Eur. J. Biochem. 25 (1972) 207—217

Mechanism of the Exchanges Catalysed by the Oxoglutarate Translocator of Rat-Heart Mitochondria

Kinetics of the Exchange Reactions between 2-Oxoglutarate, Malate and Malonate

Francis E. Sluse, Monique Ranson, and Claude Liébecq

Laboratoire de Biochimie et de Physiologie Générale, Institut Supérieur d'Education Physique de l'Université de Liège

(Received October 19/November 15, 1971)

The kinetics of the exchange reactions between 2-oxoglutarate, malate and malonate have been measured at 4 °C in preparations of rat-heart mitochondria under conditions where the oxoglutarate translocator is operating exclusively. This was possible since no activity of the tricarboxylate translocator can be measured in such preparations and since the activities of the phosphate and dicarboxylate translocators may be blocked by mersalyl, to which the oxoglutarate translocator is insensitive. The metabolism of the substrates was prevented by addition of rotenone and arsenite.

Measurements were made at three different external and three different internal concentrations of the substrates. The results show that the affinity of the oxoglutarate translocator is very much higher for external substrates than for the corresponding internal substrates. The Michaelis constants increase in the following order: external oxoglutarate < external malate < external malonate < internal oxoglutarate < internal malate < internal malonate. The results also show that the affinity of the translocator for a given substrate, either external or internal, is independent of the nature of the counter-ion. This is not true of the maximal velocities of the exchange reactions.

Of the various mechanism described by Cleland for two-substrate-two-product enzyme-catalysed reactions, only the so-called mechanism of rapid-equilibrium random bi-bi is compatible with our experimental results. This implies that the Michaelis constants determined are the dissociation constants of the various translocator-substrate(s) complexes.

The mitochondrial inner membrane contains a battery of translocators catalysing exchange reactions between anions located in the cytosol and those located in the matrix space of the mitochondria. Exchange reactions have thus been demonstrated for the adenine nucleotides and for most of the intermediates of the tricarboxylic acid cycle (see, for instance [1—4]).

The translocations follow a one-to-one stoichiometry [5—7]. The translocators show a definite selectivity for the substrates exchanged and a specific sensitivity to inhibitors (for a review, see [3]). The mechanism of the exchange translocation is completely unknown.

The physiological rôle of these translocation is obvious; they may control the rate of metabolic sequences catalysed by enzymes located in different cell compartments. A precise knowledge of the kinetics of such exchange reactions is therefore desirable.

Except in the case of adenine nucleotides, available data [8—11] are unfortunately of limited value. In the experiments where the penetration of labelled substrates has been measured [8, 9, 11] no information is available on the nature or concentration of the intramitochondrial counter-ions. In the experiments where the efflux of labelled substrate has been measured in exchange for known external counter-ions [10], no evidence has been presented that the labelled internal anion is the only extruded substrate. All these experiments have been carried out under conditions where the concentration of the external anion only has been varied.

The participation of more than one anion translocator in these reactions does not allow conclusions on the kinetic parameters of a single translocation; the parameters obtained under such conditions are necessarily composite.

The oxoglutarate translocator of rat-liver mitochondria is in fact a strict dicarboxylate translocator that catalyses exchange reactions between various dicarboxylates, such as 2-oxoglutarate, malate, malonate and succinate but does not exchange these anions for phosphate ions [12—15].

Enzymes. Malate dehydrogenase, or L-malate : NAD oxidoreductase (EC 1.1.1.37); glutamate dehydrogenase (NAD(P)), or L-glutamate : NAD(P) oxidoreductase (deaminating) (EC 1.4.1.3).

The presence of the oxoglutarate translocator has also been described in heart-muscle mitochondria by Sluse *et al.* [16] and it has been shown that its activity can be measured specifically since the activity of the tricarboxylate translocator is not measurable in these preparations and since the activity of the phosphate and dicarboxylate translocators may be blocked by mersalyl, to which the oxoglutarate translocator is insensitive.

The present paper describes the kinetics of the exchange of various dicarboxylates by the oxoglutarate translocator only, under different and known internal and external anion concentrations. The kinetic parameters have been calculated assuming a mechanism analogous to a two-substrate-two-product enzyme-catalysed reaction. A possible mechanism for the observed translocation is proposed. Preliminary accounts of the present work have been presented [17—19].

EXPERIMENTAL PROCEDURE

Materials

Special reagents were obtained from the following sources: [2-^{14}C]malonic acid, 2-oxo[5-^{14}C]glutaric acid, L-[U-^{14}C]malic acid, [U-^{14}C]sucrose and tritiated water (The Radiochemical Center, Amersham, England); rotenone (Sigma Chemical Company, Saint-Louis, Missouri, U.S.A.); mersalyl, acid form (Mann Research Laboratories, New York, U.S.A.); L-malic acid and 2-oxoglutaric acid, sodium salts (K & K Laboratories, Plainview, New York, U.S.A.); malonic acid (Merck, Darmstadt, German Federal Republic); Nagarse (Teikoku Chemical Industry, Osaka, Japan).

Preloading of the Mitochondria

Rat-heart mitochondria were prepared according to Tyler and Gonze [20] up to the third centrifugation (10 min at 8000 × *g*). They were then loaded with either malonate, 2-oxoglutarate or malate. This was achieved by incubating the whole mitochondrial suspension with the dicarboxylate anions (5-mM malate, 10-mM malonate or 1-mM oxoglutarate) in a total volume of 20 ml of a solution containing 225-mM mannitol, 75-mM sucrose, 0.05-mM EDTA and 20-mM Tris-HCl (pH 7.4) at 0 °C for 20 min. The mitochondria were centrifuged for 10 min at 8000 × *g*, resuspended in the same solution without Tris-HCl and divided in three parts. The accumulated anions were allowed to leak out of the mitochondria during successive washings in large volumes of the same mannitol-sucrose-EDTA solution at 4 °C (followed by centrifugation), the successive number of which (1, 2 or 3) lead to preparations with different concentrations of internal anions.

Proteins were determined by the biuret method, as described by von Beisenherz *et al.* [21] using serum albumin (Bovine Fraction V) as a standard.

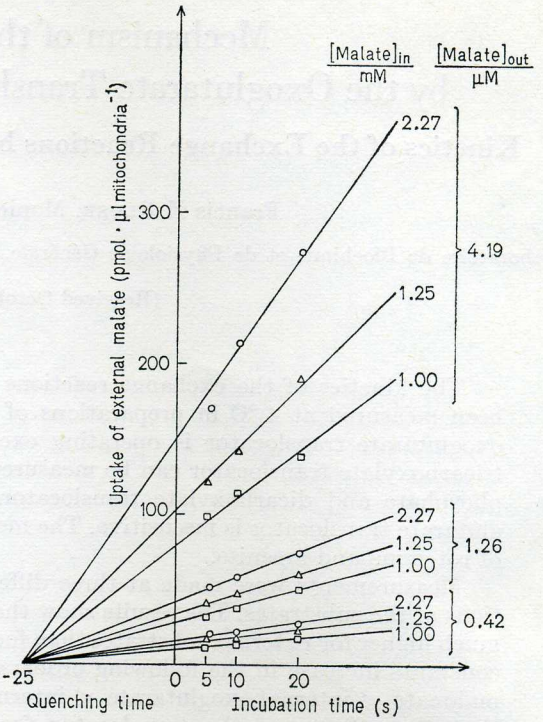


Fig. 1. *Time-course of* L-*[U-*^{14}*C]malate/*L-*malate exchange.* The quenching time corresponds to the mean time required to stop the exchange between mitochondria and external medium by centrifugation after the incubation period. It corresponds to the resolution time described by Klingenberg [3]; its determination needs careful correction for labelled material in the sucrose space (*i.e.* adherent external space + intermembrane space). Thus, subsequent exchange between sucrose space and matrix space in the mitochondrial pellet is immaterial since it is accounted for when the uptakes are corrected for the labelled malate (this experiment) in the sucrose space. The fixed quenching time of 25 s applicable to our experimental conditions was determined separately in experiments of longer duration in which the influx of labelled malonate was followed as a function of time (up to 4 min). It is the obligatory origin of all the straight lines calculated by the method of least square

Experimental Assays

The incubation medium was that used in other laboratories for permeability studies with rat-liver mitochondria [12, 13]. It contained 15-mM KCl, 5-mM $MgCl_2$, 2-mM EDTA, 50-mM Tris-Cl (pH 7.4), 22.5-mM mannitol and 7.5-mM sucrose (both derived from the stock mitochondrial suspension). No swelling is observed in this medium [16] and the sucrose space represents 60% of the mitochondrial pellet.

In a typical experiment (see Fig. 1) the mitochondria (final concentration = 1 mg protein/ml) preloaded with malate ions as indicated above were incubated for 1 min at 4 °C in 1 ml of the incubation medium supplemented with 3.3 µCi tritiated water, 4 µg rotenone, 1 µmol sodium arsenite and 0.2 µmol mersalyl. Labelled malate was then added to the sus-

pension and the mitochondria were further incubated at 4 °C for 5-, 10- or 20-s periods and finally separated from the incubation mixture by rapid centrifugation in an Eppendorf microcentrifuge (Model 3200) for 1 min. The supernatant was decanted and immediately acidified with $HClO_4$ (final concentration = 0.7 M). The mitochondrial pellet containing the trapped labelled malate was rapidly acidified with 0.7-M $HClO_4$. Further experimental details are given in the legend of the figures.

The radioactivities of the extracts of the mitochondrial pellets and of the supernatant solutions were determined in a Packard liquid scintillation counter with Insta-Gel (Packard) as scintillation medium. The total water in the pellets was calculated from the content in tritiated water. The sucrose-permeable space plus adherent medium were determined in parallel assays. The radioactivity of their labelled substrate was subtracted from the radioactivity of the total pellet to calculate the amount of labelled substrate in the matrix space of the mitochondria.

The exchanges were measured under nine experimental conditions (at three different external and three different internal concentrations of malate). Their initial velocities were determined from the slope of the straight lines of Fig. 1 and expressed in pmol/s per µl mitochondria.

The following exchange reactions were measured: (a) external 2-oxo[5-^{14}C]glutarate for internal oxoglutarate, malate or malonate, (b) external L-[U-^{14}C]-malate for internal malate, malonate or oxoglutarate, (c) external [2-^{14}C]malonate for internal malonate, oxoglutarate or malate.

Internal-Anion Concentrations. Internal malate and oxoglutarate were determined enzymatically with malate and glutamate dehydrogenases. The concentration of internal malonate was determined by allowing it to exchange for traces of external labelled malonate (at low concentration and high specific activity) until isotopic equilibrium is reached.

Justification

The initial velocities of such exchange reactions may only be determined if the concentrations of the substrates remain reasonably stable during the experimental assays. It is therefore essential that (a) the internal anions are not metabolised and do not leak out of the mitochondria, (b) the incubation medium is not contaminated by internal anions at the onset of the experiments, and (c) the magnitude of the exchange is kept low.

No oxygen uptake was measured when mitochondria were incubated at 4 °C in the presence of rotenone, arsenite and any of the tested anions. Labelled malonate, accumulated in mitochondria during a preloading period, did not leak out during a subsequent incubation in a substrate-free medium. The amount of mitochondria was such that the concentrations of both exchanged anions did not change by more than 15%; no use was made of those experiments in which such conditions were not realised.

Contamination of the external space by the anions used in the preloading part of the experiment is negligible as the result of dilution. Even in the case of experiments making use of mitochondrial preparations having been washed once only, the dilution is of the order of 200000; this figure may be obtained by calculation and was confirmed in control experiments in which the dilution of tritiated water was measured. Thus the concentration of the "internal" anions in the external space was 0.05—5% of the concentration of the external anion. This is still reduced when use is made of mitochondrial preparations washed more than once.

The external-substrate concentrations selected in the experiments reported here were of the order of the Michaelis constants for the external substrates. The internal-substrate concentrations were also of the order of the Michaelis constants for the internal substrates as a result of adequate preloading conditions.

As indicated in the Introduction, the only active translocator of our preparations is the oxoglutarate translocator.

We have assumed that our preloading experimental conditions remove from the mitochondria other possible counter-ions exchangeable for the added labelled external anions. We have also assumed that the different number of washings (from 1 to 3) do not lead to mitochondria with different properties, as evidenced by the constant sucrose space fo our preparations, and by their normal coupling properties tested in separate experiments.

Data Processing

The exchange were treated as two-substrate-two-product reactions in which the products are identical with the substrates but located in the other phase [Eqn (1)]. No account was taken for the possible participation of protons in the exchange (see [22]).

$$A_{in} + B_{out} \rightarrow A_{out} + B_{in} \qquad (1)$$

Double-reciprocal plots of initial velocities *versus* substrate concentrations were analysed according to Florini and Vestling [23] and provided straight lines that fitted Eqn (2):

$$1/v = \left(1 + \frac{K_a}{[A]} + \frac{K_b}{[B]} + \frac{K_{ab}}{[A]\,[B]}\right) \Big/ V \qquad (2)$$

where: v = initial velocity; V = maximum velocity (velocity for infinite concentration of A and B); K_a = Michaelis constant for A ($v = V/2$ if $[A] = K_a$ and $[B] = \infty$); K_b = Michaelis constant for B ($v = V/2$ if $[B] = K_b$ and $[A] = \infty$), K_{ab} = a constant.

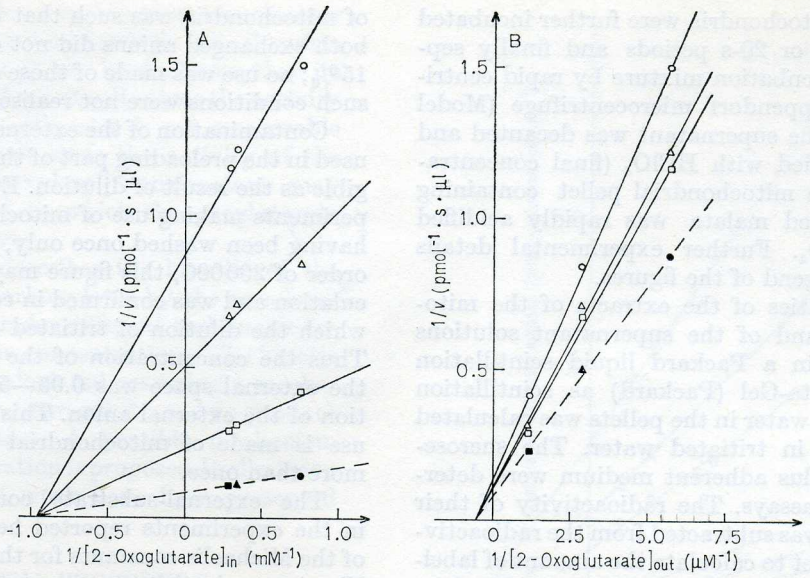


Fig. 2 *Kinetics of 2-oxo[5-^{14}C]glutarate influx in exchange for internal 2-oxoglutarate.* Heart mitochondria (final concentration = 0.68 mg protein/ml). (A) $1/v$ against 1/[internal substrate]; external oxo[5-^{14}C]glutarate = 0.17 µM (○); 0.35 µM (△) or 0.88 µM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_b$ (apparent-maximum velocities) determined in Fig. 2B and give $1/V$ by extrapolation (----). (B) $1/v$ against 1/[external substrate]; internal 2-oxoglutarate = 1.33 mM (○), 2.98 mM (△) or 3.52 mM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_a$ (apparent-maximum velocities) determined in Fig. 2A and give $1/V$ by extrapolation (----). v is expressed in pmol/s per µl mitochondria

At non-saturating concentrations of A, the apparent-maximum velocity V_b is given by Eqn (3a):

$$V_b = V\Big/\left(1 + \frac{K_a}{[A]}\right) \qquad (3a)$$

and at non-saturating concentrations of B, the apparent-maximum velocity V_a is given by Eqn (3b):

$$V_a = V\Big/\left(1 + \frac{K_b}{[B]}\right). \qquad (3b)$$

If we define K_a' and K_b' by Eqn (4a) and (4b),

$$K_a' = K_{ab}/K_b \qquad (4a)$$

and

$$K_b' = K_{ab}/K_a \qquad (4b)$$

then the apparent Michaelis constants for A (at non-saturating concentrations of B) and for B (at non-saturating concentrations of A) are given by Eqn (5a) and (5b):

$$K_a\,(\text{app.}) = K_a \cdot \frac{1 + (K_b'/[B])}{1 + (K_b/[B])} \qquad (5a)$$

and

$$K_b\,(\text{app.}) = K_b \cdot \frac{1 + (K_a'/[A])}{1 + (K_a/[A])}. \qquad (5b)$$

To these apparent Michaelis constants correspond apparent-half-maximum velocities.

When $1/v$ is plotted against 1/[A], a straight line is obtained for each of the three fixed concentrations of B. Extrapolation to the ordinate gives three intercepts equal to the reciprocal of the three apparent-maximum velocities V_a; extrapolation to the abscissa gives intercepts equal to $-1/K_a$ (app.). Moreover, the three lines must have a common intersect at a value on the abscissa equal to $-1/K_a'$.

If the three values of $1/V_a$ are plotted against 1/[B], another straight line (dashed in our figures) is obtained according to Eqn (3b), that intercepts the ordinate at $1/V$, and the abscissa at $-1/K_b$.

The same applies to plots of $1/v$ against 1/[B] and provides values of V_b, K_b (app.), V and K_a.

RESULTS

The kinetics of the following exchange reactions are presented in a series of double-reciprocal plots in which the points corresponding to the nine experimental conditions are arranged as a function of the internal-anion concentration (Part A) or as a function of the external-anion concentration (Part B).

Fig. 2: external 2-oxo[5-^{14}C]glutarate, internal oxoglutarate; Fig. 3: external L-[U-^{14}C]malate, internal oxoglutarate; Fig. 4: external [2-^{14}C]malonate, internal oxoglutarate; Fig. 5: external L-[U-^{14}C]-malate, internal malate; Fig. 6: external 2-oxo[5-^{14}C]-glutarate, internal malate; Fig. 7: external [2-^{14}C]-malonate, internal malate, Fig. 8: external [2-^{14}C]-malonate, internal malonate; Fig. 9: external 2-oxo-

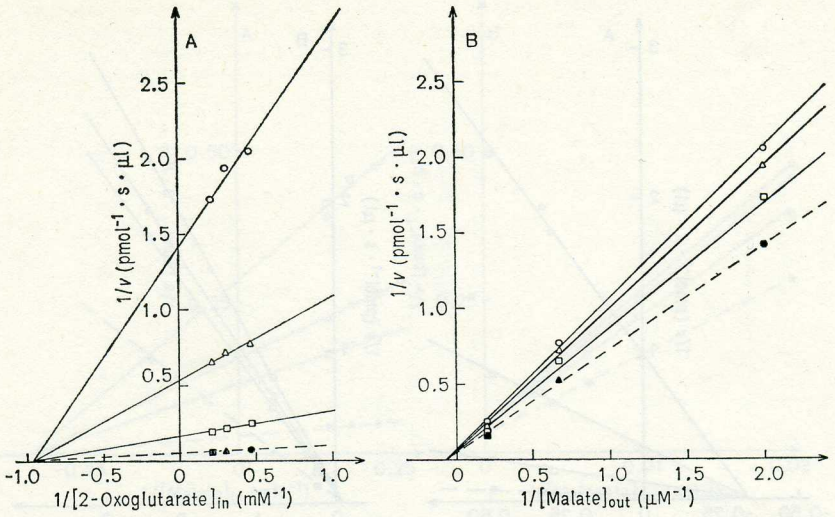


Fig. 3. *Kinetics of L-[U-^{14}C]malate influx in exchange for internal 2-oxoglutarate.* Heart mitochondria (final concentration = 0.88 mg protein/ml). (A) $1/v$ against 1[/internal substrate]; external L-[U-^{14}C]malate = 0.5 µM (○), 1.5 µM (△) or 5 µM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_b$ (apparent-maximum velocities) determined in Fig. 3B and give $1/V$ by extrapolation (----). (B) $1/v$ against 1/[external substrate]; internal 2-oxoglutarate = 2.15 mM (○), 3.28 mM (△) or 4.72 mM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_a$ (apparent-maximum velocities) determined in Fig. 3A and give $1/V$ by extrapolation (----). v is expressed in pmol/s per µl mitochondria

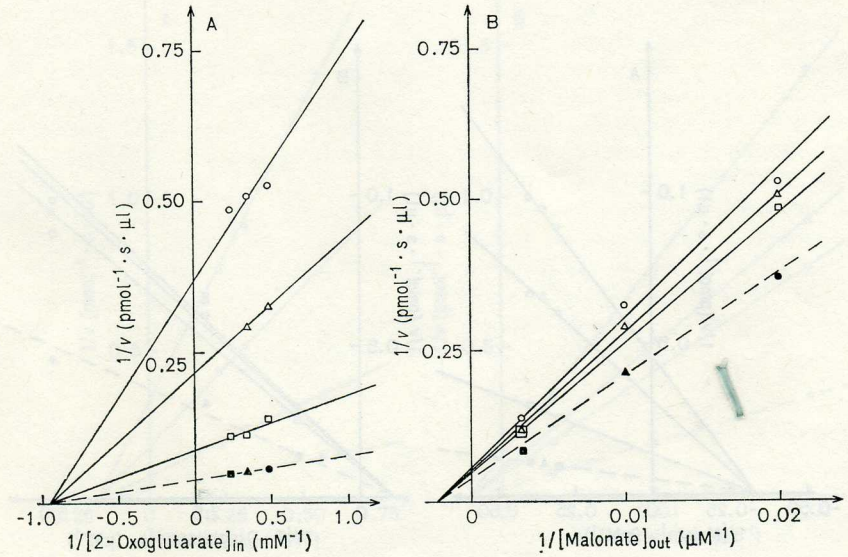


Fig. 4. *Kinetics of [2-^{14}C]malonate influx in exchange for internal 2-oxoglutarate.* Heart mitochondria (final concentration = 1.01 mg protein/ml). (A) $1/v$ against 1/[internal substrate]; external [2-^{14}C]malonate = 50 µM (○), 100 µM (△) or 300 µM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_b$ (apparent-maximum velocities) determined in Fig. 4B and give $1/V$ by extrapolation (----). (B) $1/v$ against 1/[external substrate]; internal 2-oxoglutarate = 2.07 mM (○), 2.93 mM (△) or 4.38 mM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_a$ (apparent-maximum velocities) determined in Fig. 4A and give $1/V$ by extrapolation (----). v is expressed in pmol/s per µl mitochondria

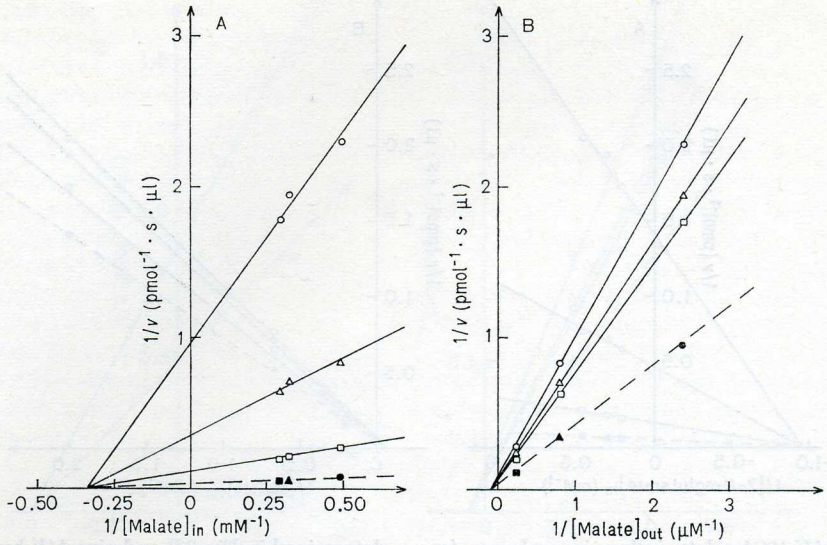


Fig. 5. *Kinetics of L-[U-^{14}C]malate influx in exchange for internal L-malate.* Heart mitochondria (final concentration = 1.05 mg protein/ml). (A) $1/v$ against 1/[internal substrate]; external L-[U-^{14}C]malate = 0.42 µM (○), 1.26 µM (△) or 4.19 µM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_b$ (apparent-maximum velocities) determined in Fig. 5B and give $1/V$ by extrapolation (----). (B) $1/v$ against 1/[external substrate]; internal L-malate = 2.03 mM (○), 3.13 mM (△) or 3.44 mM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_a$ (apparent-maximum velocities) determined in Fig. 5A and give $1/V$ by extrapolation (----). v is expressed in pmol/s per µl mitochondria

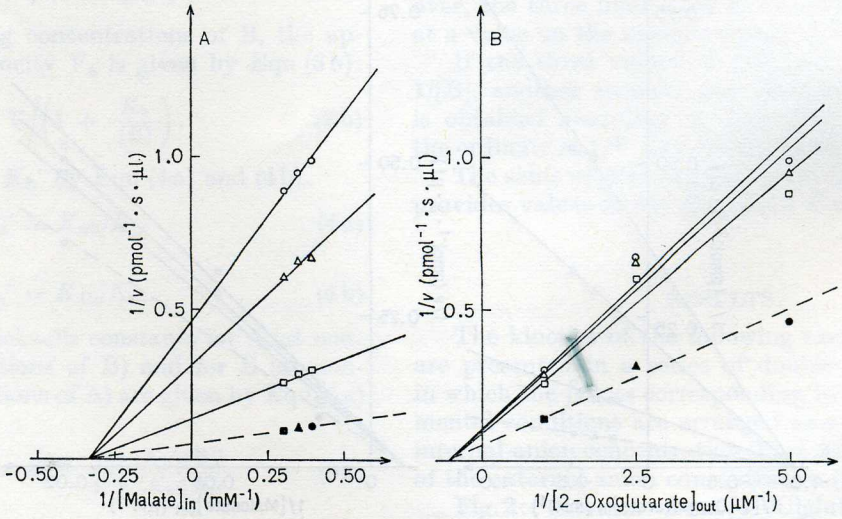


Fig. 6. *Kinetics of 2-oxo[5-^{14}C]glutarate influx in exchange for internal L-malate.* Heart mitochondria (final concentration = 0.80 mg protein/ml). (A) $1/v$ against 1/[internal substrate]; external 2-oxo[5-^{14}C]glutarate = 0.2 µM (○), 0.4 µM (△) or 1 µM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_b$ (apparent-maximum velocities) determined in Fig. 6B and give $1/V$ by extrapolation (----). (B) $1/v$ against 1/[external substrate]; internal L-malate = 2.54 mM (○), 2.86 mM (△) or 3.24 mM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_a$ (apparent-maximum velocities) determined in Fig. 6A and give $1/V$ by extrapolation (----). v is expressed in pmol/s per µl mitochondria

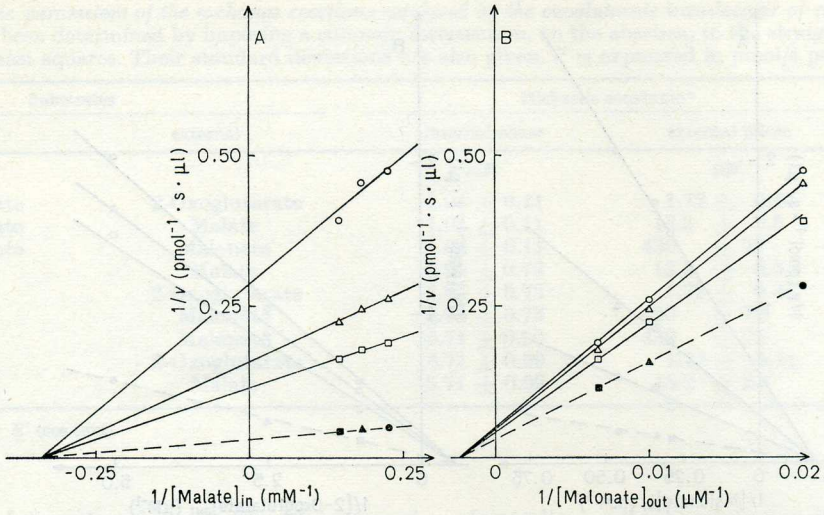


Fig. 7. *Kinetics of [2-^{14}C]malonate influx in exchange for internal L-malate.* Heart mitochondria (final concentration = 2.14 mg protein/ml). (A) $1/v$ against 1/[internal substrate]; external [2-^{14}C]malonate = 50 µM (○), 100 µM (△) or 150 µM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_b$ (apparent-maximum velocities) determined in Fig. 7B and give $1/V$ by extrapolation (----). (B) $1/v$ against 1/[external substrate]; internal L-malate = 4.43 mM (○), 5.49 mM (△) or 6.84 mM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_a$ (apparent-maximum velocities) determined in Fig. 7A and give $1/V$ by extrapolation (----). v is expressed in pmol/s per µl mitochondria

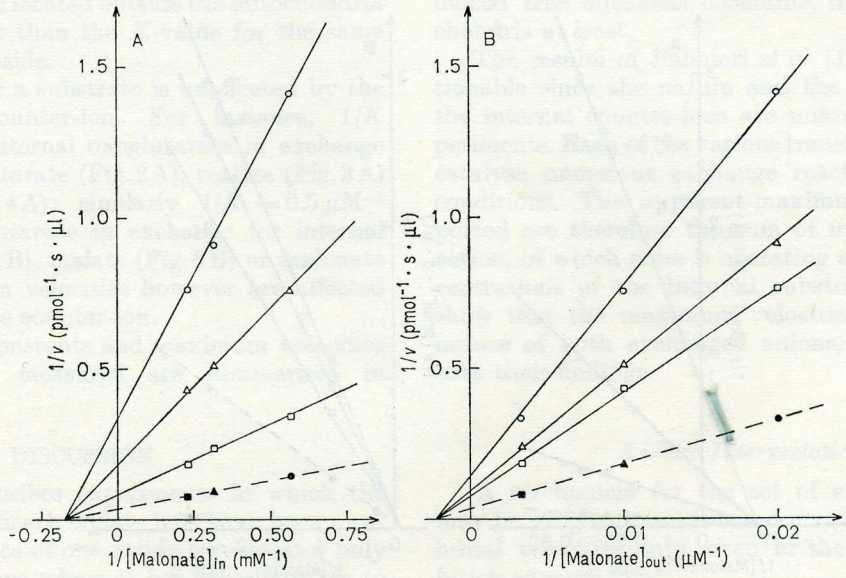


Fig. 8. *Kinetics of [2-^{14}C]malonate influx in exchange for internal malonate.* Heart mitochondria (final concentration = 1.5 mg protein/ml). (A) $1/v$ against 1/[internal substrate]; external [2-^{14}C]malonate = 50 µM (○), 100 µM (△) or 300 µM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_b$ (apparent-maximum velocities) determined in Fig. 8B and give $1/V$ by extrapolation (----). (B) $1/v$ against 1/[external substrate]; internal malonate = 1.78 mM (○), 3.17 mM (△) or 4.39 mM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_a$ (apparent-maximum velocities) determined in Fig. 8A and give $1/V$ by extrapolation (----). v is expressed in pmol/s per µl mitochondria

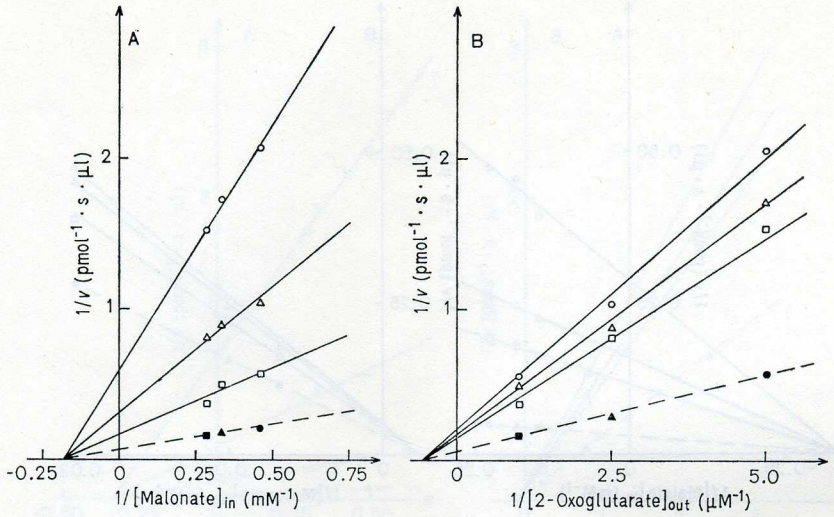


Fig. 9. *Kinetics of 2-oxo[5-^{14}C]glutarate influx in exchange for internal malonate.* Heart mitochondria (final concentration = 0.58 mg protein/ml). (A) $1/v$ against 1/[internal substrate]; external 2-oxo[5-^{14}C]glutarate = 0.2 µM (○), 0.4 µM (△) or 1 µM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_b$ (apparent-maximum velocities) determined in Fig. 9B and give $1/V$ by extrapolation (----). (B) $1/v$ against 1/[external substrate]; internal malonate = 2.17 mM (○), 2.98 mM (△) or 3.51 mM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_a$ (apparent-maximum velocities) determined in Fig. 9A and give $1/V$ by extrapolation (----). v is expressed in pmol/s per µl mitochondria

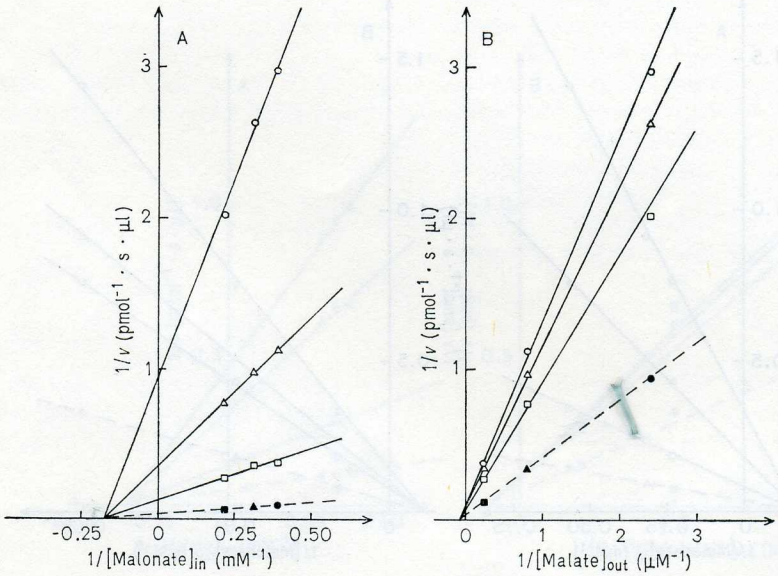


Fig. 10. *Kinetics of L-[U-^{14}C]malate influx in exchange for internal malonate.* Heart mitochondria (final concentration = 0.88 mg protein/ml). (A) $1/v$ against 1/[internal substrate]; external L-[U-^{14}C]malate = 0.42 µM (○), 1.26 µM (△) or 4.19 µM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_b$ (apparent-maximum velocities) determined in Fig. 10B and give $1/V$ by extrapolation (----). (B) $1/v$ against 1/[external substrate]; internal malonate = 2.56 mM (○), 3.30 mM (△) or 4.65 mM (□); the filled symbols (●, ▲, ■) correspond to the three values of $1/V_a$ (apparent-maximum velocities) determined in Fig. 10A and give $1/V$ by extrapolation (----). v is expressed in pmol/s per µl mitochondria

Table 1. *Kinetic parameters of the exchange reactions catalysed by the oxoglutarate translocator of rat-heart mitochondria*
The K-values have been determined by imposing a common intersection, on the abscissa, to the straight lines calculated by the method of least squares. Their standard deviations are also given. V is expressed in pmol/s per µl mitochondria

Substrates		Michaelis constants[a]		V at 4 °C
internal	external	internal phase	external phase	
		mM	µM	pmol × s^{-1} × µl^{-1}
2-Oxoglutarate	2-Oxoglutarate	1.04 ± 0.11	1.72 ± 0.44	12.2
2-Oxoglutarate	Malate	1.04 ± 0.11	13.2 ± 2.6	19.1
2-Oxoglutarate	Malonate	1.04 ± 0.11	430 ± 77	25.6
Malate	Malate	2.94 ± 0.73	13.2 ± 2.6	33.6
Malate	2-Oxoglutarate	2.94 ± 0.73	1.72 ± 0.44	19.4
Malate	Malonate	2.94 ± 0.73	430 ± 77	33.5
Malonate	Malonate	5.71 ± 0.99	430 ± 77	29.0
Malonate	2-Oxoglutarate	5.71 ± 0.99	1.72 ± 0.44	16.8
Malonate	Malate	5.71 ± 0.99	13.2 ± 2.6	34.5

[a] K (app.) = K = K' (see text).

[5-^{14}C]glutarate, internal malonate; Fig. 10: external L-[U-^{14}C]malate, internal malonate.

The figures show linear relationships between $1/v$ and $1/[A]$ or $1/[B]$, from which values for the apparent-maximum velocities, V_a or V_b, may be calculated; the maximum velocities determined in the A or B parts of the figures are equal. It appears therefore that the exchange reactions follow the law expressed by Eqn (2).

In each half-figure the four straight lines intercept the abscissa at the same point. Therefore K (app.) $= K = K'$ in all cases.

The experimental results also show that the K-value for a substrate located outside the mitochondria is very much lower than the K-value for the same substrate located inside.

The K-value for a substrate is unaffected by the nature of the counter-ion. For instance, $1/K \approx 0.95$ mM^{-1} for internal oxoglutarate in exchange for external oxoglutarate (Fig. 2A), malate (Fig. 3A) or malonate (Fig. 4A); similarly $1/K \approx 0.5$ µM^{-1} for external oxoglutarate in exchange for internal oxoglutarate (Fig. 2B), malate (Fig. 6B) or malonate (Fig. 9B). Maximum velocities however are affected by the nature of the counter-ion.

The Michaelis constants and maximum velocities of the exchanges measured are summarised in Table 1.

DISCUSSION

This paper describes experiments in which the exchange of three dicarboxylate ions have been measured in the presence of one active translocator only and under conditions where it has been possible to vary both internal and external concentrations of the substrates. Our results differ from some already published data drawn from experiments where such essential conditions have not been realised.

Robinson and Williams [10] for instance claim that the binding of an external dicarboxylate ion, in rat-liver mitochondria, is a function of the identity of both external and internal anions. This is not in accordance with our observations made with rat-heart mitochondria.

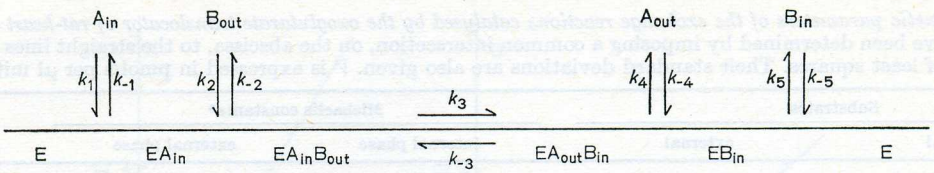
Their experiments have been made under conditions where more than one translocator may operate: it is therefore not surprising that the binding affinities in the effluxes measured be affected differently by counter-ions that activate one, or more than one, translocator in their preparations. The fact that these authors have worked at only one concentration of the internal anion is probably not misleading in this respect since the apparent Michaelis constants are indeed true Michaelis constants, in rat-heart mitochondria at least.

The results of Palmieri *et al.* [11] are also questionable since the nature and the concentration of the internal counter-ions are unknown in their experiments. Each of the various translocators may thus catalyse numerous exchange reactions under such conditions. The apparent-maximum velocities reported are therefore the sum of more than one reaction, of which none is operating at saturating concentrations of the internal substrates. Our results show that the maximum velocities depend on the nature of both exchanged anions, in contradiction with their findings.

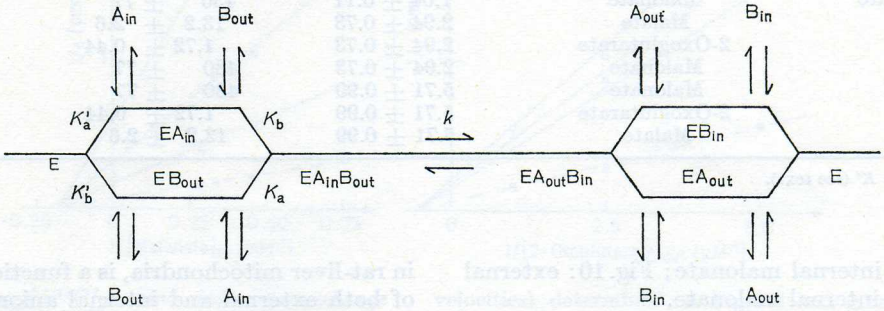
Kinetic Interpretation

A mechanism for the set of exchanges studied may be proposed and is based on an analysis of their initial velocities only, even in the absence of inhibition experiments.

Of the various mechanisms for two-substrate reactions described in the extensive studies of Cleland [24—26], only three provide kinetics compatible with Eqn (2) that our results fit. They will be briefly discussed in the assumption that they may be applied to transport reactions catalysed by translocators.



Scheme 1



Scheme 2

In one of them, called "steady-state ping-pong bi-bi", the substrate on one side of the membrane binds to the translocator and is transported on the other side where it dissociates; the other substrate then binds to the translocator and is transported in the other direction to be finally released. Such a mechanism may be excluded because K_{ab} would have to be equal to 0 [24], which is not the case.

In another mechanism, called "steady-state ordered bi-bi", one of the substrates binds to the translocator before the other and, after translocation, is released first also. This is illustrated in Scheme 1 where E is the translocator.

As shown by Frieden [27], the following equations (in which $E_t \triangleq$ total translocator) apply to such a mechanism:

$$V = [E_t] \Big/ \left(\frac{1}{k_3} + \frac{1}{k_4} + \frac{1}{k_5} + \frac{k_{-3}}{k_3 k_4} \right) \quad (6)$$

$$K_a = V/[E_t]\, k_1 \quad (7)$$

$$K_a' = k_{-1}/k_1 \quad (8)$$

and

$$K_b = \frac{V}{[E_t]\, k_2} \left(1 + \frac{k_{-2}}{k_3} + \frac{k_{-2}\, k_{-3}}{k_3\, k_4} \right). \quad (9)$$

For K_a to be equal to K_a', as observed in our experiments, k_{-1} would have to be equal to:

$$k_{-1} = 1 \Big/ \left(\frac{1}{k_3} + \frac{1}{k_4} + \frac{1}{k_5} + \frac{k_{-3}}{k_3 k_4} \right). \quad (10)$$

This could only be the result of a coïncidence repeated in all exchange reactions studied.

K_b would depend upon the nature of A since the constants k_3, k_{-3} and k_4, appearing in Eqn (9), represent velocity constants of reactions involving A or EAB. K_a would also depend upon the nature of B since the constants k_3, k_{-3}, k_4 and k_5 which appear in the V term of Eqn (7) represent velocity constants of reactions involving B or EAB. This is not the case in our experiments.

It is therefore unlikely that the mechanism of steady-state ordered bi-bi explains our results.

The third possibility is the mechanism called "rapid equilibrium random bi-bi" [24] illustrated in Scheme 2. This differs from the preceding mechanism in that there is no fixed order for the combination of the translocator with the substrates, and that the limiting step is the translocation reaction itself in which $EA_{in}B_{out}$ becomes $EA_{out}B_{in}$ ($V = k\,[E_t]$).

The physical significance of the K-constants in such a mechanism is: K_a', dissociation constant of EA_{in}; K_b', dissociation constant of EB_{out}; K_a, dissociation constant of the ternary complex $EA_{in}B_{out}$ into EB_{out} and A_{in}; K_b, dissociation constant of the same ternary complex $EA_{in}B_{out}$ into EA_{in} and B_{out}.

K_a' and K_b', dissociation constants of EA_{in} and EB_{out}, characterise the random binding of the first substrate and are independent of the subsequent binding of the second substrate; they are therefore independent of its nature. This is observed in our experiments.

K_a and K_b, dissociation constants of the ternary complex $EA_{in}B_{out}$, characterise the random binding of the second substrate and are not necessarily independent of the nature of the first substrate bound. If the binding of the first substrate does not affect the affinity of the translocator for the second substrate, then K_a and K_b are necessarily equal to K_a' and K_b'. This is also observed in our experiments.

Our experimental results may thus be explained by the mechanism of rapid-equilibrium random bi-bi. The anions located in the external and internal phases combine with the oxoglutarate translocator in a completely independent way and the Michaelis constants given in Table 1 are their dissociation constants.

We wish to thank Mlle Laurette Bertrand for her skillfull technical assistance, the *Fonds National de la Recherche Scientifique* and the *Fonds de la Recherche Scientifique Médicale* for their financial support, and Prof. H. Van Cauwenberge for instrumental facilities. Our thanks are also due to Dr G. Goffart who derived the equations used in the preparation of the Table and Prof. J.-M. Wiame for his advice in the preparation of the manuscript. F.E.S. wishes to thank Prof. J. M. Tager and Dr A. J. Meijer who introduced him to this field of research.

REFERENCES

1. Gamble, J. L., Jr., *J. Biol. Chem.* 240 (1965) 2668.
2. Chappell, J. B., *Brit. Med. Bull.* 24 (1968) 150.
3. Klingenberg, M., in *Essays in Biochemistry* (edited by P. N. Campbell and F. Dickens), Academic Press, London 1970, Vol. 6, p. 119.
4. Van Dam, K., and Meyer, A. J., *Ann. Rev. Biochem.* 40 (1971) 115.
5. Klingenberg, M., and Pfaff, E., in *Regulation of Metabolic Processes in Mitochondria* (edited by J. M. Tager, S. Papa, E. Quagliariello and E. C. Slater), Elsevier, Amsterdam 1966, p. 180.
6. Quagliariello, E., Papa, S., Meyer, A. J., and Tager, J. M., in *Mitochondria. Structure and Function.* (edited by L. Ernster and Z. Drahota), Academic Press, London 1969, Vol. 17, p. 335.
7. Papa, S., Lofrumento, N. E., Quagliariello, E., Meyer, A. J., and Tager, J. M., *J. Bioenerg.* 1 (1970) 287.
8. Kraayenhof, R., Tsou, C. S., and Van Dam, K., *Biochim. Biophys. Acta*, 172 (1969) 580.
9. Quagliariello, E., Palmieri, F., Prezioso, G., and Klingenberg, M., *FEBS Lett.* 4 (1969) 251.
10. Robinson, B. H., and Williams, G. R., *Biochim. Biophys. Acta*, 216 (1970) 63.
11. Palmieri, F., Prezioso, G., Quagliariello, E., and Klingenberg, M., *Eur. J. Biochem.* 22 (1971) 66.
12. De Haan, E. J., and Tager, J. M., *Biochim. Biophys. Acta*, 153 (1968) 98.
13. Meyer, A. J., and Tager, J. M., *Biochim. Biophys. Acta*, 189 (1969) 136.
14. Robinson, B. H., Williams, G. R., Halperin, M. L., and Leznoff, C. C., *Eur. J. Biochem.* 20 (1971) 65.
15. Chappell, J. B., and Robinson, B. H., in *The Metabolic Roles of Citrate* (edited by T. W. Goodwin), Academic Press, London 1968, p. 105.
16. Sluse, F., Meijer, A. J., and Tager, J. M., *FEBS Lett.* 18 (1971) 149.
17. Sluse, F., and Ranson, M., *Arch. Int. Physiol. Biochim.* 79 (1971) 634.
18. Sluse, F., Ranson, M., and Liébecq, C., *J. Physiol. (Paris)* in the press.
19. Sluse, F., and Ranson, M., *Abstr. Commun. 7th Meet. Fed. Eur. Biochem. Soc.* (1971) 228.
20. Tyler, D. D., and Gonze, J., *Methods Enzymol.* 10 (1967) 75.
21. von Beisenherz, G., Boltze, H. J., Bücher, T., Czok, R., Garbade, K. H., Meyer-Arendt, E., and Pfleiderer, G., *Z. Naturforsch.* 8b (1953) 555.
22. Papa, S., Lofrumento, N. E., Kanduc, D., Paradies, G., and Quagliariello, E., *Eur. J. Biochem.* 22 (1971) 134.
23. Florini, J. R., and Vestling, C. S., *Biochim. Biophys. Acta*, 25 (1957) 575.
24. Cleland, W. W., *Biochim. Biophys. Acta*, 67 (1963) 104.
25. Cleland, W. W., *Biochim. Biophys. Acta*, 67 (1963) 173.
26. Cleland, W. W., *Biochim. Biophys. Acta*, 67 (1963) 188.
27. Frieden, C., *J. Amer. Chem. Soc.* 79 (1957) 1894.

F. E. Sluse, M. Ranson, and C. Liébecq
Laboratoire de Biochimie et de Physiologie Générale
Institut Supérieur d'Education Physique de l'Université
Rue des Bonnes-Villes 1, B-4000 Liège, Belgium