  $\text{EB}$ . If the kinetics are of the steady-state type, these conditions are no longer sufficient but are



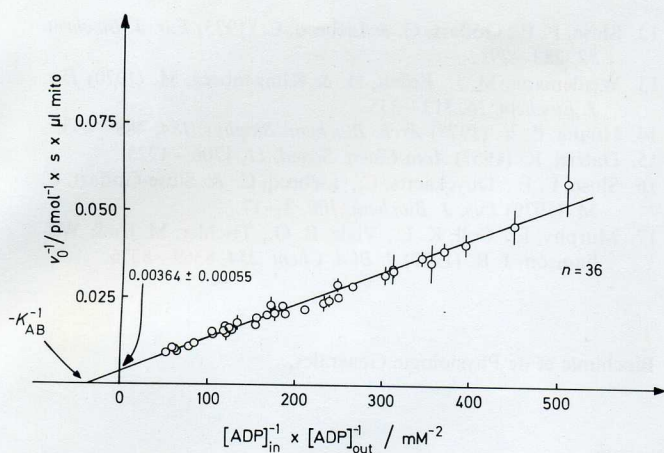


Fig. 5. Reciprocal of the initial rate of ADP exchange as a function of the reciprocal of  $[ADP]_{in} \cdot [ADP]_{out}$ . The values of  $v_0^{-1}$  were obtained from Fig. 3. The straight line was calculated by the least-squares method

still necessary. Thus, the existence of a substrate-concentration range in which Eqn (2) is verified, at least approximately, implies a strong positive cooperativity in the binding of the two substrates. This property of the translocator is different from that of the oxoglutarate translocator, to which the two substrates bind independently [8, 16].

We have demonstrated that the exchange  $ADP_{in}/ADP_{out}$  occurs via a ternary complex with the carrier. It is to be noted, however, that this exchange step may be distinct from the transport step of the substrates. Indeed a scheme such:



could, with suitable values of the rate constants, give a rate equation that approaches Eqn (2). This mechanism in which the binding of the substrate and the release of the products are sequential is thus not a ping-pong mechanism but does, however, lead to a model more similar to the single-site mobile-carrier model. Such a mechanism has been proposed by Williamson [17] for the glutamate/aspartate carrier.

The total internal- $Mg^{2+}$  concentration varies between 8 mM and 16 mM in our experiments and the total internal-ADP concentration varies between 2 mM and 6.2 mM, the lowest concentration being obtained when mitochondria are preincubated for 10 min at 25 °C in the presence of succinate and orthophosphate. At these  $Mg^{2+}$ -ion concentrations the concentration of the Mg-nucleotide complexes largely exceed the concentration of the free nucleotides. In Fig. 5 the  $K_{AB}$  value is obtained by extrapolation of data that are calculated on total  $[ADP]_{in} \times [ADP]_{out}$ , without taking into account the fact that variable parts of the  $ADP_{in}$  are complexed to  $Mg^{2+}$  ions. The linear relationship indicates that the total  $[ADP]_{in}$  is propor-

tional to the internal exchangeable-substrate concentration and this ratio does not depend on the internal  $Mg^{2+}$ -ion concentration. Thus, as the total  $[ADP]_{in} = \text{free } [ADP]_{in} + [Mg \cdot ADP]_{in}$ , we must have total  $[ADP]_{in} \approx [Mg \cdot ADP]_{in}$  and the magnesium-nucleotide complex must be a substrate for the carrier. On the other hand the external substrate is the free ADP under our experimental conditions. It is generally accepted that the free nucleotide is the only substrate for the carrier and our observation remains an intriguing feature still under investigation.

It could be suggested that the internal Mg-ADP complex binds to the carrier but that the ADP alone is transported, the  $Mg^{2+}$  remaining attached to the internal side of the carrier till it is released inside with the entered ADP as a new Mg-ADP complex.

## CONCLUSIONS

Our results completely disagree with the ping-pong mechanism which has so far been assumed for this translocator: instead of the expected kinetics we have found kinetics in which a ternary complex is the only significant one, at least in the substrate-concentration range used. Two models are possible in the light of our results: (a) one in which the carrier possesses an internal and an external site with a strong positive cooperativity, the ternary complex performing the exchange and the transport of the substrates in a single step ( $EAB \rightarrow EPQ$ ); (b) a second possibility is that the carrier does not necessarily span the membrane, is mobile and transports the substrates one by one, the formation of a ternary complex being necessary, however, for the release of the first product.

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