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Diphosphopyridine Nucleotide Specific Isocitric Dehydrogenase of Mammalian Mitochondria. II. Kinetic Properties of the Enzyme of the Ehrlich Ascites Carcinoma*

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ABSTRACT: Interactions of substrates, activators, and inhibitors are examined in the reaction catalyzed by the diphosphopyridine nucleotide specific isocitric dehydrogenase of the mitochondria of the Ehrlich ascites carcinoma. Cooperative homotropic interactions in rate–concentration functions are found for isocitrate, magnesium ion, and the effectors, adenosine diphosphate (ADP) and reduced diphosphopyridine nucleotide (DPNH). Isocitrate homotropic cooperativity is pH dependent; ADP abolishes this effect, while DPNH, a negative effector, increases isocitrate cooperativity. Reduced triphosphopyridine nucleotide inhibits the enzyme and manifests somewhat smaller cooperative interactions. ADP antagonizes the inhibition by both reduced pyridine nucleotides. 2,4-Dinitrophenol inhibits the reaction and demonstrates marked homotropic cooperative effects in the presence of ADP or high isocitrate concentration. In the absence of ADP, inorganic phosphate is found to stabilize the enzyme and to activate the reaction. The effect of pH on effector and substrate cooperativity is interpreted as allosteric ligand binding of H^+. The data support a role for the diphosphopyridine nucleotide specific isocitric dehydrogenase in regulation of metabolism and are found to be consistent with the regulatory enzyme model proposed by Monod *et al.* (Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88).

Solubilization and purification of the DPN^+-specific isocitric dehydrogenase (DICDH)[1] of mammalian mitochondria was first described by Plaut and Sung (1954). More recently, Chen and Plaut (1962, 1963) have purified further the beef heart enzyme and have demonstrated a specific activation of the enzyme by ADP manifested in an increased affinity for isocitrate; ADP was found to induce aggregation of the enzyme (Chen *et al.*, 1964), an effect prevented by DPNH, an inhibitor of enzyme activity. Chen and Plaut (1963) have drawn attention to the possibility of a role for DICDH in the control of respiration. The nonclassical, cooperative response of the rate of the DICDH reaction to concentration of substrates, modified by pH and adenine nucleotides, has been demonstrated in a number of laboratories (*Acetobacter peroxydans* (yeast), Hathaway and Atkinson, 1963; Atkinson *et al.*, 1965; *Neurospora*, Sanwal *et al.*, 1963; Sanwal and Stachow, 1965; Sanwal *et al.*, 1965; Sanwal and Cook, 1966; and rat heart and locust flight muscle mitochondria, Goebell and Klingenberg, 1964; Klingenberg *et al.*, 1965).

Monod *et al.* (1965) have proposed a model for the cooperative properties of regulatory enzymes based on changes in ligand affinities resulting from symmetrical cooperative changes in the conformation of protein subunits. The empirical Hill plot has been applied by these authors (Monod *et al.*, 1963) to quantitate cooperative effects in rate data obtained with L-threonine deaminase. This device has been used in the present study to evaluate the allosteric behavior of several substrates and effectors in the DICDH reaction catalyzed by extracts of acetone powders derived from mito-

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[1] Abbreviations used: DPN^+ and DPNH, diphosphopyridine nucleotides, oxidized and reduced, respectively; TPN^+ and TPNH, triphosphopyridine nucleotides, oxidized and reduced, respectively; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphates, respectively; DCIP, 2,6-dichlorophenolindophenol; DICDH, DPN^+-specific isocitric dehydrogenase.

chondria of the Ehrlich ascites carcinoma. The properties of the partially purified enzyme are consistent with the behavior of the respiration of the source mitochondria in the presence of isocitrate (Stein *et al.*, 1967) and support a role for the enzyme in the regulation of the citric acid cycle. The interactions of substrates, activators, and inhibitors are found to be in good agreement with the predictions of the Monod–Wyman–Changeux (1965) model.

Experimental Section

Materials. DPN^+, DPNH, TPNH, trisodium DL-isocitrate, ADP, and *Clostridium kluyveri* diaphorase were obtained from the Sigma Chemical Co. Sephadex G-25 was obtained from the Pharmacia Uppsala. 2,4-Dinitrophenol (DNP) (analytical reagent) was recrystallized from water, mp 112–113.5 (cor). DCIP was repurified by the procedure given by Savage (1957). Ammonium sulfate (enzyme grade) was obtained from the Mann Research Laboratory, Inc.

Enzyme Preparations. Extracts of acetone-dried mitochondria of the Ehrlich ascites carcinoma are prepared in 1 M potassium phosphate buffer (pH 7.2) as described previously (Stein *et al.*, 1967). The extract is adjusted to pH 6.5 with acetic acid and fractionated between 0.27 and 0.37 saturated ammonium sulfate. The pellet is taken up in a minimal volume of 0.5 M potassium phosphate buffer (pH 7.2), passed through a 0.5 × 15 cm column of Sephadex G-25 preequilibrated with the same buffer and aliquotted into separate containers for storage at −15°. Fivefold purification of DICDH activity is thus obtained in 60–70% yield, with a final specific activity of 1.2–1.4 μmoles of DPN reduced/min per mg of biuret protein (Gornall *et al.*, 1949) in the standard DICDH assay described in the previous report (Stein *et al.*, 1967). The TPN^+ specific activity in this fraction is reduced to about 1% of the DICDH unitage.

Assay of DICDH. The protocols of the experiments are indicated in the text. Isocitrate is standardized in the pig heart TPN^+-specific isocitric dehydrogenase system (Ochoa, 1957). All nucleotides and DNP are neutralized prior to use; ADP solutions are prepared fresh daily. In experiments extended over several hours or days, the stock enzyme solutions are assayed periodically to correct for possible decay of activity. Enzyme activity is measured spectrophotometrically by following either the formation of DPNH at 340 mμ or the bleaching of DCIP at 600 mμ coupled to DPNH formation by a large excess of *Clostridium kluyveri* diaphorase. The DCIP assay is used under conditions where it is desired to remove product DPNH or to avoid interference by the large absorbancy of DNP in the 340-mμ region; the assay is linear in enzyme concentration to $\Delta A_{600} = 0.25$/min. DCIP is added to a final absorbancy of 2 at 600 mμ with sufficient clostridial diaphorase to decolorize the reaction mixture in 4–5 sec with added DPNH. With the dehydrogenase operative at close to zero DPNH concentration, it is possible to observe prolonged linear rates of dye reduction, confirming Chen and Plaut's (1963) observation with the beef heart enzyme by a different assay procedure. While an inhibition of diaphorase by DNP is detected, the enzyme is added in sufficient amount to preclude any significant effect on its indicator role in the experiments on the inhibition of DICDH by DNP. Reactions are followed continuously with either a PMQ-II Zeiss spectrophotometer fitted with a Sargent logarithmic recorder, or with a Model 2000 Gilford spectrophotometric system. The reactions are uniformly started by addition of enzyme and are followed for 3–4 min. Since DICDH activity decays fairly rapidly in the absence of stabilizers, the reaction mixture generally contains 5 μmoles of phosphate, partly carried over from the stock of enzyme. The activity of the preparations used is such that almost invariably 10 μl is the largest aliquot used in the 1-ml reaction mixtures.

Treatment of Data. Following Monod *et al.* (1963) the sigmoid response of rate is evaluated graphically by means of the Hill plot. Maximal velocities are obtained at high substrate concentration or by double-reciprocal plots, when necessary. Inhibition of activity by excess substrate is noted under some experimental conditions, requiring extrapolation of the data to produce the maximal velocity required to calculate the data. For the treatment of inhibitor cooperativity where incomplete inhibition is obtained at saturating inhibitor concentration, the quantity $\log (v - v_s)/(v_0 - v)$ is calculated according to Jensen and Nester (1966). V_s, the rate at infinite inhibitor concentration, is evaluated by plotting $(1 - (v/v_0))^{-1}$ against the reciprocal of the square of inhibitor concentration.

Results

Extraction and Stabilization. Extraction of acetone-dried mitochondria as described above yields a relatively stable preparation which may be stored for weeks at −15° with little loss of activity. Storage of extracts in low phosphate or Tris buffers leads to rapid decay of DICDH activity. In the course of inactivation, activity curves are obtained characteristically with a lag period of a few minutes followed by an abrupt increase in rate. In preliminary experiments, reactivation of the partially inactivated enzyme has been achieved by preincubation of the extracts in ADP or potassium phosphate.

Effect of Phosphate on Reaction. Assay of DICDH activity in extracts of mitochondria powders prepared in low phosphate or Tris buffers, or phosphate-stabilized extracts transferred into low phosphate solution, demonstrate in the absence of ADP a requirement for phosphate in the reaction mixture. The dependence of rate on phosphate concentration under the conditions of the experiment shown in Figure 1 follows Michaelis kinetics very closely with an apparent dissociation constant of approximately 1 mM. This value is lower than the concentration of phosphate required to effect significant stabilization. A phosphate requirement has also been shown for the DICDH reaction catalyzed

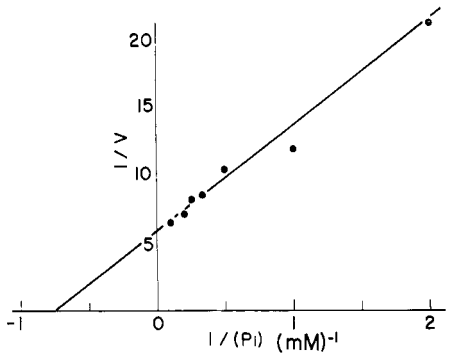

FIGURE 1: The effect of inorganic phosphate concentration on DICDH of Ehrlich ascites carcinoma mitochondria. Enzyme: purified fraction transferred into 1 mM potassium phosphate (pH 7.2). Reaction mixture: Tris·HCl (pH 7.2) (25 μmoles), isocitrate (2.5 μmoles), DPN^+ (0.41 μmole), $MnCl_2$ (0.3 μmole), and potassium phosphate (pH 7.2), varied as indicated, in final volume of 1 ml.

by crude extracts of the mitochondria of mouse liver, brain, and hepatoma. Ramakrishnan and Martin (1955) have shown a requirement for phosphate by the *Aspergillus* enzyme. Goebell and Klingenberg (1964) have noted a slight stimulation by phosphate of DICDH activity in high-speed supernatants of disrupted rat heart mitochondria.

Effect of Isocitrate. In agreement with the results of others, a cooperative effect of isocitrate on rate is obtained at alkaline pH (Figure 2) whereas at acid pH usual kinetics are obtained at low isocitrate concentration (see below). Above neutrality, inclusion of ADP in the reaction mixtures increases the apparent

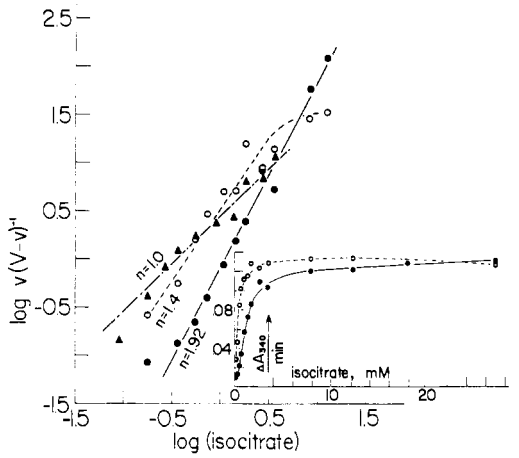

FIGURE 2: The effect of isocitrate concentration on DICDH activity at pH 7.7. Reaction mixtures contain 10 μmoles of potassium phosphate (pH 7.7), 25 μmoles of glycyl-glycine (pH 7.7), 10 μmoles of $MgCl_2$, and 0.67 μmole of DPN^+ in a final volume of 1.0 ml. Open circles: 1 mM ADP; triangles, 2.5 mM ADP.

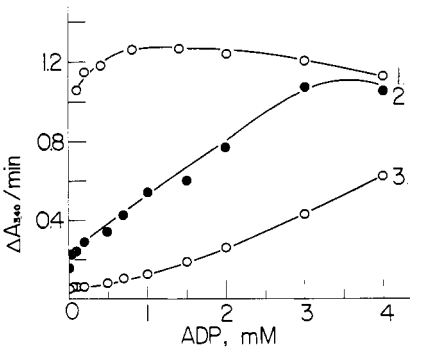

FIGURE 3: The effect of ADP concentration on DICDH activity. Reaction mixture as in Figure 2. Isocitrate concentration for curves 1, 2, and 3, 2.7, 0.54, and 0.18 mM, respectively.

affinity of the enzyme for isocitrate and abolishes its cooperativity (Figure 2). In the yeast DICDH system, AMP was shown to increase apparent isocitrate affinity, by Atkinson *et al.* (1965); in reaction mixtures decreased in magnesium concentration Cennamo *et al.* (1967) have demonstrated a heterotropic reduction by AMP of the Hill coefficient for isocitrate.

Effect of ADP. The effect of ADP concentration on the rate of the DICDH reaction is examined in the experiment in Figure 3. At low isocitrate concentration a pronounced cooperative effect is obtained.

Effect of Magnesium. At low DPN^+ concentration, evidence is obtained for homotropic cooperativity (n = 1.8) of magnesium. However in reaction mixtures low in isocitrate, dependence of activity on magnesium concentration appears normal (n = 0.9) (unpublished experiments).

Effect of DPN^+. Examination of the dependence of rate on the concentration of DPN^+ (Figure 4) shows little if any of the cooperative effect presented by the locust flight muscle (Klingenberg *et al.*, 1965) and yeast (Atkinson *et al.*, 1965) enzymes. Consideration of the properties of Mg and DPN^+ in this system is deferred to a later report.

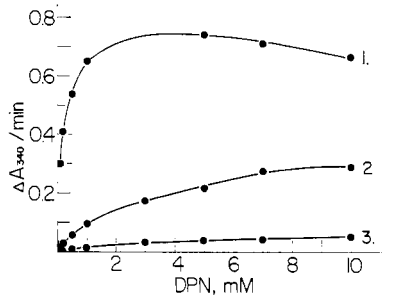

FIGURE 4: The effect of DPN^+ concentration on DICDH activity. Reaction mixture as Figure 2. The reaction mixtures for curves 1, 2, and 3 contain 2.7, 0.54, and 0.09 mM isocitrate, respectively.

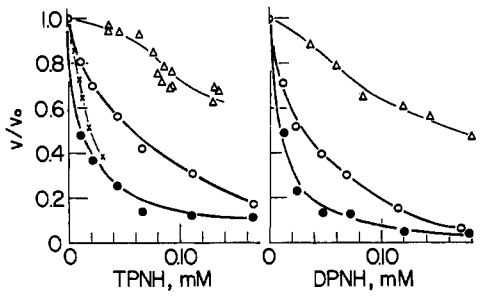

FIGURE 5: Inhibition of DICDH by reduced pyridine nucleotides. Reaction mixtures as in Figure 2, with the following additions in micromoles. TPNH closed circles: isocitrate, 0.9; open circles: isocitrate, 0.9, ADP, 1.0; crosses: isocitrate, 0.9, DPNH, 0.02; triangles: isocitrate, 4.1. The Hill coefficients are in this order 0.8, 1.1, 1.4, and 1.6. DPNH closed circles: isocitrate, 0.9; open circles: isocitrate, 0.9, ADP 1.0; triangles; isocitrate, 2.1. The Hill coefficients are, in this order, 0.9, 1.0, and 1.7. The Hill coefficient is evaluated by the quantity log $(v - v_s)/(v_0 - v)$ for the curves indicated by triangles and crosses.

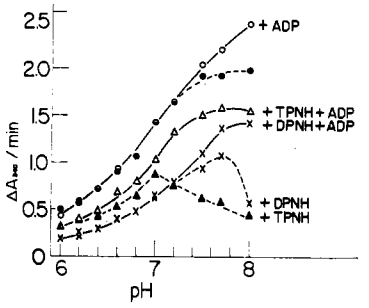

FIGURE 6: pH dependence of the inhibition of DICDH by reduced pyridine nucleotides. Reaction mixtures contain 50 μmoles of potassium phosphate at indicated pH, 10 μmoles of $MgCl_2$, 1.65 μmoles of isocitrate, 0.67 μmole of DPN^+ in a final volume of 1 ml. Added as indicated, ADP, TPNH, and DPNH, 1.0, 0.16, and 0.13 mM, respectively.

Effect of Reduced Pyridine Nucleotides. DPNH and TPNH inhibit the initial rate of the DICDH reaction (Figure 5); a pH dependence is obtained in this effect as well as in its reversal by ADP (Figure 6). Change in rate with DPNH concentration is shown in Figure 5. At 2.1 mM isocitrate a cooperative homotropic effect of DPNH is obtained, while the kinetics of inhibition at 0.9 mM isocitrate appear normal.

DPNH added to the reaction mixture has a marked effect on the dependence of rate on isocitrate concentration. At pH 6.6 (Figure 7) the normal Michaelian dependence is converted by DPNH to a cooperative function, while at pH 7.7 (Figure 8) isocitrate cooperativity is further increased. It will be noted that the substrate inhibition obtained at pH 6.6 (Figure 7) is absent at pH 7.7 (Figure 2).

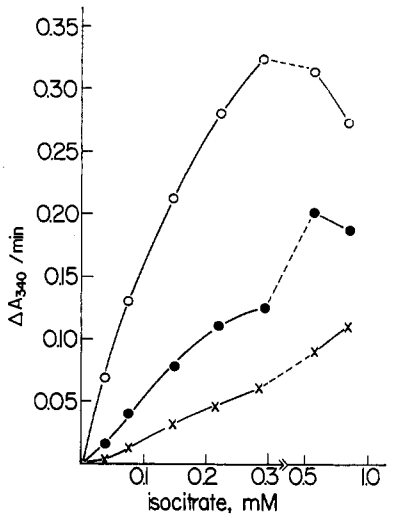

FIGURE 7: Induction of isocitrate cooperativity by DPNH. Reaction mixtures contain 50 μmoles of potassium phosphate (pH 6.6), 10 μmoles of $MgCl_2$, and 0.67 μmole of DPN^+ in a 1.0-ml final volume. DPNH concentration: open circles, 0; closed circles, 0.065 mM; crosses, 0.19 mM. The Hill coefficients calculated for the data to 0.3 mM isocitrate are: open circles, 1.0; closed circles, 1.8; crosses, 2.1.

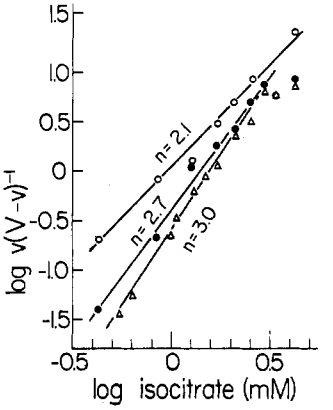

FIGURE 8: The effect of DPNH on isocitrate cooperativity. Reaction mixtures as in Figure 2. DPNH concentration is: open circles, 0; closed circles, 0.12 mM; triangles, 0.20 mM. The relative V_{max} values determined by experiment are: open circles, 1.0; closed circles, 0.63; triangles, 0.49.

Evidence for the homotropic cooperativity of TPNH is shown in Figure 5. As compared to DPNH, a higher (4 mM) concentration of isocitrate is required to elicit this response, the Hill coefficient for TPNH at 2 mM isocitrate being about 0.9–1.0 (not shown). The heterotropic effect of TPNH on isocitrate cooperativity is shown in Figure 9; with increasing TPNH concentra-

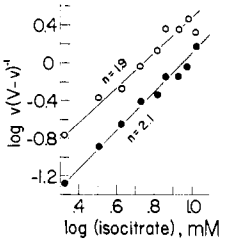

FIGURE 9: Inhibition of DICDH by TPNH at pH 7.7. Reaction mixtures contain 0.03 ml of crude extract, 100 μmoles of potassium phosphate buffer (pH 7.7), 10 μmoles of $MgCl_2$, and 0.67 μmole of DPN^+ in a 1.0-ml final volume. Closed circles, 0.011 mM TPNH.

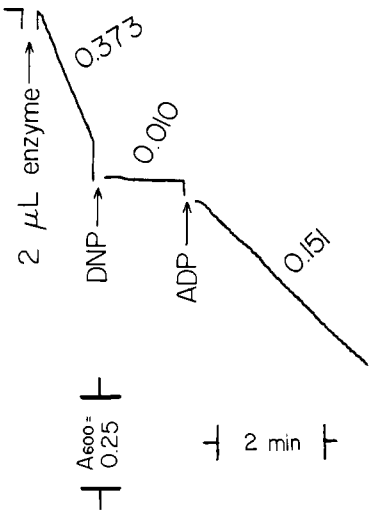

FIGURE 10: Inhibition of the DICDH reaction by DNP and reversal of inhibition by ADP. Reaction mixtures contain 5 μmoles of potassium phosphate (pH 7.2), 25 μmoles of glycyl-glycine (pH 7.2), 0.3 μmole of $MnCl_2$, 0.54 μmole of isocitrate, and 0.67 μmole of DPN^+ in a 1.0-ml final volume. Diaphorase system is included as indicated in the Experimental Section. Reaction is started with enzyme; at the indicated times 2.0 μmoles of DNP and 4.0 μmoles of ADP are added. Numbers above tracing indicate slopes in $-\Delta A_{600}$/min.

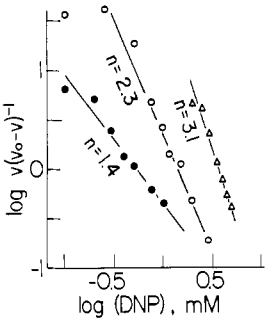

FIGURE 11: Inhibition of DICDH by DNP. The effect of DNP concentration. Reaction mixtures as in Figure 2. Components of diaphorase system included as indicated in Experimental Section. Closed circles: 1.35 mM isocitrate; open circles: 1.35 mM isocitrate and 1.0 mM ADP; triangles: 5.9 mM isocitrate.

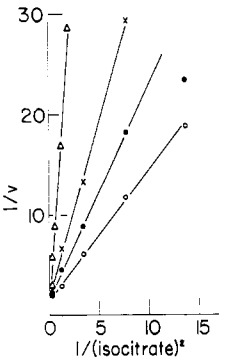

FIGURE 12: Inhibition of DICDH by DNP. Reaction mixture as in Figure 2; components of diaphorase system included as described in Experimental Section. Concentration of DNP for open circles, closed circles, crosses, and triangles is 0, 0.1, 0.4, and 1.0, respectively. The calculated Hill coefficients are, in the same order, 2.1, 2.1, 2.0, and 2.2. Not shown are data at 3.0 mM DNP, $n = 2.2$.

tion the maximum Hill coefficient obtained for isocitrate is 2.3 (not shown). An effect of TPNH in accelerating the progressive decay of reaction rate has been reported by Chen and Plaut (1963) and confirmed repeatedly in these studies. A synergistic effect of DPNH on the inhibition by TPNH is demonstrated in Figure 5. Here DPNH increases both the cooperativity and affinity of TPNH.

Effect of DNP. The reaction shown in Figure 10 demonstrates inhibition of the DICDH reaction by DNP and antagonism of the inhibition by ADP with no apparent lag in the effect of either reagent. No effect on the inhibition is obtained with either AMP or ATP. Behavior of DNP as a negative allosteric effector is shown in Figure 11; marked homotropic cooperativity is obtained at high isocitrate concentration or on addition of ADP to the reaction mixture. Figure 12 shows the effect of DNP in decreasing the apparent affinity of isocitrate without modifying the V_{max} of the reaction. In the range 0.1–3.0 mM DNP there appears to be no significant effect on isocitrate cooperativity.

Discussion

Allosteric Interactions. The allosteric properties of isocitrate, ADP, and DPNH are in accord with the predictions of the K model of regulatory enzyme function proposed by Monod *et al.* (1965). In the reversible conformational transition T $\rightleftharpoons$ R, postulated by these

authors, the finding of isocitrate cooperativity (Figure 2) defines T, by convention the low isocitrate affinity state, as the predominant form at equilibrium. In increasing the apparent affinity of isocitrate, ADP reduces isocitrate cooperativity (Figure 2) and itself shows homotropic cooperativity (Figure 3). The obverse behavior is manifested by DPNH, *i.e.*, heterotropic increase in isocitrate cooperativity (Figure 8) and homotropic cooperativity at moderately high isocitrate concentration (Figure 5). These effects permit the assignments of preferential affinity, ADP for the R state and DPNH for the T state. The antagonism of DPNH inhibition by ADP is interpreted as the interplay of the two effectors on the position of the conformational equilibrium.

At pH 6.6, the usual dependence of rate on isocitrate concentration and the effect of DPNH in generating isocitrate cooperativity (Figure 7) suggest that at acid pH the equilibrium favors the R conformation. Since isocitrate cooperativity is taken as evidence for the T $\rightarrow$ R shift, it is inferred that DPNH at acid pH displaces the equilibrium to the T state. The finding of diminished sensitivity of DPNH inhibition to ADP at low pH (Figure 6) is consistent with this interpretation. These effects are most easily rationalized by proposing the conformational shift to be accompanied by net H^+ release, $H^+ + T \rightleftharpoons RH$, in the direction suggested above. This implies that the acid strength of a specific group, independent of other allosteric binding sites, is modified as a result of the change in conformation; the hydrogen ions in question would then assume the role of allosteric effectors. A consequence of this proposal is the requirement that under appropriate conditions other allosteric ligands will produce a change in the affinity of the enzyme for H^+. A possible demonstration of this effect is contained in the data presented by Klingenberg *et al.* (1965), which show an increase in the pH optimum in the DICDH reaction with increasing isocitrate concentration. Dixon and Webb (1964; Figure IV.39) have suggested that such functions might indicate a change in the pK of a group at an enzyme site.

The effect of ADP in increasing pH optimum and rendering it insensitive to isocitrate concentration (Klingenberg *et al.*, 1965) is interpreted as additional evidence for the allosteric nature of this interaction. Investigation of the effect of pH on the cooperativity of binding of allosteric inhibitors is required for verification of these observations. For the case of DICDH the model of Monod *et al.* (1965) predicts that the homotropic cooperativity of DPNH should increase as the pH is decreased.

The allosteric interactions of TPNH with isocitrate and ADP are in qualitative agreement with the properties of DPNH; however, the heterotropic effect of DPNH on TPNH cooperativity (Figure 5) appears to be at variance with the predictions of the model. The addition of 0.02 mM DPNH to reaction mixtures 2 mM in isocitrate increases the apparent affinity of TPNH (s_{50} of inhibition by TPNH reduced from 0.03 to 0.015 mM, not shown). This behavior is expected if no direct interaction occurs between DPNH and TPNH and if both reduced pyridine nucleotides have a higher affinity for the T conformation. However, the model demands a reduction in the Hill coefficient for TPNH, whereas an increase is obtained. This mode of interaction may be general since it has been demonstrated with a preparation obtained from beef brain mitochondria as well (unpublished observations).

A further problem is encountered in the interpretation of the properties of DNP by the model of Monod *et al.* (1965). While the heterotropic effects of isocitrate and ADP (Figure 11) may be interpreted as indicating stabilization of the T conformation by DNP, in the absence of ADP the predicted heterotropic effect on isocitrate is not observed (Figure 12). Atkinson *et al.* (1965) present results of calculations indicating that effectors modifying the affinity of allosteric ligands will themselves show homotropic cooperativity without the necessity of invoking interaction between the effector sites. In terms of the model discussed here, such a proposal would imply the assumption that DNP antagonizes the binding of ADP and isocitrate in a manner insensitive to conformation state. It would appear reasonable to suppose that such intraoligomeric interaction is not incompatible with the Monod–Wyman–Changeux model. Additional information is required to confirm a hypothesis for the conformation-selective affinity of DNP.

Regulatory Implications. Isocitrate cooperativity and the effects of phosphate and adenine nucleotides implicate DICDH in the regulation of respiration to the requirements of oxidative phosphorylation. In addition, the negative allosteric properties of reduced pyridine nucleotides permit an independent response of the enzyme activity to the redox state of the mitochondrion.

Inhibition by TPNH suggests that in addition to energy demand, DICDH activity may also be regulated by the state of biosynthetic reduction. Allowing the reverse, energy-linked transhydrogenase reaction (Danielson and Ernster, 1963a,b) to account for a significant fraction of intramitochondrial TPN^+ reduction, then this inhibition could afford a means of regulation of the level of TPNH. In this regard, the interactions with ADP and DPNH are suggestive of a complex interrelationship between the oxidative and reductive systems of regulation.

It is pertinent to note here the equilibrium constant of the aconitase reaction, which favors the citrate side by a factor of about 20 (Krebs, 1953). This effect, in maintaining the isocitrate concentration at levels lower than otherwise would be expected, would tend to limit the range of isocitrate affinity required to maintain regulation of enzyme activity.

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