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Kinetic Relationships of Some Factors Affecting the Time of Onset of Mitochondrial Swelling*

Jerald L. Connelly,† Gary W. Evans, and Curtis H. Hallstrom‡

ABSTRACT: Evidence is presented which demonstrates that time of onset (T_0) of rat liver mitochondrial swelling is an appropriate and useful parameter in experiments designed to determine kinetic relationships between factors affecting T_0. The competitive inhibition of succinate-increased T_0 by malonate as shown by $1/T_0$ *vs.* 1/[succinate] plots provides a model for further kinetic experiments and, in addition, demonstrates the requirements for substrate oxidation in substrate-supported increased T_0. A competitive relationship between P_i, as swelling agent, and substrate, as protective agent, has been demonstrated.

Adenosine diphosphate, required in addition to respiratory substrate for optimum protection, acts independently of P_i concentration.

It is currently accepted that several energy-requiring mechanisms in the mitochondrion are closely, if not intimately related. These include, in addition to phosphorylation of ADP, ion movement across the mitochondrial membranes, and NADH–NADP transhydrogenation. Furthermore, it has been observed that these functions are often reflected by variations in the internal or gross structure of the mitochondrion. Such findings are not altogether surprising since the early studies of Raaflaub (1953), and many more recent studies, demonstrated that gross structural variation, swelling, and contraction, are inducible and controllable by agents such as ATP, ADP, P_i, and respiratory substrates, which are common to energy-producing and -requiring processes.

It is likely, then, that a thorough knowledge of the mechanisms involved in the control of mitochondrial structural change would contribute to the understanding of the related energy-linked processes. Accordingly, this laboratory has undertaken to ascertain the chemistry of the mechanisms involved in the mitochondrial processes which act to retard the swelling process. Connelly and Hallstrom (1967) reported that ADP and respiratory substrate have primary roles in processes which delay mitochondrial swelling under conditions in which oxidative phosphorylation is prevented by oligomycin. Previous reports (Connelly and Hallstrom, 1966a, 1967) indicated an inverse relationship between P_i concentration and time of onset (T_0) of swelling. These observations suggested that P_i may be encouraging a shorter T_0 by affecting, in some manner, the protective roles of substrate, ADP, or both. This

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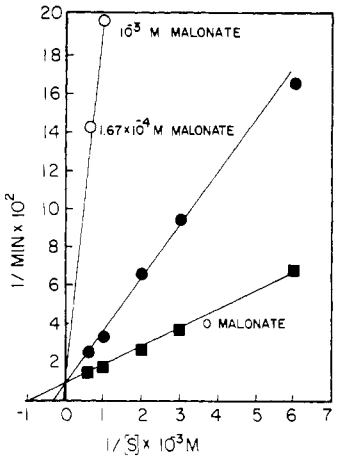

FIGURE 1: Kinetic relationship of malonate inhibition of succinate-increased T_0. Media are the same as described in Table I with 1 μg/ml of oligomycin in all systems. Malonate and succinate were present from the beginning at the concentrations noted.

report describes the evaluation of this problem by the application of kinetic techniques which employ T_0 as an fundamental parameter.

Experimental Section

The preparation of rat liver mitochondria and the conduct of swelling studies was essentially the same as previously described (Connelly and Lardy, 1964; Connelly and Hallstrom, 1966a). The experimental approach to determining the kinetic relationship between inorganic phosphate and substrate and between inorganic phosphate and ADP is described in the legends of Figures 3 and 4.

Of general concern in the overall question of mechanism relative to T_0 is the viability of mitochondria prior to and following T_0. This is particularly pertinent to the participation of substrate and could be indicated by respiratory rates and respiratory control ratios. While this aspect is treated more

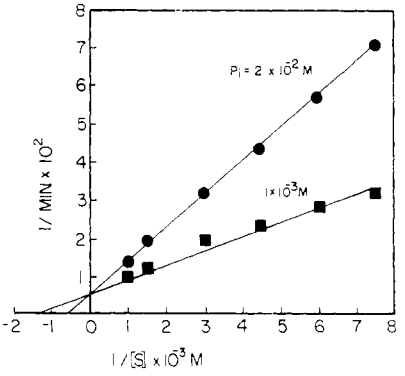

FIGURE 2: The kinetic relationship of inorganic phosphate and succinate. Media contained 138 mM sucrose, 16.7 mM K^+ histidine (pH 7.0), 1×10^{-4} M ADP, 1 μg/ml of oligomycin, 0.1 ml of mitochondrial suspension (2.5 mg of protein), and enough water plus additions for a total volume of 6 ml. Additions were 1×10^{-3} M and 2×10^{-2} M NaP_i (pH 7.0) and succinate present from the beginning at the concentrations noted. Experiments were generally conducted at pH 7.0 rather than pH 7.5 so that a lower P_i concentration could be used without obtaining unreasonably large T_0 values. Similar but less distinct kinetic relationships were obtained at pH 7.5 using P_i concentrations of smaller difference.

TABLE I: The Effect of Malonate on Systems Increasing T_0.[a]

Additions	T_0 − Malonate (min)	T_0 + Malonate (min)
None	7	4
Oligomycin	7	7
Succinate	11	5
ADP	19	11
ADP + oligomycin	16	11
Succinate + oligo	23	7
Succinate + ADP	51	17
Succinate + ADP + oligo	56	14

[a] The media contained 138 mM sucrose, 16.7 mM K^+ histidine (pH 7.5), 5 mM NaP_i (pH 7.5), 0.1 ml of mitochondrial suspension (2.5 mg of protein), and enough water plus additions for a total volume of 6 ml. Additions as indicated in the table are: 1 μg/ml of oligomycin, 0.167 mM Na^+ succinate, and 0.5 mM NaK^+ ADP. All additions were made prior to addition of the mitochondria. Time of onset of swelling is expressed in minutes.

thoroughly elsewhere (D. R. Myron and J. L. Connelly, unpublished work) it is worth noting here that (1) in the presence of oligomycin the rate of substrate disappearance does not change with substrate concentration but T_0 increases proportionally, and (2) under these conditions (oligomycin) the significance of control ratios is unknown.

Results

The observation that oligomycin increases the T_0 of swelling in the presence of substrate (Connelly and Hallstrom, 1967; Chappell and Greville, 1961) necessitates an explanation of the substrate role under these conditions where respiration, as well as phosphorylation, is inhibited. Lardy *et al.* (1965) reported inhibition of succinate oxidation by 72% and glutamate oxidation by 94% in the presence of oligomycin A. Thus, the possibility remained that the substrate role in delaying T_0 could be mediated through the residual substrate oxidation. Malonate, which inhibits succinic dehydrogenase, was used as a tool to determine whether succinate oxidation is required to give its effects on T_0. Table I shows that malonate effectively eliminates the influence of succinate in all systems, suggesting that succinate is increasing T_0 through its conversion into fumarate.[1] Application of the Lineweaver–Burk plots (Dixon and Webb, 1964) to these studies resulted in the classical competitive inhibition relationship (Figure 1).

The results of this experiment encouraged the utilization of T_0 as an appropriate parameter for the evaluation of kinetic relationships among other factors affecting T_0. Of primary interest is the relationship between P_i as swelling agent and substrate and ADP as protective agents. In exploratory ex-

[1] Subsequent to this work several approaches have verified the need for substrate utilization in the delay of T_0. This phenomenon is nonspecific for oxidizable substrates. Pertinent to the use of malonate is the inhibition of the protective effects of citrate by fluorocitrate. A full report of the role of substrate in mitochondrial integrity is submitted for publication.

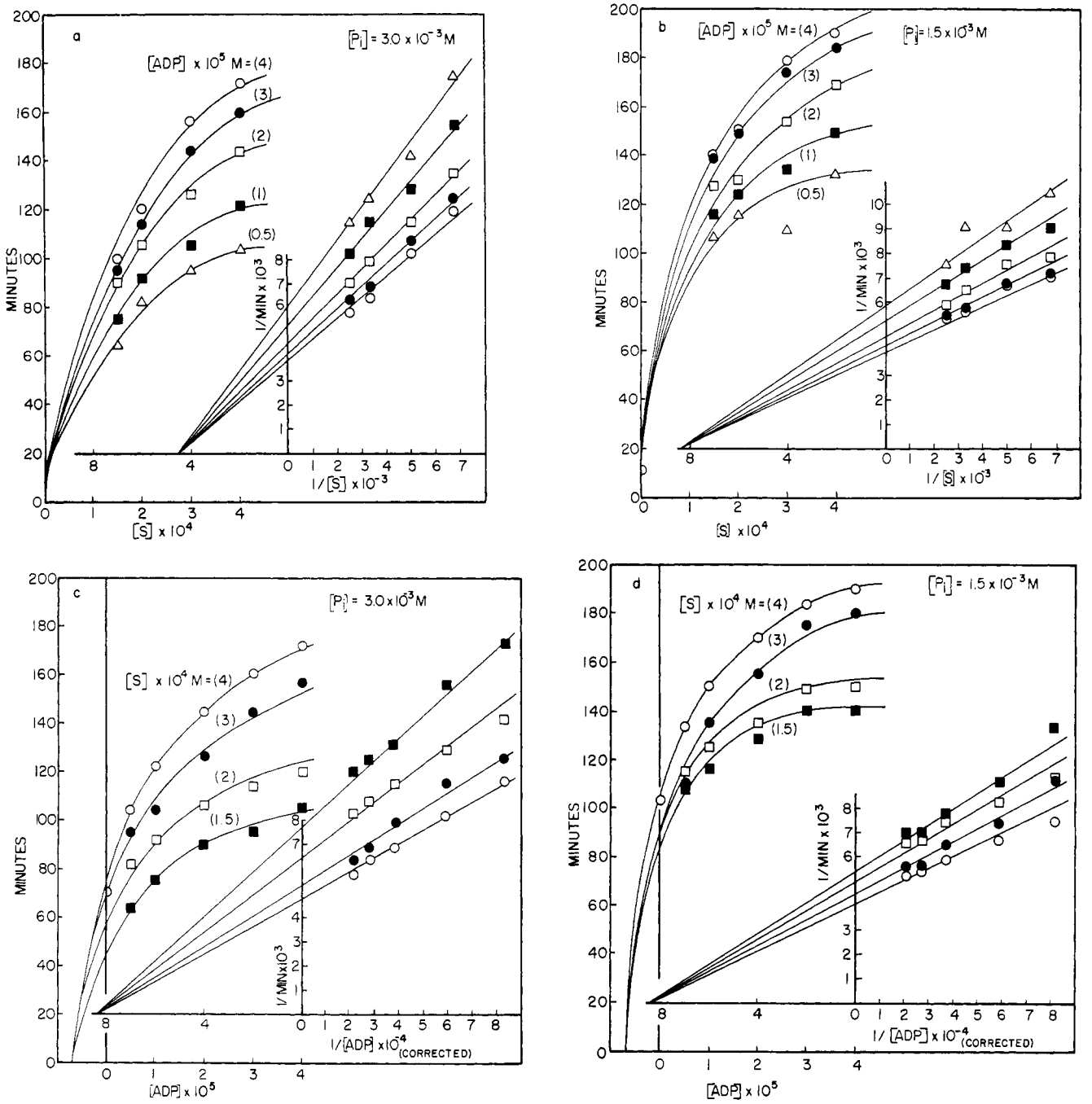

FIGURE 3: The kinetic interrelationship of inorganic phosphate, glutamate, and ADP. Media are the same as in Figure 2 with 3×10^{-3} M P_i (3a, 3c) and 1.5×10^{-3} M P_i (3b, 3d). Substrate (glutamate) and ADP concentrations are as indicated. In accordance with the primary data of plots 3c and 3d, inverse plots were constructed using an ADP concentration equal to added ADP plus 0.7×10^{-5} M. This correction, demanded by consistent intercept of these experimental curves with the x axis, apparently account for the significant contribution of endogenous ADP. The value indicated by data from several experiments agrees quite closely with the levels of ADP in mitochondria determined directly (Pressman, 1958).

periments, it was most convenient to hold ADP concentration constant (Connelly and Hallstrom, 1966b) and to examine the relationship between P_i and substrate. Furthermore, due to the impracticability of obtaining T_0 values in the absence of P_i, it was necessary to determine the nature of inhibition by conducting the experiment at two different but constant P_i concentrations. The results shown in Figure 2 show that this interrelationship follows the Michaelis–Menten kinetics for apparent competitive inhibition. Solving simultaneous equations (Dixon and Webb, 1964, eq VIII-16) at the two inhibition concentrations yields a K_m for succinate of 7.2×10^{-4} M and a K_i for P_i of 1.3×10^{-2} M.

This preliminary finding indicated the feasibility of applying kinetics to the investigation of interrelationships among P_i, ADP, substrate, and T_0. To this end experimental conditions were designed to show whether P_i influences the protective roles of substrate and ADP and, if so, in what way. Figure 3a–d depicts the relationship between substrate and ADP at two levels of P_i. At either concentration of P_i in the presence of oligomycin, the kinetic relationship between ADP and

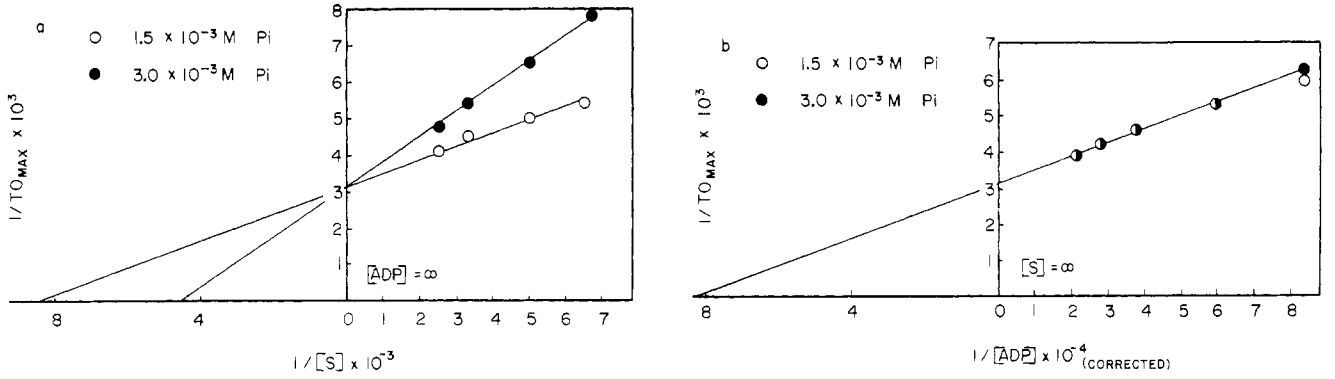

FIGURE 4: Secondary plots of the influence of P_i on substrate: T_0 and ADP:T_0 relationships. Intercepts of the y axis of Figures 3a and 3b, where $[S] = \infty$, are plotted against [ADP] as shown in Figure 4b. Intercepts of the y axis of Figures 3c and 3d, where $[ADP] = \infty$, are plotted against $[S]$ as shown in Figure 4a. This technique of analysis is essentially that described by Mahler and Cordes (1966).

glutamate[2] is consistant with that of a two-substrate system for which the common intercept of the double-reciprocal plots is on the base line. By comparing Figures 3a and 3b, it can be seen that the $K_{m,app}$ of glutamate varies directly with P_i to an extent equal at all concentrations of ADP employed. On the other hand, variation in the concentration of P_i appears to have little effect on the $K_{m,app}$ of ADP (Figure 3c,d). These findings are verified and supplemented by the secondary plots (Figure 4a,b) which demonstrate that the influence of P_i on substrate utilization is of the classical competitive inhibition type and that P_i is without effect on the function of ADP. The identity of the limiting intercepts ($1/T_{0,max}$) of these two plots serves as a measure of internal consistency and attests to the reliability of the primary data.

Discussion

The utilization of T_0 appears to have considerable merit in evaluating kinetic relationships among factors affecting mitochondrial integrity. Treatment of this parameter and associated factors with direct and double-reciprocal plotting consistantly demonstrates that the kinetics involved are of the Michaelis–Menten type. This is especially supported by the classical competitive-type plots relating T_0, succinate, and malonate[3] (Webb, 1966).

The mechanism of action of P_i in swelling, under conditions used in this work, is not fully understood. However, the findings reported here firmly link the role of this swelling agent to one of the factors (substrate utilization) responsible for the maintenance of mitochondrial integrity. Slater and Bonner (1952) using heart muscle preparations showed that succinic dehydrogenase was competitively inhibited by P_i. Although such a direct effect might account for the influence of P_i on succinate-increased T_0, the similar inhibition of glutamate-increased T_0 calls for an alternative mechanism. One possibility is that added P_i induces the removal of a substrate-generated energy source which directly regulates T_0. This explanation is compatible with phosphorylation of an intermediate contributing to the work function (see Scheme I of Lardy *et al.*, 1964) supporting increased T_0 or with the competitive disruption of a substrate-generated chemiosmotic gradient (Mitchell, 1966). Such an explanation is suggestive of a competition, for respiratory-generated energy, between those mechanisms which lengthen T_0 and other energy-requiring processes in the mitochondrion. An explanation of this nature is supported by observed competitive relationships between phosphorylation and the energy-linked transhydrogenase (Lee and Ernster, 1966) or ion accumulation (Rossi and Lehninger, 1964).

While the mode of action of ADP is not known, the complete independence from P_i concentration of this aspect of protection implies that free inorganic phosphate is not itself an integral component of the mechanism. Although there is no direct experimental evidence, the possibility remains that the ADP structure is exerting directive influence through allosteric means.

[2] The phenomena noted in Figure 3a–d have been observed for succinate as well as glutamate. Data for the latter are presented to indicate that the mechanism involved is not substrate specific and is operable with either flavin- or NAD-linked respiration.

[3] The results of the malonate experiments are of further significance since they indicate a requirement for succinate oxidation, not inhibition of oxidation (Chappell and Greville, 1959), in the maintenance of mitochondrial integrity. These observations are consistent with those of Di Sabato and Fonnesu (1959) and Kaufman and Kaplan (1960) who also observed malonate reversal of succinate delayed T_0.

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Synthesis and Decay Rates of Major Classes of Deoxyribonucleic Acid Like Ribonucleic Acid in Sea Urchin Embryos*

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ABSTRACT: A method is described for measuring the instantaneous rates of synthesis and the spectrum of stabilities of unstable RNA by analyzing the kinetics of entry of radioactive adenosine into ATP and into RNA. This method was applied to sea urchin embryos during their development when the level of accumulation of heterogeneous, unstable RNA with DNA-like base compositions decreases substantially. The RNA appeared to fall into two classes determined on the basis of stability. One-third of the steady-state level of unstable RNA has a half-life of 5–10 min and the rest a half-life of 60–90 min.

This spectrum does not change between blastula and pluteus stages, while the instantaneous rate of synthesis of RNA per nucleus is decreasing.

Recent experiments have shown that the accumulation per nucleus of DNA-like RNA[1] decreases gradually during the development of sea urchin embryos from cleavage to pluteus stages (Emerson and Humphreys, 1970). Previous studies indicate that most DNA-like RNA synthesized in animal cells is unstable; most of it appears to be restricted to the nucleus and decays with an average half-life estimated at from 3 to 30 min (Attardi *et al.*, 1966; Soiero *et al.*, 1968; Scherrer *et al.*, 1966; Penman *et al.*, 1968). A small fraction associated with polysomes has an estimated half-life of several hours (Penman *et al.*, 1963; Attardi *et al.*, 1966; Penman *et al.*, 1968). The extent of heterogeneity in the stabilities of these two classes of RNA and the possibility of other classes of unstable RNA (Penman *et al.*, 1968) have not been scrutinized. Because most or all DNA-like RNA is unstable, its level of accumulation is related to both its rates of synthesis and its rates of decay. In order to analyze the developmental changes in levels of accumulation, we had to determine the instantaneous rates of synthesis and the spectrum of stabilities of all DNA-like RNA molecules synthesized in the embryos. This was done by determining the kinetics of entry of radioactive adenosine into the ATP precursor pool and into RNA. Sea urchin embryos were favorable material for the study of the complete spectrum of unstable RNA species because unstable RNA represented at least 85% of the newly synthesized RNA accumulated during several hours of labeling (Emerson and Humphreys, 1970; and Results).

Material and Methods

Culturing and Processing of Embryos. Embryos of *Lytechinus pictus* were cultured at 16.5° on a rotary shaker as an 0.5% suspension (v/v) in artificial sea water, with penicillin (80 unit/ml) and streptomycin (50 μg/ml). Embryos cultured beyond mesenchyme blastula stage were resuspended at 18 and 40 hr in fresh sea water at a concentration of 0.05% embryos. The experiments were performed on mesenchyme blastulae (18–22.5 hr after fertilization) or early plutei (44–50 hr). Cultures used had at least 98% fertilization and normal development.

Blastulae were suspended at 2.5% concentration, and incubated with 10 μCi/ml of [8-^{3}H]adenosine (28 Ci/mmole; Schwartz). Sample aliquots of 0.8 ml were taken at various times after addition of the radioactive label. Plutei, suspended at 0.5%, were incubated with 2.5 μCi/ml of tritiated adenosine; 4-ml samples were taken. The sample of embryos was squirted into excess sea water cooled on ice and washed twice through a 3:1 mixture of acid sea water (0.02 M acetate, pH 4.5) and isotonic sucrose to remove external isotope. The number of embryos per sample (about 20,000) was determined by the method of Hinegardner (1967). The number of nuclei at the beginning and end of each period of incubation was determined by counting stained nuclei in fixed preparations (Emerson and Humphreys, 1970). The pelleted embryos were suspended in ice-cold 0.5 N perchloric acid (always used cold), homogenized with 25 strokes of tight-fitting Dounce homogenizer (Kontes Glass Co.), cooled on ice for 15 min, and centrifuged for 10 min at 27,000*g*. The supernatant was used immediately for isolation of nucleotides, and the pellet resuspended in 2 ml of 0.5 N perchloric acid by vigorously squirting it through an 18-gauge syringe needle, and recentrifuged. The pellet was washed 4 more times with perchloric acid and used to determine radioactivity in RNA.

Counting of Radioactivity. Samples solubilized in 0.3 N

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[1] DNA-like RNA has a base composition similar to DNA and is heterogeneous in size (Emerson and Humphreys, 1970).