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# Steady-State Kinetics of the Overall Oxidative Phosphorylation Reaction in Heart Mitochondria

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**Abstract**

The steady-state velocity dependence of the overall mitochondrial oxidative phosphorylation reaction on the concentrations of extramitochondrial ADP and $P_i$ and of several of the catalytic components was investigated, using the $O_2$ uptake step as the indicator reaction and conditions of saturation with $O_2$, malate, and pyruvate. The studies were carried out with tightly coupled bovine heart mitochondria incubated in the presence of hexokinase, glucose, and $Mg^{2+}$. The data were corrected to conditions of hexokinase saturation with factors determined in hexokinase dependence studies. The concentrations of catalytic components were varied, in effect, by application of highly specific, tight-binding inactivators of the components. The principal objectives were (a) to distinguish individual reactions coupled by freely diffusible intermediate reactants, (b) to determine the relationships (coupling relationships) between these reactions in regard to how a change in the degrees to which one limits the rate of the overall reaction affects the degree to which the others limit the rate, and (c) to use the findings to determine how the individual reactions are coupled. The feasibility of achieving these objectives was suggested by the observations (a) that the initial steady-state velocity of the overall reaction varies in fairly close accord with a rectangular

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hyperbola (i.e., with Michaelis–Menten kinetics) whether it is a catalytic component or a substrate that is varied, (b) that apparent Michaelis constants of the substrates and catalytic components may be used as indicators of the coupling relationships between the individual reactions, and (c) that two types of coupling relationships exist between the individual reactions: "sequential" (characteristic of reactions linked in simple sequence) and "nonsequential" (mechanism uncertain), in which a change in the degree to which one individual reaction of a pair is rate limiting results in an inverse change and in no change, respectively, in the degree to which the other is rate limiting. Six individual reactions were distinguished: the energy-yielding rotenone-, antimycin-, and cyanide-sensitive steps of the respiratory chain and the energy-consuming $P_i$ transport, phosphorylation, and AdN (adenine nucleotide) transport reactions. The results indicate (a) that the coupling relationship is sequential between the $P_i$ transport and rotenone-sensitive reactions, the $P_i$ transport and cyanide-sensitive reactions, the AdN transport and rotenone-sensitive reactions, the AdN transport and cyanide-sensitive reactions, and the AdN transport and phosphorylation reactions, and (b) that the coupling relationship is nonsequential between the AdN and $P_i$ transport reactions, the $P_i$ transport and phosphorylation reactions, the $P_i$ transport and antimycin-sensitive reactions, and the AdN transport and antimycin-sensitive reactions. In the sequential group of individual reaction pairs, the individual reactions of all but the AdN transport–phosphorylation reaction pair appear to be linked in a partially nonsequential manner. It is proposed that the nonsequential and partially nonsequential coupling relationships come about as a result of one individual reaction of a pair removing freely diffusible intermediate reactants at two or more points which are situated symmetrically and unsymmetrically, respectively, about the other.

## Introduction

The overall mitochondrial oxidative phosphorylation reaction, as studied here, is the process in which extramitochondrial ADP and $P_i$ are converted to extramitochondrial ATP with energy derived from extramitochondrial malate and pyruvate. In preliminary studies on the steady-state kinetics of this reaction coupled to the hexokinase reaction, we noted that the initial velocity varies in fairly close accord with a rectangular hyperbola whether it is a catalytic component or a substrate that is varied. Thus, we observed that reciprocal plots of initial velocity vs. component concentration are quite linear whether the component is ADP, $P_i$, hexokinase, or the AdN (adenine nucleotide) carrier. This is in accord with the predictions of theoretical

studies by Hearon (1) and Waley (2) in which the steady-state kinetics of simple multienzyme reactions of the type

$$A \overset{E_1}{\rightleftharpoons} I_1 \overset{E_2}{\rightleftharpoons} I_2 \overset{E_3}{\rightleftharpoons} P \qquad (1)$$

were approximated by considering the individual reactions to follow first-order kinetics. In the reaction shown, a substrate A is converted to a product P by way of three reversible enzyme-catalyzed reactions linked by the freely diffusible intermediate reactants $I_1$ and $I_2$. The system is considered to be free of allosteric interactions and to achieve a steady-state by virtue of continuous flux of A in and P out (open system). In the steady state the net forward velocities of the individual reactions would be equal and the intermediate reactants would be consumed as rapidly as they are formed (1, 2).

Hearon (1) obtained results indicating that the net forward velocities of reactions of this type can be expected to vary with the concentrations of either substrates or catalytic components in strict accord with rectangular hyperbolas. An important conclusion of this work was that the same can be expected of much more complex multienzyme reactions. Waley (2) made similar observations in respect to the relationship between velocity and enzyme concentration, using relatively simple techniques and assuming the individual reactions to follow first-order kinetics only in regard to the steps involving the intermediate reactants as substrates.

Although these approximations cannot be considered to predict the kinetics of real multienzyme reactions with a high degree of accuracy, they nevertheless appear to do so in the case of the overall oxidative phosphorylation reaction when the concentrations of substrates and catalytic components are varied over experimentally feasible ranges. It is generally recognized (3–9) that exact accordance of the rate behavior of a real multienzyme reaction of this type with a rectangular hyperbola might be expected only when the varied component is a substrate and only in the limiting case where the individual reaction in which the varied substrate is directly involved is the only one limiting the rate (i.e., in the case where all the other individual reactions are at thermodynamic equilibrium).

According to Waley's treatment, the reciprocal of the velocity $v$ of overall reaction (1) is given by the equation

$$\frac{1}{v} = \frac{1}{Q}\left(\frac{R}{e_1} + \frac{S}{e_2} + \frac{T}{e_3}\right) \qquad (I)$$

in which $e_1$, $e_2$, and $e_3$ are the concentrations of the enzymes and $Q$, $R$, $S$, and $T$ are functions of the rate constants and of the concentrations of A and P. Waley pointed out that the rate of the overall reaction depends on the concentrations of all the enzymes, that there is no need for a certain

rate-limiting step, but that if, for instance, $e_1$ were made small enough, the term $R/e_1$ would be large compared with $S/e_2$ and $T/e_3$, and thus would govern the rate (2). He showed that the most efficient use of enzymes occurs when $R/e_1^2 = S/e_2^2 = T/e_3^2$, from which it is evident that this occurs when the concentrations of the enzymes and reactants are such that the individual reactions are equally rate limiting in the overall reaction. Since biological systems tend to be efficient in the use of enzymes (10), it is therefore reasonable to expect that the various individual reactions of the overall oxidative phosphorylation reaction are more or less equally rate limiting under normal conditions, and that regardless of the component that is varied, the velocity of the overall reaction will not vary in strict accord with a rectangular hyperbola.

As is evident from Eq. (I), the relationship between individual reactions of multienzyme systems in which the individual reactions are linked in simple sequence is such that a change in the degree to which one is rate limiting results in oppositely directed changes in the degrees to which all the others are rate limiting. Thus, if the $E_2$-catalyzed reaction of overall reaction (1) were made more rate limiting, such as by decreasing the concentration of the enzyme, the concentrations of the intermediate reactants would change during the approach to the new steady state such that the $E_2$-catalyzed reaction would be farther from thermodynamic equilibrium and the $E_1$- and $E_3$-catalyzed reactions closer to thermodynamic equilibrium. In preliminary studies on the overall oxidative phosphorylation reaction, considered to consist of a number of tightly coupled individual reactions linked by freely diffusible intermediate reactants, this "sequential" coupling relationship between individual reactions of pairs was observed only in certain cases. In some cases the relationship was found to be such that a change in the degree to which one is rate limiting has little or no effect on the degree to which the other is rate limiting. The existence of this "nonsequential" coupling relationship suggested that a detailed study of the relationships of this type between the individual energy-yielding and energy-consuming reactions might be useful in deciding how these reactions are coupled. Accordingly, the principal objectives of this study were (a) to distinguish as many individual reactions as possible, (b) to determine which of these two types of relationships exist between them, and (c) to use the findings to determine how the reactions are coupled.

In the attempt to achieve these objectives the steady-state velocity dependence of the overall reaction on the concentrations of external ADP and $P_i$ and of several of the catalytic components was determined, using the $O_2$ uptake step as the indicator reaction and conditions of saturation with $O_2$, malate, and pyruvate. The concentrations of the catalytic components were varied, in effect, by application of graded amounts of highly specific,

tight-binding inhibitors (inactivators) of the components. In all, three energy-yielding reactions (the rotenone-, antimycin-, and cyanide-sensitive steps of the respiratory chain) and three energy-consuming reactions (the $P_i$ transport, phosphorylation, and AdN transport reactions) were distinguished. Coupling relationships between these reactions were determined from changes in apparent $K_m$'s obtained experimentally for ADP, $P_i$, and, in some cases, the catalytic components. That apparent $K_m$'s may be used for the purpose can be seen from the facts (a) that the degree to which a given individual enzyme-catalyzed reaction is rate limiting in a multienzyme reaction is determined by the effective concentration of the enzyme in relation to the effective concentrations of the enzymes of all the other individual reactions (2, 4), and (b) that the effective concentration of a given enzyme is determined by the actual concentration and potential activity of the enzyme, the concentrations of all substrates of the enzyme, and the concentrations or magnitudes of all other agents or factors that influence the activity of the enzyme (4, 10, 11). In consequence of these facts, if a treatment of a particular multienzyme system results in a change in the degree to which a given individual reaction of the system is rate limiting in the overall reaction catalyzed by the system, the concentrations of the substrates and enzyme of this individual reaction required for half-maximum velocity of the overall reaction will change, increasing if the reaction becomes more rate limiting and decreasing if it becomes less rate limiting.

Apparent $K_m$'s were determined by closed system, initial velocity techniques similar to those commonly used in steady-state kinetic studies on single-enzyme reactions. The use of these techniques was feasible because, owing to the intermediate reactants being confined to very small spaces, the overall oxidative phosphorylation reaction, despite being very complex, rapidly approaches a near steady state with little consumption of external substrates when the concentration of mitochondria is low and the reaction is initiated by adding ADP. However, because of the high affinity of the mitochondrion for ADP and ATP (12, 13), it was necessary to use low concentrations of added ADP and to regenerate the ADP with an auxiliary enzyme (hexokinase) system. The use of saturating concentrations of the auxiliary enzyme was not economically feasible at the lowest of the ADP levels required for determination of the apparent $K_m$ of ADP. Therefore, to meet the requirement (5–9) that the auxiliary enzyme be saturating under all conditions of substrate concentration, we used a high but nonsaturating concentration of the enzyme and corrected the initial velocity data to conditions of hexokinase saturation with factors determined in hexokinase dependence studies.

As is ordinarily done in steady-state kinetic studies on single-enzyme reactions, the components that were varied were done so over limited

concentration ranges and apparent $K_m$'s of the components were obtained from reciprocal plots of initial velocity vs. component concentration on the assumption that the plots were linear (i.e., on the assumption that the velocity varied with the concentrations of the components in exact accord with rectangular hyperbolas). Since, as noted above, this assumption is not strictly valid for real multienzyme reactions, the apparent $K_m$'s obtained for the varied components cannot be considered to be highly accurate indicators of the concentrations of these components required for half-maximum velocity of the overall oxidative phosphorylation reaction. However, as required to achieve the objectives of this study, they can be expected to be reasonably reliable indicators of the directions and magnitudes of changes in these concentrations due to changes in the degrees to which the individual reactions in which the components are directly involved are rate limiting in the overall reaction.

## Materials and Methods

Bovine heart mitochondria were isolated according to a slight modification of the Nagarse procedure previously described (14). Homogenates were prepared in media containing 250 mM sucrose, 0.1 mM EDTA, 10 mM TrisCl (pH 7.8 at 0°C), and 0.2 mg of Nagarse proteinase per gram of tissue. The mitochondria were washed twice and suspended finally at a concentration of 70 mg protein/ml in media containing sucrose and TrisCl at the concentrations given above. Use of the mitochondria (stored in capped vials at 0°C) was commenced immediately after isolation. Heart mitochondria isolated according to this procedure exhibit high respiratory control ratios (15, 16) and deteriorate very slowly when maintained at 0°C.

Mitochondrial $0_2$ consumption, pH of the incubation mixture (8 ml), and mitochondrial optical density (800 nm) were monitored simultaneously under rapid stirring in a closed, thermostated (30°C) reaction chamber (13). The standard reaction medium contained 225 mM sucrose, 10 mM PIPES, 2.5 mM each of malate and pyruvate, 0.1 mM EGTA, 10 mM glucose, and 1 mM $MgCl_2$. In all cases the pH of the incubation mixture was 6.5 and the concentration of mitochondria was 0.25 mg protein/ml. Unless indicated otherwise, $P_i$, hexokinase, and inhibitors were added to the reaction medium prior to the mitochondria, the concentration of hexokinase was 10 Darrow–Colowick (17) units/ml, salts were $K^+$ salts, the mitochondria were preincubated for 2 min, and State 3 (phosphorylating) respiration was initiated by adding ADP ($Na^+$ salt). Initial velocities were determined from the initial linear portions of the oxygen electrode records. Apparent kinetic constants were determined from reciprocal plots (Lineweaver–Burk) and are presented

in the nomenclature of Cleland (18). In calculating them (11) the plots were assumed to be linear and the slopes and intercepts of the plots were determined by linear regression analysis. All velocities are expressed as microatoms of oxygen per minute per milligram of mitochondrial protein.

Triethyltin was obtained from Ventron Corporation (Danvers, Massachusetts), DCCD (*N,N'*-dicyclohexylcarbodiimide) from Aldrich Chemical Company (Milwaukee, Wisconsin), and yeast hexokinase (lyophilized, Type F-300), malate, pyruvate, PIPES, ADP, rotenone, antimycin, and oligomycin from Sigma Chemical Company (St. Louis, Missouri). Aurovertin was generously donated by C. Baldwin (Dow Chemical Company, Zionsville, Indiana), piericidin A by N. Takahashi (University of Tokyo), and carboxyatractyloside by A. Bonati (Inverni della Beffa, Milan) and S. Luciani (University of Padua).

## Results and Discussion

### *Preliminary Studies*

A number of preliminary experiments were carried out to determine procedures and conditions of incubation that permit the acquisition of valid initial velocity data. These studies established that under conditions of hexokinase saturation, the initial velocity of the overall reaction is proportional to mitochondrial concentration up to and beyond 0.25 mg protein/ml, and that 2.5 mM each of malate and pyruvate is virtually saturating under all the conditions of ADP and $P_i$ concentration employed. Owing to the need for accumulation of intramitochondrial $P_i$, it was necessary to preincubate the mitochondria in the presence of $P_i$ for at least a few seconds before initiating State 3 respiration. Not doing this lengthens the period of time required for State 3 respiration to reach a near steady state by 3 to 5 sec, depending on the amount of $P_i$ added. Addition of ADP before $P_i$ was found to result in significant interference from adenylate kinase activity.

To ascertain whether or not the response time of the oxygen electrode (Yellow Springs Instrument Company) was adequate under the standard conditions of incubation, normal (YSI 5352) and high-sensitivity (YSI 5937) oxygen electrode membranes were compared under conditions of high and low ADP and $P_i$ concentrations. Although substitution of the high-sensitivity membrane for the normal slightly increased the abruptness of the electrode responses to initiation of State 3 respiration, it had virtually no effect on the slopes of the initial linear portions of the oxygen uptake records. Therefore, the relatively stable normal-sensitivity membranes were considered adequate and used routinely.

As pointed out in the Introduction, it was necessary to regenerate ADP.

The hexokinase reaction is suitable for this purpose because its thermodynamic equilibrium is far in the direction of ADP formation and the enzyme is readily available in suitable form. Since the object in using it was to maintain the concentrations of extramitochondrial ADP and ATP very close to their initial levels for a period of time sufficient to obtain a valid estimate of the initial steady-state velocity of the overall oxidative phosphorylation reaction, it was necessary to include saturating amounts of the enzyme in the incubation mixtures (i.e., amounts sufficient to maintain the hexokinase reaction very close to thermodynamic equilibrium, where the concentrations of ADP and ATP might be expected to be very close to their initial levels).

Preliminary studies revealed that the amount of hexokinase required for saturation increases markedly as $P_i$ concentration is increased and as AdN concentration is decreased. In deciding how to deal with these changes, it was important to understand how they come about. Considering the overall oxidative phosphorylation + hexokinase reaction as a two-step multienzyme reaction, the increase as $P_i$ concentration is increased is relatively easy to understand, since $P_i$ might be expected to affect only the mitochondrial reaction at the low concentrations employed (0.4–2.0 mM). Since $P_i$ is a substrate in the mitochondrial reaction, an increase in $P_i$ concentration would increase the effective concentration of the mitochondria. This would result in the mitochondrial reaction being less rate limiting in the overall reaction and in the hexokinase reaction being more rate limiting (i.e., farther from thermodynamic equilibrium). Consequently, the amount of hexokinase required for saturation of the overall reaction would increase.

It is important to note that the coupling relationship expected between the mitochondrial and hexokinase reactions is identical to that expected between the individual reactions of simple multienzyme reactions of the type exemplified by overall reaction (1), this being so despite a marked difference in the means by which the individual reactions are coupled. Whereas the individual reactions of overall reaction (1) are linked by "accumulation type" intermediates, the mitochondrial and hexokinase reactions are linked by a "coenzyme (transfer) type" intermediate (i.e., AdN). Although not distinguishable on the basis of coupling relationship, these different means of coupling can be distinguished on the basis of certain unique characteristics of coenzymic coupling which appear to be relevant to the understanding of why the amount of hexokinase required for saturation of the overall mitochondrial + hexokinase reaction increases as the concentration of AdN is decreased. One such characteristic of coenzymic coupling is that the concentration of the coenzyme can be varied independently of the reactions coupled, these reactions affecting only the ratio of the two forms of the coenzyme determined by the presence or absence of whatever is transferred. Another is that a coenzyme is both a substrate and a product in both of the reactions it

couples. Consequently, if the enzymes of two reactions coupled in this manner have different affinities for the coenzyme, a change in the concentration of the coenzyme will change the effective concentrations of the enzymes to different extents, the effective concentration of the enzyme having the lower affinity undergoing a relatively large change. This is likely the basis for the marked increase in the level of hexokinase required for saturation of the overall mitochondrial + hexokinase reaction when the concentration of AdN is decreased. Thus, as a result of the relatively low AdN affinity of hexokinase, the effective concentration of this enzyme likely decreases to a relatively large extent when the concentration of AdN is decreased, resulting in the hexokinase reaction limiting the overall reaction to a relatively large extent and thus in the need for a relatively large amount of hexokinase to achieve saturation of the overall reaction.

The preliminary studies showed that under the conditions of low ADP concentration required for determination of the apparent $K_m$ of ADP, very high levels of hexokinase are required for virtual saturation of the overall reaction. For example, under conditions of 2.0 mM $P_i$ and 10 $\mu$M added ADP, addition of at least 100 units of the enzyme/ml of incubation mixture was found necessary. Routine use of this amount of hexokinase was not economically feasible, and it appeared that such a high concentration of the enzyme preparation might affect the mitochondrial reaction by means other than regeneration of ADP. Therefore, a high but nonsaturating concentration of hexokinase (10 units/ml) was selected for routine use and the initial velocities observed at this concentration were corrected to conditions of hexokinase saturation with factors determined in hexokinase dependence studies.

As is evident from the foregoing, it was necessary to determine the correction factors under the same conditions of added ADP to be used in the experiments the factors were to correct. The ADP concentrations chosen were 10, 13.3, 20, and 40 $\mu$M. Since in the experiments the factors were to correct, the effective concentration of the mitochondria was to be varied by varying the concentrations of a number of agents that affect only the mitochondrial reaction directly, and since changes in the effective concentration of the mitochondria could be expected to result in changes in the degree to which the hexokinase reaction limits the overall reaction, it was necessary in determining the correction factors to vary the effective concentration of the mitochondria at each of the chosen ADP levels in such a way that only the mitochondrial reaction was affected directly. This was achieved by varying the concentration of $P_i$ over the range 0.4 to 2.0 mM. The hexokinase dependence of the initial steady-state velocity of the overall mitochondrial + hexokinase reaction was determined at each of various combinations of the chosen ADP and $P_i$ concentrations by varying the concentration of hexokinase over the range 2 to 10 units/ml of incubation mixture under conditions which

were otherwise standard (see Materials and Methods). The initial velocities under conditions of hexokinase saturation were estimated by extrapolating reciprocal plots of initial velocity vs. hexokinase concentration to infinite hexokinase concentration. The plots appeared to be linear within experimental error and were therefore assumed to be linear in making the extrapolations. The degrees of correction necessary in initial velocity estimates obtained under standard conditions (10 units hexokinase/ml) at each of the chosen ADP concentrations are presented in Fig. 1. It can be seen that the magnitude of the correction increases markedly as the concentration of ADP is decreased, and that at a particular level of ADP the magnitude increases with the velocity in fairly close accord with a rectangular hyperbola. Except

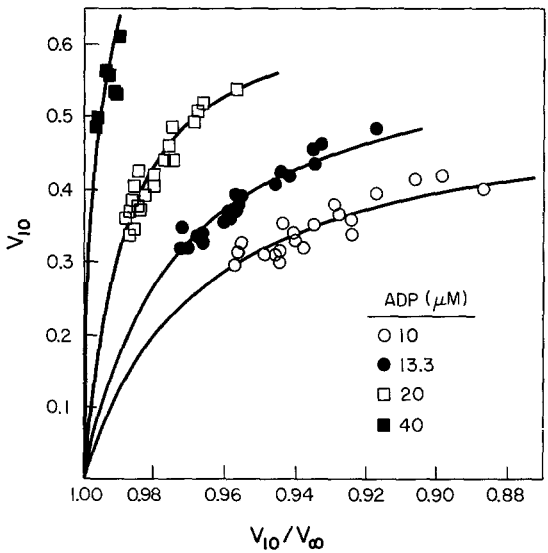



**Fig. 1.** Data for correction of the initial State 3 velocities of respiration to conditions of hexokinase saturation. The data were obtained in four experiments, in each of which ADP was varied as indicated and $P_i$ was varied over the range 0.4 to 2.0 mM. For each combination of ADP and $P_i$, determinations were made under conditions of 2, 2.65, 4, and 10 units hexokinase/ml incubation mixture. $v_{10}$ is the initial velocity under the standard conditions of incubation (10 units of hexokinase/ml incubation mixture) and $v_\infty$ is the intial velocity under conditions of infinite hexokinase concentration. Both values were obtained from best-fit straight lines of reciprocal plots of initial velocity vs. hexokinase concentration. The curves shown are rectangular hyperbolas and were obtained by forming reciprocal plots of $v_{10}$ vs. $1 - v_{10}/v_\infty$ and determining the best-fit straight lines. The curves were used by dividing the initial velocity ($v_{10}$) values obtained under the standard conditions by their corresponding $v_{10}/v_\infty$ values.

where evident otherwise, all the data presented below were corrected in accordance with these results.

It was found impractical to use ADP at concentrations lower than about 10 $\mu$M and $P_i$ at concentrations lower than about 0.5 mM. As is evident from Fig. 1, decreasing the concentration of ADP beyond 10 $\mu$M results in the correction factors becoming very large. In the case of $P_i$, decreasing the concentration beyond about 0.5 mM results in the initial linear portion of the oxygen electrode record becoming too small to permit the assumption that a condition closely approximating a steady state has been achieved. In any case, it was necessary to use fairly high concentrations of ADP and $P_i$ to avoid making the energy-consuming reactions so rate limiting as to result in uncoupling of these reactions from the energy-yielding reactions, this being particularly necessary in experiments involving the use of inhibitors of the energy-consuming reactions. Needless to say, uncoupling could not be tolerated, particularly uncoupling that varies in extent as the velocity of the overall reaction is varied. Determination of the effects of the various inhibitors used in this study on ADP/O (determined in the absence of hexokinase, glucose, and $Mg^{2+}$) revealed that uncoupling (decrease of ADP/O) can be avoided by avoiding inhibition of the energy-consuming reactions to the extent that initiation of State 3 respiration results in less than approximately a fivefold increase in velocity. In most of the experiments reported, the lowest velocity was well above this point (i.e., well within the velocity range over which ADP/O is constant and thus well within the velocity range over which $O_2$ consumption is proportional to ATP formation).

Because of the narrow feasible ranges of ADP and $P_i$ concentration and of the limited time available to conduct the individual experiments, it was necessary to carry out the measurements rapidly and precisely. This was achieved by using a large volume (8 ml) of the reaction mixtures and a micrometer syringe which was modified for making rapid and reproducible additions to the reaction mixtures with polyethylene tubing. In a few experiments the precision of the measurements was estimated by immediately repeating the measurements twice. In each case the mean of the standard deviations of the measurements fell within the range 0.5 to 1.0%. The usual procedure was to repeat a particular measurement immediately only if we suspected that a mistake had been made, and then, after completing a set of measurements, to repeat the entire set as many times as we could. We were thus able to decrease random error while obtaining a check on, and diminishing the consequences of, aging effects. Aging effects were usually observed, but were very small in relation to the effects of the treatments.

In most of the experiments both ADP and $P_i$ were varied so that the coupling relationship between the AdN and $P_i$ transport reactions and the

effects of the various treatments on it could be assessed. In early experiments four combinations each of ADP and $P_i$, ranging from 10 to 40 $\mu$M and 0.5 to 2 mM, respectively, were used. This ordinarily permitted only one repetition of the sets of measurements. It was soon learned that the same results can be obtained with less difficulty by using only three concentrations each of the substrates and repeating the sets of measurements twice. This combination was used in most of the experiments.

### *Effects of ADP and $P_i$ Concentration*

As shown in Fig. 2, reciprocal plots of the initial velocity of the overall oxidative phosphorylation reaction vs. ADP at different fixed levels of $P_i$ (Fig. 2A) and vs. $P_i$ at different fixed levels of ADP (Fig. 2C) are quite linear over

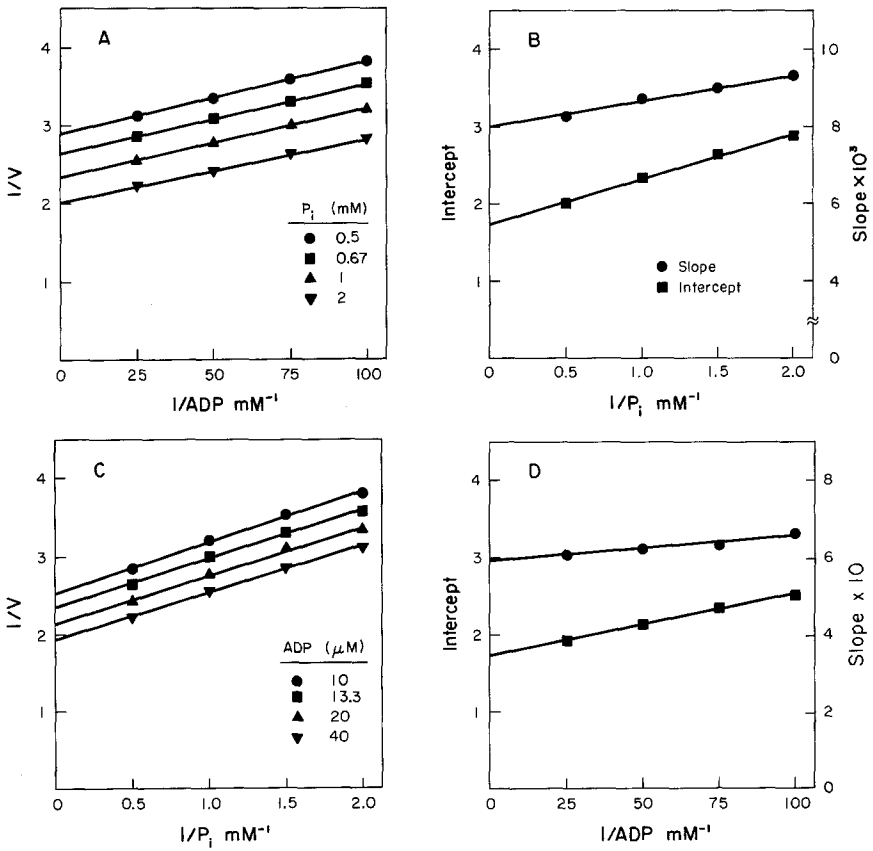



**Fig. 2.** Reciprocal plots of initial velocity vs. ADP (A) and $P_i$ (C) concentration, and replots of the slopes and intercepts (B and D).

the limited ranges of concentration employed and form patterns similar to those obtained in steady-state kinetic studies on bisubstrate single-enzyme reactions that follow Michaelis–Menten kinetics. In fact, over the limited concentration ranges the rate dependence of the overall reaction on ADP and $P_i$ is described quite well by the single-enzyme rate equation

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \qquad \text{(II)}$$

in which $v$ is the initial velocity, $A$ and $B$ the concentrations of the substrates, $V$ the limiting velocity, $K_a$ and $K_b$ the limiting Michaelis constants of the substrates, and $K_{ia}$ the inhibition constant of substrate A (11, 18). This can be seen from Figs. 2B and 2D, which show that replots of the slopes and intercepts of the primary plots are also quite linear.

In the notation of Eq. (II) the $K_m$'s obtained for one of the substrates under conditions of finite concentration of the other are referred to as apparent $K_m$'s. Since the limiting $K_m$ is the value which the apparent $K_m$ approaches as the concentration of the other substrate approaches infinity, and the $K_i$ the value which the apparent $K_m$ approaches as the concentration of the other substrate approaches zero, the limiting $K_m$ and $K_i$ of a given substrate give the range over which the apparent $K_m$ of the substrate changes as the concentration of the other is varied from zero to infinity (18). This assumes, of course, that in accordance with Michaelis–Menten kinetics, the reciprocal plots are linear throughout this concentration range. Although, as noted in the Introduction, this assumption is not strictly valid for multienzyme reactions consisting of individual reactions linked by freely diffusible intermediates, it may be used for the purposes of this study, since the principal objectives were merely to distinguish individual reactions and to determine the coupling relationships between them. Thus, as is evident from the above definitions and the fact that a change in the concentration of a substrate or the enzyme of a given individual reaction would result in an inverse change in the degree to which the reaction is rate limiting (2), limiting kinetic constants obtained with the use of this assumption can be used as indicators of the coupling relationships between individual reactions. It is evident that if the $K_i$'s of ADP and $P_i$ are smaller than the respective $K_m$'s of these substrates (i.e., $K_i/K_m < 1$), a sequential relationship between the AdN and $P_i$ transport reactions is indicated, and that if the $K_i$'s equal the $K_m$'s, (i.e., $K_i/K_m = 1$), a nonsequential relationship is indicated.

Table I summarizes in the form of limiting kinetic constants the results of 25 experiments of the type shown in Fig. 2. Since the $K_i$'s are smaller than the $K_m$'s, the data indicate a sequential coupling relationship between the AdN and $P_i$ transport reactions. However, the $K_i$'s and $K_i/K_m$ ratios are not as small as might be expected if the AdN and $P_i$ transport reactions were linked

**Table I.** Limiting Kinetic Constants of the Overall Oxidative Phosphorylation Reaction[a]

| $V$ ($\mu$atom O/min/mg) | $K_{ia}$ (mM) | $K_a$ (mM) | $K_{ib}$ ($\mu$M) | $K_b$ ($\mu$M) |
|---|---|---|---|---|
| 0.64 ± 0.05 | 0.12 ± 0.09 | 0.33 ± 0.04 | 1.4 ± 0.9 | 4.4 ± 1.1 |

[a]The values are means ± SD of 25 experiments. The experiments represent different mitochondrial preparations and were carried out under the standard conditions of this study. Substrate $A = P_i$; substrate $B$ = ADP.

in simple sequence. This can be seen by considering the results that might be expected if we were to examine experimentally the steady-state kinetics of a simple multienzyme reaction of the type shown in overall reaction (1). In doing so, we will assume that the concentration of the product P is somehow maintained virtually at zero and that the effective concentrations of $E_2$ and $E_3$ are initially sufficiently high relative to the effective concentration of $E_1$ that the $E_2$- and $E_3$-catalyzed reactions are virtually at thermodynamic equilibrium throughout the range over which the substrate of the $E_1$-catalyzed reaction, $A$, is to be varied. Since under these conditions the $E_1$-catalyzed reaction would be the only one limiting the rate of the overall reaction, we could expect variation of A to result in the velocity of the overall reaction changing in more or less exact accord with the Michaelis–Menten equation and thus in a reciprocal plot of $A$ vs. the velocity forming a straight line.

Now, if were to decrease the effective concentration of an enzyme of the sequence other than $E_1$, say $E_2$, by decreasing the concentration of a second substrate $B$ of this enzyme, and were to determine the steady-state velocity of the overall reaction as a function of $A$ at successively lower fixed levels of $B$, we could expect to obtain reciprocal plots which become progressively less linear, concave up, as the level of $B$ is decreased, and which converge to the right of the vertical axis. Convergence to the right would occur as a result of the $E_1$-catalyzed reaction becoming more rate limiting and the $E_2$-catalyzed reaction becoming less rate limiting as the level of $A$ is decreased. In the limiting case in which $A$ approaches zero, the plots would merge as a result of the $E_2$-catalyzed reaction being virtually at thermodynamic equilibrium at even the lowest of the levels of $B$. Similarly we could expect reciprocal plots of $B$ vs. velocity obtained at various fixed levels of $A$ to converge to the right. In this case the convergence would occur as a result of the $E_2$-catalyzed reaction becoming more rate limiting and the $E_1$-catalyzed reaction becoming less rate limiting as the level of $B$ is decreased and would be associated with the $E_1$-catalyzed reaction approaching thermodynamic equilibrium. Since the plots would converge to the right regardless of the ranges over which $A$ and $B$ are varied, we could expect that if the reciprocal plots were assumed to be

linear and limiting kinetic constants were calculated according to Eq. (II), negative $K_i$'s and a negative $K_i/K_m$ ratio would invariably be obtained. As is evident from the observations of Waley (2), findings similar to these can be expected of individual reactions pairs of much more complex multienzyme reactions in which the individual reactions of the pairs are linked in simple sequence, even if the individual reactions of the pairs are separated by numerous other individual reactions.

Since the overall oxidative phosphorylation reaction exhibits kinetic characteristics similar to single-enzyme reactions when the concentrations of the substrates are varied over experimentally feasible ranges (Fig. 2), and since in the present study this reaction was linked in simple sequence to the hexokinase reaction, the above observations can be illustrated with the experimental system at hand. Furthermore, since in the determinations of correction factors the effective concentrations of the catalytic components of both the overall oxidative phosphorylation reaction and the hexokinase reaction were varied by direct means under conditions of constant concentration of the coupling coenzyme (the former by varying the concentration of $P_i$, the latter by varying the actual concentration of the enzyme), this can be done with the data on hand. Thus, as shown in Fig. 3, when the overall oxidative phosphorylation + hexokinase reaction is considered to be a bisubstrate reaction with $P_i$ and hexokinase as the substrates and correction data obtained at a single AdN concentration are plotted in reciprocal form, reciprocal plots which converge to the right of the vertical axis are obtained.

According to the above observations, reciprocal plots of the initial velocity of the overall oxidative phosphorylation reaction vs. the concentration of a component of one individual reaction obtained at different fixed concentrations of a component of any one of the others can be expected to converge strongly to the right of the vertical axis if the individual reactions are linked in simple sequence. Furthermore, the above observations indicate that regardless of how the individual reactions are coupled, the reciprocal plots can be expected to be nonlinear, concave up, and that in the case of the individual reactions being linked sequentially, the plots can be expected to become less linear, concave up, as the concentration of the fixed component is decreased. Although evident in Fig. 3, which presents average results from four experiments, this curvature is difficult to detect in the individual experiments that Fig. 3 summarizes and is not evident in any of the other experiments of this study. This is presumably due to the curvature being two small to detect by the methods employed.

The only curvature that is consistently evident is a concave-down curvature of the $P_i$ reciprocal plots. In contrast to the anticipated concave-up curvature, this curvature appears to be unaffected by changes in the degree to which the $P_i$ transport reaction limits the overall reaction, because it can be

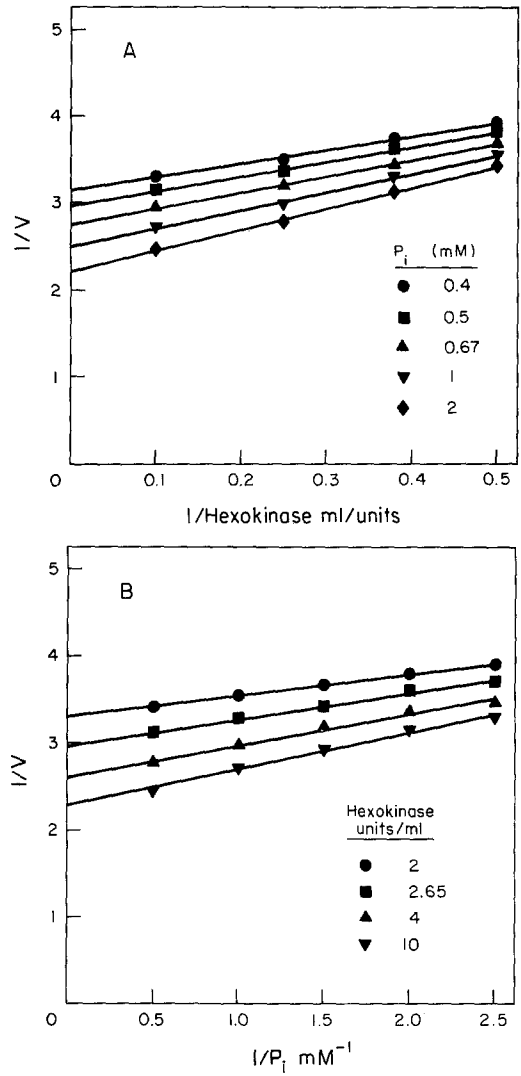



**Fig. 3.** Reciprocal plots of initial velocity vs. hexokinase (A) and $P_i$ (B) concentration. State 3 respiration was initiated by adding 10 $\mu$M ADP. Each data point represents the mean of the values obtained in four identical experiments.

eliminated by treating the mitochondria with an amount of the $P_i$ transport inhibitor mersalyl just sufficient to give a detectable inhibition of the overall reaction. Although, as might be expected, mersalyl markedly increases the apparent $K_m$ of $P_i$, it has no effect on the maximum velocity of the overall reaction, suggesting that it inhibits the $P_i$ transport reaction by decreasing the

affinity of the $P_i$ carrier for external $P_i$. Both the concave-down curvature of the $P_i$ plots and its elimination by mersalyl can be explained in a manner consistent with this by assuming that the $P_i$ carriers of heart mitochondria normally have different affinities for external $P_i$, and that mersalyl, in decreasing the affinities of the carriers, equalizes them. For a reason to be given below, the details of these studies will not be presented at this time.

There are at least two previous steady-state kinetic studies in which reciprocal plot patterns of the type shown in Fig. 3 were encountered experimentally, the first by Morrision and Ebner (19) on the inhibition of a galactosyltransferase-catalyzed reaction by $\alpha$-lactalbumin, and the second by Ebel and Lardy (20) on the inhibition of the activity of an isolated mitochondrial ATPase by aurovertin. In these studies reciprocal plots of initial velocity vs. concentration of certain substrates at different fixed levels of inhibitor were observed to converge strongly to the right of the vertical axis. A simple explanation for these results would be that the reactions concerned are carried out in two or more steps which are linked by freely diffusible intermediate reactants and which are not affected equally by the inhibitors. However, additional observations appear to rule out this explanation, particularly in the case of the galactosyltransferase reaction, and other explanations are available (19, 21, 22).

The $K_i/K_m$ ratios obtained in the experiments of Table I varied between zero and unity. This variation is considerably larger than that observed among successive determinations with a single mitochondrial preparation and thus appears to have been due in large part to something that varies among mitochondrial preparations. This observation and a number of others to be described below and in a subsequent communication have led us to believe that the fundamental coupling relationship between the AdN and $P_i$ transport reactions is fully nonsequential, and that the observed deviations of the $K_i/K_m$ ratios from unity were caused by an apparent allosteric effect of intramitochondrial $P_i$ on the AdN carrier which we have detected in determinations of the dissociation constant of the AdN–AdN carrier complex by the very sensitive inner-membrane-contraction method (13). These studies indicate that intramitochondrial $P_i$, arsenate, and sulfate decrease the affinity of the AdN carrier for external ADP and ATP, the decrease being particularly large in the case of ATP. Substantial support for the existence of this effect of intramitochondrial $P_i$ has been obtained in studies on the effects of inhibition by mersalyl and of changing the pH of the incubation mixture on the apparent $K_m$'s of ADP and $P_i$ in the overall oxidative phosphorylation reaction. Of numerous treatments, only these were found to change the $K_i/K_m$ ratio appreciably, and in each case the change can be readily explained by assuming that intramitochondrial $P_i$ decreases the affinity of the AdN carrier for extramitochondrial AdN's. Because of the need for additional, relatively

direct studies on the effects of intramitochondrial $P_i$ on the affinity of the AdN carrier, these observations will not be presented at this time. That this effect of $P_i$ would decrease the $K_i/K_m$ ratio of ADP and $P_i$ can be seen by considering a similar phenomenon observed in the case of bisubstrate single-enzyme reactions that occur by the rapid equilibrium random mechanism. In this mechanism, if addition to the enzyme of one of the substrates has no effect on the affinity of the enzyme for the other, the $K_i$'s and $K_m$'s will be equal, whereas if addition of one of the substrates decreases the affinity of the enzyme for the other, the $K_i$'s will be smaller than the $K_m$'s (10).

The observed variation of the $K_i/K_m$ ratio among experiments can be explained by assuming that the affinity of the allosteric $P_i$ binding site was higher in some of the mitochondrial preparations than in others, and that in consequence the site was operating at a higher degree of saturation in some of the preparations than in others. According to this explanation, in the experiments in which a $K_i/K_m$ ratio near unity was found, the site was of particularly high affinity and was consequently virtually saturated throughout the concentration range over which $P_i$ was varied.

### *Effects of Carboxyatractyloside*

Carboxyatractyloside is a potent and highly specific inhibitor of the AdN transport reaction (23). It appears to bind in 1:1 stoichiometry directly and irreversibly to the AdN carrier, completely inactivating it (24). Consequently, one could expect partial inhibition of the overall oxidative phosphorylation reaction with this agent to result in changes in the velocity and in the $K_m$ of $P_i$ similar to those observed when the concentration of external ADP is decreased, since, like decreasing the concentration of ADP, treating the mitochondria with carboxyatractyloside would decrease the effective concentration of the AdN carrier. Furthermore, one could expect the inhibition to result in the $K_m$ of ADP increasing, since decreasing the effective concentration of the carrier would result in the AdN transport reaction being more rate limiting. As can be seen from Table II, the only discrepancy between these expectations and the experimental results is that carboxyatractyloside has little or no effect on the $K_m$ of $P_i$, whereas decreasing the concentration of ADP decreases the $K_m$ of $P_i$ (indicated by the $K_i$'s being appreciably smaller than the $K_m$'s). However, this can be expected if we assume the existence of the apparent allosteric effect of $P_i$, since the $K_m$'s for $P_i$ in Table II are limiting $K_m$'s and thus pertain to conditions of ADP saturation (i.e., conditions under which changes in the affinity of the AdN carrier for external AdN's are likely to have no effect on the AdN transport reaction). It should be noted that regardless of what is assumed concerning the existence of the apparent allosteric effect of $P_i$, the observed effects of carboxyatractyloside

**Table II.** Effects of Carboxyatractyloside on the Limiting Kinetic Constants[a]

| Carboxyatractyloside (nmol/mg) | $V$ (μatom O/min/mg) | $K_{ia}$ (mM) | $K_a$ (mM) | $K_{ib}$ (μM) | $K_b$ (μM) |
|---|---|---|---|---|---|
| None | 0.67 ± 0.03 | 0.13 ± 0.14 | 0.36 ± 0.03 | 1.2 ± 1.3 | 4.4 ± 1.6 |
| 0.4 | 0.62 ± 0.03[b] | 0.16 ± 0.10 | 0.36 ± 0.02 | 2.0 ± 1.1[b] | 5.2 ± 1.7[b] |
| 0.6 | 0.58 ± 0.04[b] | 0.18 ± 0.12[b] | 0.37 ± 0.03 | 2.5 ± 1.3[b] | 6.0 ± 2.1[b] |
| 0.8 | 0.52 ± 0.04[b] | 0.17 ± 0.10 | 0.38 ± 0.04 | 2.8 ± 1.1[b] | 7.4 ± 2.6[b] |

[a]The values are means ± SD of four experiments. Substrate $A$ = $P_i$; substrate $B$ = ADP.

[b]Significantly different from "none" ($P < 0.05$, paired $t$-test).

(Table II) indicate a fully nonsequential coupling relationship between the AdN and $P_i$ transport reactions.

Carboxyatractyloside appears to inactivate more than half the AdN carriers in a quantitative fashion under the conditions of the present study. This is indicated by linear inhibition of inner-membrane contraction induced by external AdN's. In previous studies with atractyloside (13, 25), evidence was obtained suggesting that the magnitude of inner-membrane contraction is directly proportional to the degree of saturation of the AdN carrier, and that extrapolation of the linear portion of the atractyloside inhibition curve to zero contraction gives a fairly accurate estimate of the concentration of the carrier. These findings were used in the present study to determine the kinetics of the overall oxidative phosphorylation reaction in respect to AdN carrier concentration as well as to external ADP and $P_i$ concentration. The experiments of Table II were of this type and their additional results are summarized in Figs. 4 and 5.

The concentration of the AdN carrier was estimated as shown in Fig. 4 and was determined to be 1.25 nmol/mg mitochondrial protein. In the kinetics portions of the experiments, the concentration of carboxyatractyloside was varied between 0 and 0.8 nmol/mg protein (approximately the range over which the inhibitor binds quantitatively, as indicated by the data of Fig. 4) to establish effective carrier concentrations of 1.25, 0.85, 0.65, and 0.45 nmol/mg protein. Figures 5A and 5C present ADP and $P_i$ reciprocal plots at different fixed concentrations of active carrier, and Figs. 5B and 5D present replots of the slopes and intercepts of these plots. The plots indicate that in accord with the theoretical approximations noted in the Introduction, a hyperbolic relationship exists between the velocity of the overall reaction and the concentration of the AdN carrier similar to those that exist between the velocity and the concentrations of ADP and $P_i$, and that over experimentally feasible concentration ranges, the initial velocity of the reaction varies in close

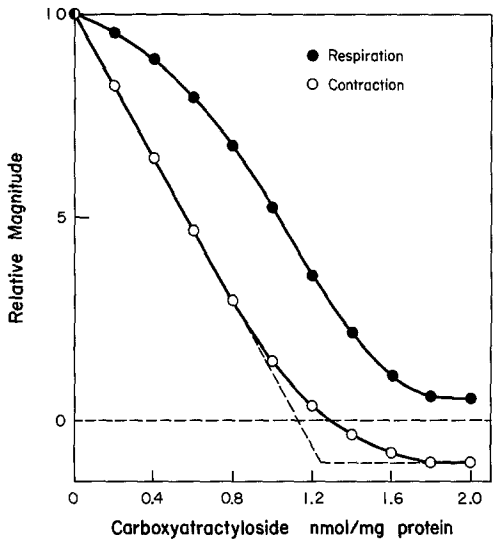



**Fig. 4.** Determination of AdN carrier concentration by the AdN-induced inner-membrane-contraction method (13, 25). Each data point represents the mean of the values obtained in four identical experiments. The conditions of incubation were standard except that the concentration of EGTA was 3 mM. The concentrations of ADP and $P_i$ were 100 $\mu$M and 1 mM, respectively. The magnitude of contraction is extended into the negative range to take into account a slight carboxyatractyloside-insensitive, ADP-induced decrease in mitochondrial optical density which occurs simultaneously with the carboxyatractyloside-sensitive, ADP-induced increase in mitochondrial optical density associated with inner-membrane contraction.

accord with Eq. (II) when either ADP and the carrier or $P_i$ and the carrier are considered to be the substrates. As might be expected from Table II, determinations of kinetic constants, considering the overall reaction as a bisubstrate reaction with these combinations as the substrates, showed that, of the substrates ADP and $P_i$, only ADP has an appreciable effect on the $K_m$ of the AdN carrier, decreasing it over the approximate range 0.6 to 0.3 nmol/mg protein as the concentration of ADP is increased from 10 uM to saturation.

### *Effects of Rotenone, Piericidin A, Antimycin, and Cyanide*

Rotenone (26–29) and piericidin A (28) inhibit electron transport in the first energy-coupling segment of the respiratory chain and antimycin (29,30) and cyanide (29, 31) in the second and third segments, respectively. As shown in Table III, rotenone, piericidin A, and cyanide have essentially identical effects on $V$ and the $K_m$'s of ADP and $P_i$, in each case inhibition being

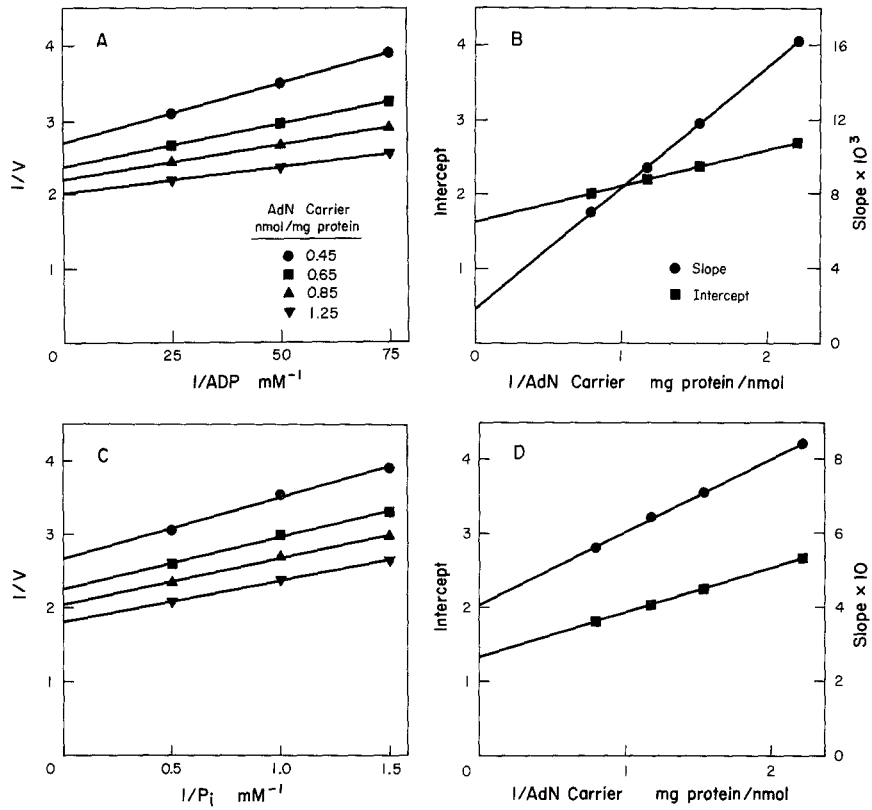



**Fig. 5.** Effects of AdN carrier concentration on the reciprocal plots of initial velocity vs. ADP (A) and $P_i$ (C) concentration. Replots of the slopes and intercepts of the ADP and $P_i$ reciprocal plots are presented in B and D, respectively. The various carrier concentrations indicated are effective concentrations and were established by inactivating fractions of the carrier pool by adding carboxyatractyloside and assuming complete binding of the inhibitor to the carrier. The size of the carrier pool was estimated as shown in Fig. 4. Each data point of the ADP reciprocal plots represents the mean of the 12 values obtained in the four experiments of Table II under conditions of 0.67, 1.0, and 2.0 mM $P_i$, and each data point of the $P_i$ reciprocal plots represents the mean of the 12 values obtained in these experiments under conditions of 13.3, 20, and 40 $\mu$M ADP.

associated with a decrease in $V$ and decreases in the limiting $K_m$'s of both ADP and $P_i$, the decrease in the $K_m$ of $P_i$ being one-half to two-thirds the decrease in the $K_m$ of ADP. In contrast, antimycin inhibition results only in a decrease in $V$.

If the respiratory chains of a given mitochondrion were considered to consist of series of fixed components with no points for distribution of electrons among chains, one could expect that inactivation of any essential

**Table III.** Effects of Rotenone, Piericidin A, Antimycin, and Cyanide on the Limiting Kinetic Constants[a]

| Number of experiments | Inhibitor | $V$ ($\mu$atom O/min/mg) | $K_{ia}$ (mM) | $K_a$ (mM) | $K_{ib}$ ($\mu$M) | $K_b$ ($\mu$M) |
|---|---|---|---|---|---|---|
| 3 | None | 0.67 ± 0.05 | 0.05 ± 0.06 | 0.32 ± 0.03 | 0.8 ± 1.0 | 4.6 ± 0.9 |
| | Rotenone | 0.46 ± 0.04[b] | 0.05 ± 0.07 | 0.25 ± 0.01[b] | 0.7 ± 0.9 | 2.9 ± 0.6[b] |
| 1 | None | 0.68 | 0.20 | 0.32 | 2.1 | 3.3 |
| | Piericidin A | 0.45 | 0.18 | 0.27 | 1.6 | 2.4 |
| 3 | None | 0.63 ± 0.04 | 0.14 ± 0.14 | 0.33 ± 0.05 | 1.7 ± 1.5 | 4.1 ± 0.8 |
| | Antimycin | 0.50 ± 0.03[b] | 0.15 ± 0.14 | 0.32 ± 0.03 | 1.9 ± 1.8 | 4.2 ± 0.8 |
| 3 | None | 0.65 ± 0.03 | 0.13 ± 0.13 | 0.34 ± 0.04 | 1.8 ± 1.3 | 5.5 ± 2.0 |
| | Cyanide | 0.45 ± 0.04[b] | 0.10 ± 0.08 | 0.27 ± 0.06[b] | 1.2 ± 0.7 | 3.6 ± 1.5[b] |

[a]The values are means ± SD of the number of experiments indicated. The concentrations of the inhibitors were: rotenone, 0.07 nmol/mg protein; piericidin A, 0.05 nmol/mg protein; antimycin, 0.13 nmol/mg protein; cyanide, 10 $\mu$M. Because the piericidin A stock solution was exposed to $O_2$ during the experiment (a condition under which it is slowly inactivated), the piericidin A experiment was carried out using only two concentrations each of ADP (10 and 40 $\mu$M) and $P_i$ (0.5 and 2.0 mM) and the sets of measurements were repeated seven times. In the cyanide experiments, KCN was added to the incubation mixture 1 min after the mitochondria, and $P_i$ and ADP 3.5 and 4.0 min, respectively, after the mitochondria. Substrate $A$ = $P_i$; Substrate $B$ = ADP.

[b]Significantly different from "none" ($P < 0.05$, paired $t$-test).

component of a given chain would inactivate the entire chain, and thus that all tight-binding inhibitors of essential components would have the same effects on the $K_m$'s of ADP and $P_i$ in the overall oxidative phosphorylation reaction. The results of this study are clearly not consistent with this and suggest that freely diffusible intermediate reactants exist between the rotenone- and antimycin-sensitive steps and between the antimycin- and cyanide-sensitive steps. Evidence for the linkage of the rotenone- and antimycin-sensitive steps in this manner has been given previously by Klingenberg and Kröger (32, 33), and evidence for the linkage of the antimycin- and cyanide-sensitive steps in this manner has been given previously by Wohlrab (34).

The experiments of Table III indicate that the coupling relationship of the rotenone- and cyanide-sensitive reactions to the AdN and $P_i$ transport reactions is in all cases sequential, and that the coupling relationship of the antimycin- sensitive reaction to the AdN and $P_i$ transport reactions is in each case nonsequential. As can be seen from Fig. 6, which presents reciprocal plots of the data obtained in the rotenone experiments of Table III, the "rotenone" and "control" reciprocal plots of initial velocity vs. ADP (Fig. 6A) converge very slightly to the right of the vertical axis and those of initial velocity vs. $P_i$ (Fig. 6B) converge slightly to the left. As pointed out above, these plots could be expected to converge strongly to the right if the rotenone-sensitive reaction were coupled to the AdN and $P_i$ transport reactions in simple sequence. Therefore, the coupling relationship in each case appears to be partially nonsequential. Since cyanide has virtually the same effects as rotenone, the same can be said of the relationships of the cyanide-sensitive reaction to the AdN and $P_i$ transport reactions.

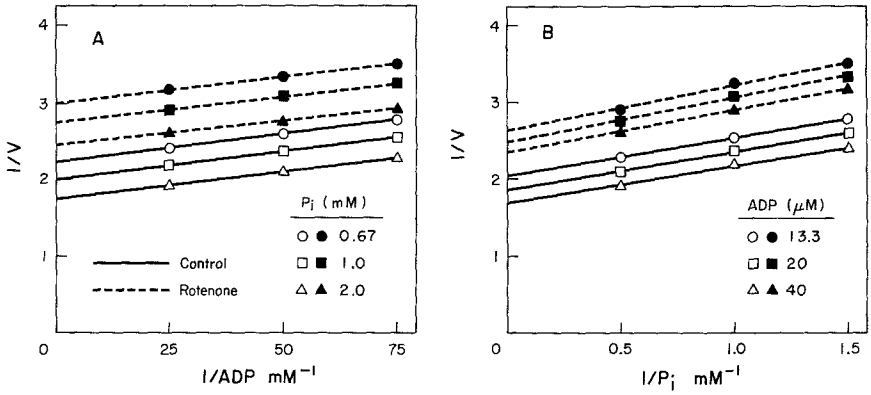



**Fig. 6.** Effects of rotenone on reciprocal plots of initial velocity vs. ADP (A) and $P_i$ (B) concentration. Each data point represents the mean of the values obtained in three identical experiments. The conditions and limiting kinetic constants are given in Table III.

In the carboxyatractyloside study we observed that for a given mitochondrial preparation, virtually the same value for total concentration of AdN carrier can be obtained from the initial velocity data as from the inner-membrane-contraction data. Thus, we found that if by trial and error we simply find the value that gives the best fit of a straight line to reciprocal plots of initial velocity vs. effective concentration of the carrier, we have a value which is virtually identical to the one obtained by the inner-membrane-contraction method. This finding is in accord with the theoretical approximations noted in the Introduction and indicates that the concentrations of other catalytic components can be determined with a fair degree of accuracy from the effects of highly specific, tight-binding inactivators of the components on the velocity of the overall reaction. By carrying out the determinations under different conditions of ADP and $P_i$ concentration, one can at the same time determine the coupling relationships of the reactions catalyzed by the components to the AdN and $P_i$ transport reactions.

Because antimycin binds rapidly and tightly to a single component of the respiratory chain (30, 35), the antimycin-sensitive component was selected for a study of this type. In preliminary experiments it was found that changing the concentration of either ADP or $P_i$ has no effect on the $K_m$ of this component, as expected from the lack of effect of antimycin inhibition on the $K_m$'s of ADP and $P_i$ (Table III). Therefore, in the subsequent, more accurate experiments, the results of which are summarized in Fig. 7 and Table IV, this

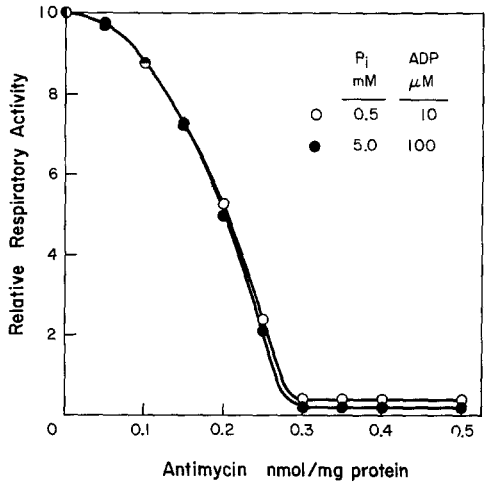



**Fig. 7.** Effect of antimycin on the initial velocity of the overall oxidative phosphorylation reaction under conditions of high and low ADP and $P_i$ concentration. Each data point represents the mean of the values obtained in three experiments. The kinetic constants obtained in these experiments are presented in Table IV.

**Table IV.** Estimates of the Concentration and $K_m$ of the Antimycin-Sensitive Component[a]

| ADP ($\mu$M) | $P_i$ (mM) | $V_{max}$ ($\mu$atom O/min/mg) | Concentration (nmol/mg) | $K_m$ (nmol/mg) |
|---|---|---|---|---|
| 10 | 0.5 | 0.36 ± 0.03 | 0.14 ± 0.02 | 0.007 ± 0.003 |
| 100 | 5.0 | 0.71 ± 0.04 | 0.13 ± 0.02 | 0.007 ± 0.003 |

[a]The values are means ± SD of three experiments. The methods employed are given in the text. $V_{max}$ is the velocity of the overall oxidative phosphorylation reaction under conditions of saturation with the antimycin-sensitive component.

was demonstrated by varying the concentrations of ADP and $P_i$ together to make possible more repeat determinations within the individual experiments. From the results the concentration of the antimycin-sensitive component was estimated to be approximately 0.13 nmol/mg protein and the $K_m$ of the component 0.007 nmol/mg protein (Table IV). These values were obtained using only the portion of the velocity data obtained under conditions of 0, 0.05, and 0.10 nmol antimycin/mg protein and assuming complete binding of the added antimycin. Thus, in experiments in which the concentration of the antimycin-sensitive component was estimated to be 0.13 nmol/mg protein, the effective concentrations of the component were assumed to be 0.13, 0.08, and 0.03 nmol/mg protein, and reciprocal plots of these values vs. the initial velocities associated with them form a straight line from which the $K_m$ of the component was estimated. In the individual experiments the $K_m$ was estimated using the mean of the two concentration estimates obtained under the different conditions of ADP and $P_i$ concentration.

Since it is unlikely that all the added antimycin was actually bound in these studies, the concentration estimates are likely high. Previous estimates obtained with intact bovine heart mitochondria are considerably higher than those obtained here. Thus, Berden and Slater (30), using a fluorometric technique, obtained a value of about 0.27 nmol/mg protein, and the estimate of cytochrome $c_1$ concentration of Blair et al. (36) indicates the concentration of the component to be 0.21 nmol/mg protein. The latter estimate is based on the finding of Rieske et al. (35) that the concentration of the antimycin-sensitive component equals the concentration of cytochome $c_1$ in heart mitochondria.

As in evident from Fig. 7 and similar studies by Klingenberg and Kröger (32, 33), the shape of respiratory inhibition curves in which respiratory activity is expressed relative to the control is affected by a given change in conditions of incubation only if the change results in a change in the degree to which the inhibited reaction limits the overall reaction. This would not hold, of course, if the change in conditions were to somehow affect the binding of

the inhibitor, or if, as likely occurs in the case of carboxyatractyloside inhibition when the extent of the inhibition is large (Fig. 4), inhibition were to result in uncoupling of the inhibited reaction from the indicator reaction. In the case of antimycin inhibition, the small separation of the curves observed under the different conditions of ADP and $P_i$ concentration (Fig. 7) appears to have been due entirely to the presence of a small amount of antimycin-insensitive respiration which was not affected by the change in the concentrations of ADP and $P_i$.

### *Effects of Oligomycin, Aurovertin, DCCD, and Triethyltin*

Oligomycin (37), aurovertin (38), DCCD (39), and triethyltin (40) are fairly specific inhibitors of the phosphorylation step of the overall oxidative phosphorylation reaction. As shown in Table V, these inhibitors have essentially identical effects on the kinetics of the overall reaction, decreasing $V$ and the limiting $K_m$ of ADP. An exception may be triethyltin, which apparently has the additional effect of slightly increasing the $K_m$ of $P_i$.

Although the mechanisms by which these agents inhibit the phosphorylation reaction are not known, it is generally thought that they bind at different sites within the ATPase complex which catalyzes the phosphorylation reaction. Since all four have essentially the same effects on the kinetic parameters determined, there is no indication of there being different steps within the phosphorylation reaction coupled by freely diffusible intermediates (i.e., intermediate reactants that are free to diffuse from one ATPase complex to another within a given mitochondrion). However, this does not rule out the presence of such steps (determination of whether or not such steps exist would require determination of the effects on the $K_m$ of one of the inhibitor-sensitive components of varying the effective concentrations of the other inhibitor-

**Table V.** Effects of Oligomycin, Aurovertin, DCCD, and Triethyltin on the Limiting Kinetic Constants[a]

| Number of experiments | Inhibitor (nmol/mg) | $V$ (μatom O/min/mg) | $K_{ia}$ (mM) | $K_a$ (mM) | $K_{ib}$ (μM) | $K_b$ (μM) |
|---|---|---|---|---|---|---|
| 1 | None | 0.62 | 0.18 | 0.33 | 2.7 | 4.9 |
| | 0.15 Oligomycin | 0.49 | 0.16 | 0.35 | 1.3 | 2.7 |
| | 0.16 Aurovertin | 0.47 | 0.16 | 0.32 | 1.4 | 2.7 |
| 1 | None | 0.62 | 0.06 | 0.32 | 0.8 | 4.0 |
| | 15 DCCD | 0.49 | 0.05 | 0.31 | 0.3 | 2.0 |
| | 6.7 triethyltin | 0.48 | 0.04 | 0.37 | 0.3 | 2.2 |

[a]Substrate $A$ = $P_i$; substrate $B$ = ADP.

sensitive components). Regardless of what the case may be, it is appropriate to equate inhibition with a decrease in the effective concentration of the ATPase complex, and thus with an increase in the degree to which the phosphorylation reaction is rate limiting in the overall reaction. The results (Table V) indicate that the coupling relationship between the AdN transport and phosphorylation reactions is sequential, and that the coupling relationship between the $P_i$ transport and phosphorylation reactions is nonsequential. As can be seen from Fig. 8, which presents reciprocal plots of the initial velocity data obtained in the oligomycin experiment of Table V, the convergence to the right of the "oligomycin" and "control" reciprocal plots of initial velocity vs. ADP is sufficiently strong to suggest that the AdN transport and phosphorylation reactions are linked in simple sequence. Thus, it can be seen (Fig. 8A) that if the overall oxidative phosphorylation reaction were studied as a bisubstrate reaction, considering external ADP and the oligomycin-sensitive component to be the substrates, reciprocal plot patterns similar to those of Fig. 3 would be obtained.

These findings were tested in the same manner in which the relationship of the antimycin-sensitive reaction to the AdN and $P_i$ transport reactions was tested, using aurovertin to vary the effective concentration of the ATPase complex. Although aurovertin binds rapidly (41) and specifically to the ATPase, a number of studies (42–45) suggest that the binding is very complex, that there are two kinds of binding sites which vary in number and affinity depending on the conditions of incubation. However, a relatively recent study (46) suggests that in intact mitochondria aurovertin binds to only one of the two kinds of sites. Therefore, the presence of only one kind of

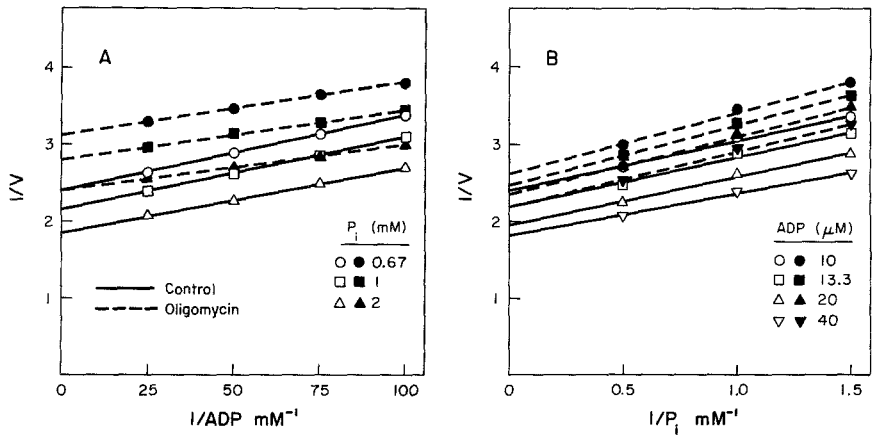



**Fig. 8.** Effects of oligomycin on reciprocal plots of initial velocity vs. ADP (A) and $P_i$ (B) concentration. The conditions and limiting kinetic constants are given in Table V.

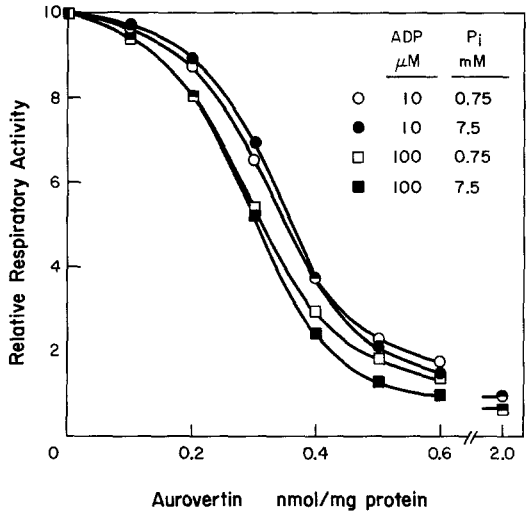



**Fig. 9.** Effects of aurovertin on the initial velocity of the overall oxidative phosphorylation reaction under conditions of high and low ADP and $P_i$ concentrations. Each data point represents the mean of the values obtained in two identical experiments. The mitochondria were preincubated for 3 min before initiating State 3 respiration. Kinetic constants derived from these and two additional experiments of the same kind are presented in Table VI.

site will be assumed here. In any case, the same results in regard to coupling relationships can be expected, since all that is needed for this determination is that the effective concentration of the aurovertin-sensitive component be decreased in a reproducible and specific manner.

Four comprehensive experiments were conducted, two of which include determination of complete aurovertin inhibition curves (Fig. 9). In each the effects of low and high concentrations of ADP and $P_i$ in four combinations were compared. The concentration and $K_m$ of the aurovertin-sensitive component were estimated from the initial velocity data obtained under conditions of 0, 0.1, and 0.2 nmol aurovertin/mg mitochondrial protein, assuming complete binding of the added aurovertin and using the same method employed in the antimycin study. Table IV shows that a concentration of approximately 0.30 nmol/mg protein is obtained by this method, a value in good agreement with the value 0.27 nmol/mg protein obtained by Bertina et al. (47) with intact rat heart mitochondria by a fluorometric technique. The $K_m$ of the aurovertin-sensitive component was found (a) to increase markedly as the concentration of ADP is increased, (b) to decrease slightly as the concentration of $P_i$ is increased under conditions of low ADP, and (c) to undergo no change as the concentration of $P_i$ is increased under conditions of

**Table VI.** Estimates of the Concentration and $K_m$ of the Aurovertin-Sensitive Component and the Effects of ADP and $P_i$ Concentration on the $K_m$[a]

| ADP ($\mu$M) | $P_i$ (mM) | $V_{max}$ ($\mu$atom O/min/mg) | Concentration (nmol/mg) | $K_m$ (nmol/mg) |
|---|---|---|---|---|
| 10 | 0.75 | 0.38 ± 0.06 | 0.35 ± 0.04 | 0.036 ± 0.017 |
| 10 | 7.5 | 0.48 ± 0.05[b] | 0.31 ± 0.03 | 0.030 ± 0.016[b] |
| 100 | 0.75 | 0.56 ± 0.08[b] | 0.30 ± 0.03 | 0.068 ± 0.033[b] |
| 100 | 7.5 | 0.84 ± 0.10[b] | 0.31 ± 0.04 | 0.069 ± 0.036[b] |

[a]The values are means ± SD of four experiments. The methods employed and assumptions made are given in the text. The mitochondria were preincubated for 3 min before initiating State 3 respiration. $V_{max}$ is the velocity of the overall oxidative phosphorylation reaction under conditions of saturation with the aurovertin-sensitive component.

[b]Significantly different from the value obtained under conditions of 10 $\mu$M ADP and 0.75 mM $P_i$ ($P < 0.05$, paired $t$-test).

high ADP (Table VI). With the exception of (b), these observations are in agreement with the experiments of Table V indicating that the coupling relationship between the AdN transport and phosphorylation reactions is sequential and that the coupling relationship between the phosphorylation and $P_i$ transport reactions is nonsequential. Observation (b) can be explained by assuming the existence of the allosteric effect of $P_i$ on the AdN carrier. Thus, under conditions of low ADP, but not under conditions of high (saturating) ADP, an increase in the concentration of $P_i$ and the consequent decrease in the affinity of the AdN carrier results in an increase in the degree to which the AdN transport reaction is rate limiting. Since the coupling relationship between the AdN transport and aurovertin-sensitive reactions is sequential, this results in a decrease in the degree to which the aurovertin-sensitive reaction is rate limiting, and thus in a decrease in the $K_m$ of the catalytic component of this reaction.

## Conclusions

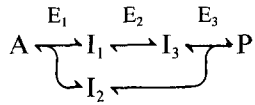
The results of this study appear to be consistent with the conclusions (a) that the coupling relationship is sequential between the AdN transport and rotenone-sensitive reactions, the AdN transport and cyanide-sensitive reactions, the $P_i$ transport and rotenone-sensitive reactions, the $P_i$ transport and cyanide-sensitive reactions, and the AdN transport and phosphorylation reactions, and (b) that the coupling relationship is nonsequential between the AdN and $P_i$ transport reactions, the AdN transport and antimycin-sensitive reactions, the $P_i$ transport and antimycin-sensitive reactions, and the $P_i$

transport and phosphorylation reactions. In the sequential group are two reaction pairs, the $P_i$ transport–rotenone-sensitive pair and the $P_i$ transport–cyanide-sensitive pair, in which the individual reactions are clearly not coupled in simple sequence by the criterion of Fig. 3 and thus appear to be coupled in a partially nonsequential manner. In fact, of this group, only the AdN transport and phosphorylation reactions appear to be linked in simple sequence by this criterion. Present information indicates that these reactions are linked coenzymically by the freely diffusible ADP and ATP of the matrix (48).

The mechanisms by which the relatively numerous nonsequential and partially nonsequential relationships come about is presently uncertain. However, some light can be shed on this problem by considering the principal means by which the individual reactions of the overall oxidative phosphorylation reaction might be expected to affect each other in regard to the degrees to which they limit the overall reaction. Consider, for example, the AdN and $P_i$ transport reactions. Disregarding the apparent allosteric effect of $P_i$, the coupling relationship between these reactions appears to be fully nonsequential. This means that changing the effective concentration of the catalytic component of one of these reactions by such direct means as changing the concentration of the external substrate has no effect on the apparent $K_m$ of the external substrate or the catalytic component of the other, and thus that changing the degree to which one is rate limiting by such means has no effect on the degree to which the other is rate limiting. In attempting to understand how this comes about, it is important to realize that in multienzyme reactions in which the individual reactions are linked by freely diffusible intermediates, changing the effective concentration of the enzyme of one individual reaction changes the effective concentrations of the enzymes of all the others.This is clearly evident from the fact that doing this changes the net forward velocities of all the other individual reactions. Since it is likely that the AdN and $P_i$ transport reactions are linked in this manner, it seems likely that changes in the effective concentration of the catalytic component of one of these reactions somehow elicit changes in the effective concentration of the catalytic component of the other which are just sufficient to prevent this other reaction from changing in regard to how much it limits the overall reaction. And since it is likely that the intermediate reactants that link the AdN and $P_i$ transport reactions are confined to the mitochondria, it seems likely that the transmission of these changes in effective concentration of catalytic component between the two reactions occur through changes in the concentrations of internal intermediate reactants. In other words, it seems likely that when for example, the effective concentration of the $P_i$ carrier is increased by direct means, resulting in the $P_i$ transport reaction being less rate limiting, the

effective concentration of the AdN carrier, through an increase in the ratio of internal ATP (a substrate in the AdN transport reaction) to internal ADP (a product in the AdN transport reaction), increases during the approach to the new steady state to the extent that by the time the new steady state has been reached, the AdN transport reaction is no more limiting than it was before the effective concentration of the $P_i$ carrier was increased.

A coupling arrangement that could give this result is one in which the individual reactions of the pair have characteristics and locations in respect to each other and to other individual reactions such that one individual reaction of the pair removes freely diffusible intermediate reactants at two or more points which are situated symmetrically about the other. This can be seen by considering the simple model reaction




(2)

which is identical to overall reaction (1) except that two products are formed in the first individual reaction of the sequence, one of which is a substrate in the third individual reaction. With this modification, the $E_3$-catalyzed reaction removes freely diffusible intermediates at two points which are situated symmetrically about the $E_2$-catalyzed reaction. That the coupling relationship between the $E_2$- and $E_3$-catalyzed reactions might, in consequence, be fully nonsequential is best seen (in the absence of quantitative methods) by assuming $I_3$ to be identical to $I_2$ and considering what would happen during the approach of the overall reaction to a new steady state if the degree to which either the $E_2$- or $E_3$-catalyzed reaction is rate limiting were changed by direct means. For example, if the $E_3$-catalyzed reaction were made less rate limiting by increasing the actual concentration of $E_3$, an immediate (pre-steady state) result would be that the $E_2$-catalyzed reaction becomes more rate limiting. However, during the approach of the overall reaction to the new steady state (i.e., to the new state in which the concentrations of the intermediate reactants are constant), the concentration ratio of $I_1$ to $I_2$ would increase (due to $I_2$ being consumed faster than it is produced), resulting in the $E_2$-catalyzed reaction becoming less rate limiting and in the $E_3$-catalyzed reaction becoming more rate limiting. Since any tendency for the $E_2$-catalyzed reaction to become more rate limiting as a result of the $E_3$-catalyzed reaction being made less rate limiting would tend to increase the ratio of $I_1$ to $I_2$, it seems clear that these changes would proceed to the point of complete reversal of the increase in the degree to which the $E_2$-catalyzed reaction is rate limiting. Nevertheless, before we can be fully certain of this, some means must be found to treat the system quantitatively.

If this explanation of the nonsequential coupling relationship is correct, the partially nonsequential coupling relationships observed in this study can be explained by assuming that one individual reaction of a given pair exhibiting this relationship removes products at more than two points which are situated unsymmetrically about the other individual reaction of the pair. Preliminary attempts to explain the observed coupling relationships in terms of these mechanisms and of various models of the overall oxidative phosphorylation reaction have been quite successful in the cases of certain chemiosmotic models. The results of these attempts will be detailed in a later communication if the testable predictions of the models should prove to be correct under thorough testing.

Since the concentrations and $K_m$'s of three catalytic components were estimated in this study, a comparison of the individual reactions catalyzed by these components in regard to the degrees to which they are rate limiting can be made. Thus, the degrees to which the antimycin-, aurovertin-, and carboxyatractyloside-sensitive reactions are rate limiting, as indicated by the concentrations of the catalytic components expressed as multiples of their $K_m$'s are approximately 19, 9, and 2, respectively, under conditions of 10 $\mu$M ADP, and 19, 4, and 4, respectively, under conditions of ADP saturation. In making this comparison, the concentration of $P_i$ need not be considered, because changes in this parameter have no effect whatever on the degree to which the antimycin-sensitive reaction is rate limiting and affect the degrees to which the aurovertin- and carboxyatractyloside-sensitive reactions are rate limiting only slightly and apparently only through the apparent allosteric effect of $P_i$ on the AdN carrier.

A particularly interesting observation of this study is the nonsequential coupling relationship. As pointed out above, nonsequential coupling between two individual reactions is indicated if inhibition of one has no effect on the factor by which inhibition of the other decreases the rate of the overall reaction (e.g., see Fig. 7). This characteristic has been observed previously in studies by Baum et al. (49) on submitochondrial particles, by Stubbs et al. (50) on isolated hepatocytes, and by Lemasters and Sowers (51) on rat liver mitochondria. In the very recent study of Lemasters and Sowers, it was noted that decreasing the rate of the overall oxidative phosphorylation reaction by decreasing the concentration of $P_i$ has no effect on the factor by which atractyloside inhibits the overall reaction. From this the investigators concluded that the AdN transport reaction does not become less rate limiting when the rate of the overall reaction is decreased by decreasing the concentration of $P_i$. This conclusion is consistent with our observations and indicates the existence of a fully nonsequential coupling relationship between the AdN and $P_i$ transport reactions in rat liver mitochondria.

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