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Diphosphopyridine Nucleotide Specific Isocitric Dehydrogenase of Mammalian Mitochondria. I. On the Roles of Pyridine Nucleotide Transhydrogenase and the Isocitric Dehydrogenases in the Respiration of Mitochondria of Normal and Neoplastic Tissues*

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ABSTRACT: The respiration of pyridine nucleotide depleted mitochondria in the presence of isocitrate has been measured. Evidence has been obtained for the oxidation of isocitrate by both the triphosphopyridine nucleotide specific isocitric dehydrogenase–transhydrogenase (I) and the diphosphopyridine nucleotide specific isocitric dehydrogenase (II) pathways. Pathway II is independent of triphosphopyridine nucleotide, requires isocitrate and diphosphopyridine nucleotide concentrations higher than those of I, is stimulated by adenosine diphosphate, and is inhibited by 2,4-dinitrophenol in a manner closely similar to the behavior of the soluble enzyme. The respiration pattern is tissue specific: I predominates in heart, II in brain, and Ehrlich ascites mitochondria; liver mitochondria are intermediate with about equal capacity for I and II. Distribution of the two isocitric dehydrogenases in extracts of various mitochondria has been measured. Evidence for pyridine nucleotide transhydrogenase in Ehrlich ascites carcinoma has been obtained by pyridine nucleotide analog assay and respiration studies.

In the course of a reinvestigation into the problem of the occurrence of pyridine nucleotide transhydrogenase in mitochondria of neoplastic tissues we have identified in extracts of mitochondria of the Ehrlich ascites carcinoma an active DPN-specific isocitric dehydrogenase[1] which appears to be responsible for a major part of the respiration coupled to the oxidation of isocitric acid in this system. Properties of DICDH have been examined in recent studies from several laboratories. Chen and Plaut (1963) have reinvestigated the purified beef heart enzyme and have described an activating effect of ADP on the reaction. Hathaway and Atkinson (1963) and Atkinson *et al.* (1965) have shown a sigmoid dependence of activity on isocitrate concentration for the yeast enzyme and investigated the kinetic properties of the response of activity to the effectors citrate and AMP. Sanwal *et al.* (1963) have shown a parabolic dependence of the reciprocal rate on the reciprocal of isocitrate concentration in the reaction catalyzed by the *Neurospora crassa* enzyme and have discussed (Sanwal *et al.*, 1964) the enzyme in terms of the model proposed by Monod *et al.* (1963). Goebell and Klingenberg (1964) and Klingenberg *et al.* (1965) have investigated the pH dependence and effect of isocitrate and DPN^+ concentration on the DICDH activity of high-speed supernatants of disrupted mitochondria of some mammalian tissues and of locust flight muscle. Studies on the kinetic behavior of the enzyme from the various sources have suggested to all authors a role for DICDH in the regulation of citric acid cycle activity.

The present studies were prompted in part by the failure of Hawtrey and Silk (1961) to detect pyridine nucleotide transhydrogenase activity in their preparations (Hawtrey and Silk, 1960) of mitochondria of the Ehrlich ascites carcinoma and their finding in this system of significant DICDH activity dependent on unusually high DPN^+ concentration. It will be shown that assay of the Hawtrey and Silk preparation by the

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[1] Abbreviations used: DPN^+ and DPNH, diphosphopyridine nucleotide, oxidized and reduced, respectively; TPN^+ and TPNH, triphosphopyridine nucleotide, oxidized and reduced, respectively; APDPN and APTPN, the acetylpyridine analogs of DPN^+ and TPN^+, respectively; TNDPN, the thionicotinamide analog of DPN^+; ADP, adenosine diphosphate; AMP, adenosine monophosphate; DICDH and TICDH, the DPN^+- and TPN^+-specific isocitric dehydrogenases.

pyridine nucleotide analog method (Stein *et al.*, 1959) does demonstrate the presence of unsymmetrical pyridine nucleotide transhydrogenase in the same order of magnitude of the activity of DICDH and TICDH measured by following spectrophotometrically the reduction of DPN^+ and TPN^+ with the pyridine nucleotide depleted mitochondrial preparations.

The experiments presented in this paper are addressed to the question of the relative roles played by the TICDH–pyridine nucleotide transhydrogenase (Kaplan *et al.*, 1956) and the DICDH pathways in respiration of a number of tissues. It will be shown that respiration may proceed by either pathway in tissue specific patterns and the conditions for eliciting response differ for the two pathways; the DICDH-linked respiration is identified by the similarity of its properties to those of the soluble enzyme. An examinaton of some of the properties of the DICDH of the mitochondria of the Ehrlich ascites carcinoma will be presented in the following publication (Stein *et al.*, 1967).

Experimental Section

Materials. DPN^+, DPNH, TPNH, cytochrome C, ADP, and trisodium DL-isocitrate were obtained from the Sigma Chemical Co. Chromatographically pure TPN was prepared from DPN^+ by a modification[2] of the method of Wang *et al.* (1954). APDPN and APTPN were prepared by the method of Kaplan and Ciotti (1956), TNDPN as described by Stein *et al.* (1963).

Mitochondrial Preparations. Ascites carcinoma mitochondria were prepared essentially as described by Hawtrey and Silk (1960). Ascitic fluid was obtained from 20 to 40 mice inoculated intraperitoneally 6 days previously with suspensions of Ehrlich (Lettre') ascites cells. The homogenizing steps were carried out in a home-made glass homogenizer of 200-ml capacity with a 2-in. precision bore, fitted with a Teflon pestle with approximately 0.015-in. clearance driven by a Con-Torque stirrer motor at top speed. Mitochondria of brain were prepared by the procedure of Jöbsis (1963), mitochondria of liver, heart, and lung by the procedure of Schneider and Hogeboom (1950). Mitochondria in dilute suspension were depleted of pyridine nucleotides (Hunter and Ford, 1955) in 0.15 M sucrose and 0.05 M potassium phosphate pH 7.2 medium in a shaking water bath at 37° for 45 min. After incubation the mixtures were centrifuged and resuspended in ice-cold 0.25 M sucrose.

Loss of endogenous pyridine nucleotides has been noted in liver (Hunter and Ford, 1955) and heart (Lester and Hatefi, 1958) mitochondria incubated in phosphate buffers. Hawtrey and Silk (1961) have shown a pyridine nucleotide requirement for the reduction of DCIP by isocitrate in mitochondria of the Ehrlich ascites carcinoma aged in phosphate; as prepared in the present study, no pyridine nucleotide fluorescence is detectable in the ascites mitochondria even prior to the depletion procedure (see Results, Table I). As judged by the dependence of isocitrate-linked respiration on pyridine nucleotide addition, virtually complete removal of endogenous pyridine nucleotides obtains in the phosphate-aged mitochondria of all tissues examined (see Results, Figures 3–5).

In agreement with the results of others (see Hunter, 1961) no respiratory control is evident in any of the aged preparations when tested with succinate or malate. Further, added DPNH is oxidized rapidly by all the aged preparations; as measured on the oxygen electrode, the DPNH oxidase activity is considerably greater than the rate of oxygen uptake elicited by isocitrate under optimal conditions.

The protein contents of the mitochondrial suspensions was measured by the procedure of Lowry *et al.* (1951) using their modification for the measurement of protein in particulates, or, more frequently, by the biuret method of Gornall *et al.* (1949).

Acetone Powders. The method used is essentially that of Plaut and Sung (1954), adapted for processing mitochondrial suspensions derived from samples of tissue as small as 2–3 g. The mitochondrial pellet in the centrifuge tube is dispersed with a glass rod in two to three volumes of cold, distilled water; approximately 20 volumes of acetone (−15 to −20°) is added with rapid stirring to one volume of suspension. The mixture is centrifuged briefly, the residue is taken up in acetone, washed with six to eight portions of acetone on a Hirsch funnel, and finally dried in a vacuum desiccator at room temperature under continuous pumping for 30 min. The powders, stored at −15 to −20°, appear to retain most of their activity for 1–2 years of storage. Extracts are prepared by homogenizing the powders, 100–150 mg in 3 ml of 1 M potassium phosphate buffer (pH 7.2), and centrifuging at 10,000 rpm for 20 min after a 1-hr extraction period. DICDH activity in these extracts is relatively stable to storage, decaying only a few per cent in the course of 3–5 hr at 0° or for several weeks at −15°.

Spectrophotometric Measurements on Whole Mitochondria. Spectrophotometric measurements were made at room temperature in a Zeiss PMQ-II spectrophotometer equipped with a microcell adaptor, routinely in 1-ml cuvets with a 1-cm light path. Details of the protocols are described in the text. When appropriate, mitochondrial suspensions in 0.25 M sucrose were treated with an equal volume of ice-cold 1% digitonin (Merck) and stored on ice 10–20 min before assay. The rates reported are not corrected for any light-scattering effect on absorbancy due to turbidity of the samples. The reference cuvet contains all additions save for isocitric acid and reduced pyridine nucleotide in the dehydrogenase and transhydrogenase reactions, respectively. The dehydrogenase reactions were followed at 340 mμ; the millimolar absorbancy coefficient was taken as 6.2. The transhydrogenase reaction, measured by reduction of acetylpyridine coenzyme analogs by reduced nicotinamide coenzymes, was

[2] Unpublished experiments.

followed at 375 mμ (Stein *et al.*, 1959); the difference $A_{mM}^{375\,m\mu}$ APDPNH − $A_{mM}^{375\,m\mu}$ DPNH was taken as 6.3 and was calculated from the data of Siegel *et al.* (1959).

Dehydrogenase Assays in Extracts. The standard reaction mixture for assay of DICDH is in μmoles/1 ml of final volume: isocitrate, 2.5; DPN, 0.7; $MnCl_2$, 0.3; potassium phosphate, pH 7.2, 5.0; glycylglycine, pH 7.2, 25.0; and ADP, 1.0. TICDH activity is assayed in the same reaction mixture, with omission of ADP and substitution of 0.2 μmole of TPN^+ for DPN^+. The reactions were followed at 340 mμ in a Zeiss PMQ-II spectrophotometer fitted with a Sargent logarithmic recorder or with a Gilford Model 2000 recording spectrophotometric system. One unit of enzyme activity is defined conventionally as the amount of enzyme which will reduce 1 μmole of pyridine nucleotide in 1 min. Isocitrate solutions are standardized in enzymatically active form with the TPN-specific isocitric dehydrogenase reaction (Ochoa, 1957).

Respiration. Oxygen uptake is measured in a cell thermostatted at 31° provided with a rotating platinum electrode (Hagihara, 1961)[3] and fitted with a 1-mv Texas Instruments potentiometric recorder. The response of the system is calibrated by the oxidation of aliquots of standardized DPNH solution using aged mitochondrial preparations. All reactions are carried out in a 1.5-ml reaction mixture volume with the following concentrations: KCl, 0.75 M; Tris-HCl, pH 7.2, 0.05 M; potassium phosphate, pH 7.2, 0.01 M; $MgCl_2$, 0.015 M; nicotinamide, 0.067 M; and cytochrome C, 0.003 mM.

Results

Some Properties of the Ascites Mitochondria Preparations. Table I presents data on several oxidative reactions catalyzed by mitochondria of the Ehrlich ascites carcinoma prepared by the method of Hawtrey and Silk (1960). The cytochrome C content appears to be in the normal range; nevertheless, definite stimulation of DPNH and TPNH oxidase activities are obtained by the addition of cytochrome C. The cytochrome C augmented activity shows a sensitivity to amytal and antimycin A similar to that of the endogenous rate. The pyridine nucleotide content of the undepleted sample is nil and its respiratory control (not shown) is low or absent. Measured by the pyridine nucleotide analog method, the activity of the transhydrogenase reaction is comparable to the two isocitric dehydrogenase activities. A small stimulation of TPNH oxidase activity by DPN^+ presents additional evidence for the transhydrogenase reaction in ascites mitochondria. In agreement with previous results (Stein *et al.*, 1959), the "reverse" transhydrogenase reaction DPNH plus APTPN is quite slow.

Table II shows the analysis of transhydrogenase and isocitric dehydrogenases activities of pyridine nucleotide depleted mitochondria. Pretreatment with

TABLE I: Some Properties of Ehrlich Ascites Carcinoma Mitochondria.

	mμmoles min^{-1} mg of $Protein^{-1}$ (Lowry *et al.*, 1951)	
	Preparation	
	7–31[a]	8–3
DPNH oxidase[b]	79.8	35.5
DPNH oxidase[b] + amytal (10^{-3} M)	5.44	–
DPNH oxidase[b] + antimycin (3 μg/ml)	13.7	–
DPNH oxidase[b] + cytochrome C (6 μM)	151	75.9
DPNH oxidase[b] + cytochrome C (6 μM) + amytal (10^{-3} M)	22.8	–
DPNH oxidase[b] + cytochrome C (6 μM) + antimycin (3 μg/ml)	28.0	–
TPNH oxidase[b] + cytochrome C (6 μM)	4.60 5.38	2.08 2.22
TPNH oxidase[b] + cytochrome C + DPN^+ (0.07 mM)	10.5	3.83
Succinoxidase[c]	–	218[d]
Isocitrate + DPN^+[d]	36.7	–
Isocitrate + TPN^+[d]	16.3	–
TPNH + APDPN[e]	16.2	–
DPNH + APTPN[e]	1.2	–
Cytochrome C[f]	–	0.84[i]
Cytochrome C:cytochrome A[g]	–	1.7[i]
Pyridine nucleotides[h]	–	Nil[d]

[a] Depleted in a shaking bath at 30° in 0.125 M sucrose and 0.05 M potassium phosphate (pH 7.2). [b] Reaction in 0.1 M potassium phosphate (pH 7.2). Reduced pyridine nucleotides added to a concentration yielding absorbancy at 340 mμ of about 1. Reaction followed by the change in absorbancy. [c] Determined on the oxygen electrode (expressed as microatoms of oxygen). [d] Determined in 0.1 M potassium phosphate buffer (pH 7.2), 0.6 mg of trisodium DL-isocitrate/ml, 0.01 M magnesium chloride, 0.001 M potassium cyanide, and DPN^+ and TPN^+ (0.5 and 0.2 mg/ml), respectively. [e] Determined in 0.1 M potassium phosphate buffer (pH 6.5), 0.001 M potassium cyanide, and 0.2 mg/ml of oxidized and reduced pyridine nucleotide, respectively. Mitochondria in 0.25 M sucrose are pretreated with digitonin as indicated in the Experimental Section. [f] Expressed in millimicromoles per milligram of protein. [g] Expressed as a ratio. [h] Determined fluorometrically. [i] See Acknowledgment.

[3] Dr. Berton C. Pressman's aid in constructing this equipment is gratefully acknowledged.

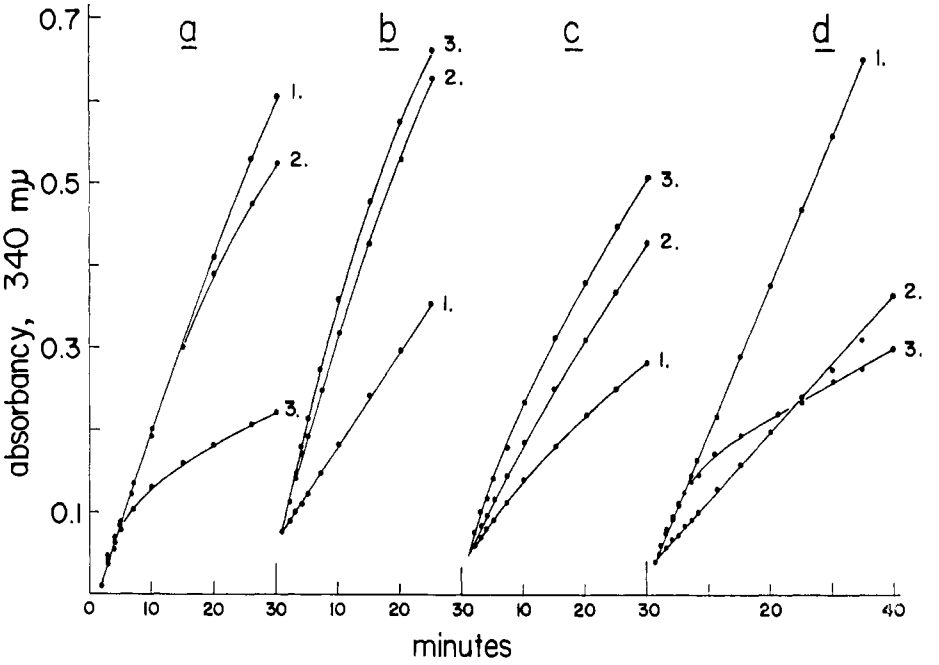

FIGURE 1: Reduction of DPN^+ and TPN^+ by pyridine nucleotide depleted mitochondria of the Ehrlich ascites carcinoma. Cuvets contain 300 μmoles of potassium phosphate buffers, 30 μmoles of $MgCl_2$, and 2.0 mg of DL-isocitrate. (curve 1) 0.5 mg of TPN. (curve 2) 2.0 mg of DPN^+. (curve 3) 0.04 mg of TPN^+ plus 2.0 mg of DPN^+ in a final volume of 3 ml. Reaction mixtures in a–c contain 3 μmoles of KCN and 1.32 mg of mitochondria as Lowry protein. Reaction mixtures in d contain 0.1 ml of acetone powder extract. The pH of the buffers is 7.5, 7.0, 6.5, and 7.5 for a, b, c, and d, respectively.

TABLE II: Transhydrogenase and Isocitric Dehydrogenases in Mitochondria[a] of the Ehrlich Ascites Carcinoma.

Assay Time	Pretreatment with Digitonin	No. of Samples	mμmoles/Minute per Milligram of Protein ± Standard Deviation: TPNH + APDPN[b]	Isocitrate + DPN^+ (a)[b]	Isocitrate + TPN^+ (b)[b]	a:b[c]
Freshly prepared	+	6	10.2 + 5.0[d]	30.9 ± 10.2	19.2 ± 4.6	1.61 ± 0.83
	−	4	−	33.0 ± 3.8	13.5 ± 2.2	2.65 ± 0.26
1 day after preparation	+	7	−	14.8 ± 7.0	16.7 ± 4.7	0.88 ± 0.31
	−	4	−	15.1 ± 6.0	10.2 ± 1.5	1.52 ± 0.77

[a] Mitochondria pretreated in hypotonic sucrose and phosphate (Table I, footnote *a*). [b] Assay systems as given in Table I. [c] Treatment of the ratios of activities of the individual samples. [d] A larger number of transhydrogenase activity determinations, including samples for which no corresponding isocitric dehydrogenase values are available, gives the average 10.0 ± 3.8, $n = 16$.

digitonin is required to demonstrate transhydrogenase activity and appears to stimulate slightly TICDH activity. Following the dehydrogenase reactions through the coupling of the oxidation of reduced pyridine nucleotides to the reduction of DCIP by the endogenous diaphorase activity, Hawtrey and Silk (1961) found a stimulation of DICDH activity by digitonin, with no effect on TICDH. Interpretation of the discrepancy of their results with the data in Table II is difficult in view of the different analytical procedures used in the two laboratories. In the assay systems described, the activity of the DPN^+ dehydrogenase exceeds the corresponding TPN^+ activity; similar results are obtained with the extracts of the ascites mitochondria (see below).

The dehydrogenase reactions in whole mitochondria are shown in Figure 1; as will be noted, under these conditions no evidence is obtained for the transhydro-

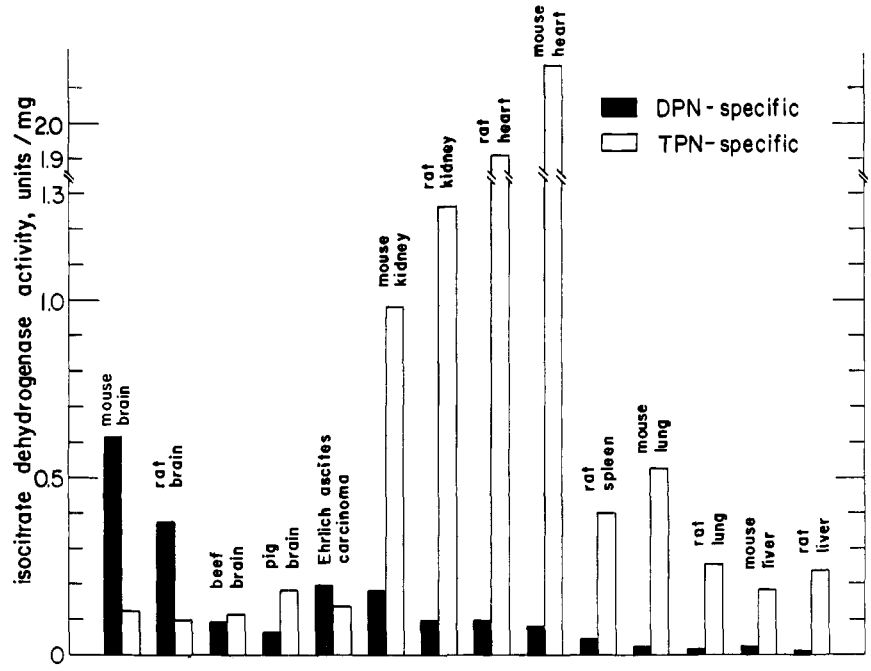

FIGURE 2: Distribution of DICDH and TICDH in extracts of acetone-dried mitochondria. Activity is based on biuret protein of the extracts. See Experimental Section for assay protocols.

genase reaction, *viz.*, an increment in the rate of reduction of DPN^+ on addition of catalytic quantities of TPN^+. In fact, such addition of TPN^+ in the presence of substrate levels of DPN^+ leads to substantially decreased rate of DPN^+ reduction. If the pH of the reaction mixture is reduced (Figure 1b,c), the inhibitory effect of TPN^+ addition is progressively abolished. Mitochondrial structure need not be invoked to explain the TPN^+ effect, since it is shown by extracts of the acetone-dried mitochondria (Figure 1d). Chen and Plaut (1963) have described an inhibitory effect of TPNH on DICDH activity; this effect will be considered at greater length in the following paper (Stein *et al.*, 1967).

Distribution of Isocitric Dehydrogenases in Mitochondria. Figure 2 shows the distribution of DICDH and TICDH activities in the extracts of mitochondria of several tissues. Estimation of the relative content of the two isocitric dehydrogenases in these extracts will be influenced by the sharper pH dependence of the DPN^+ enzyme (Plaut and Sung (1954) verified for the ascites enzyme in unpublished experiments) and the effect of ADP and isocitrate on the pH optimum of the DICDH reaction (Chen and Plaut, 1963; Klingenberg *et al.*, 1965). With the stable extracts obtained in this study, reproducible ratios of the two activities would be expected; in fact the profiles of Figure 2 are in general accord with the findings of Goebell and Klingenberg (1964). The interest in this data lies in the comparison of the relative activities of the two dehydrogenases in a series of tissues with their respective capacities for respiration along the alternate pathways represented by the two enzymes.

It will be noted that the activity profiles fall into two characteristic groups, one with DICDH activity equal to or greater than TICDH, the other with TICDH activity at least five to ten times greater than DICDH. Interestingly, it is the latter group which has shown the highest activities in the unsymmetrical pyridine nucleotide transhydrogenase assay (Stein *et al.*, 1959). Of this group, heart, liver, and kidney (unpublished data) mitochondria show significant TPN^+-dependent oxygen uptake in the presence of isocitrate.

Respiratory Studies. Typical tracings of oxygen consumption by the mitochondria of mouse carcinoma and of several rat tissues are reproduced in Figures 3–5. Utilizing the rapid response of the rotating platinum electrode (Hagihara, 1961) it is possible to evaluate the change in respiration within 10–15 sec of the various additions. The data for the mitochondria of the Ehrlich ascites carcinoma in Figure 3a,b show a DPN^+-linked oxygen uptake dependent on high isocitrate concentration and stimulated by the addition of ADP. Under these conditions, addition of TPN^+ inhibits respiration, in a manner reminiscent of the effect of TPN^+ on reduction of DPN^+ shown in Figure 1a,d. The effect of DPN^+ concentration on respiration is shown in Figure 6. Of particular interest is the inhibition of respiration by 2,4-dinitrophenol (DNP) and reversal of inhibition by ADP (Figure 3b). Dependence of inhibition on ADP and 2,4-dinitrophenol concentration is shown in Figure 7; the concentration of DNP required to effect 50% inhibition increases with increasing ADP concentration. Low con-

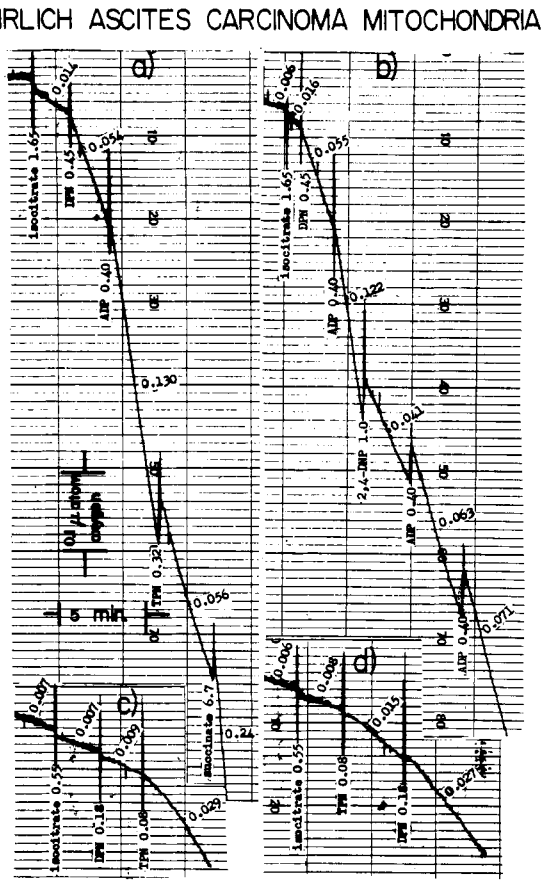

FIGURE 3: Oxygen uptake by pyridine nucleotide depleted mitochondria of the Ehrlich ascites carcinoma. To each reaction mixture is added 3.4 mg of biuret protein. The vertical spikes are mixing artifacts, additions are indicated below these with the final concentration of addenda in micromoles per milliliter. The oxygen uptake values for the total reaction mixture expressed in microatoms per minute are indicated above the tracings.

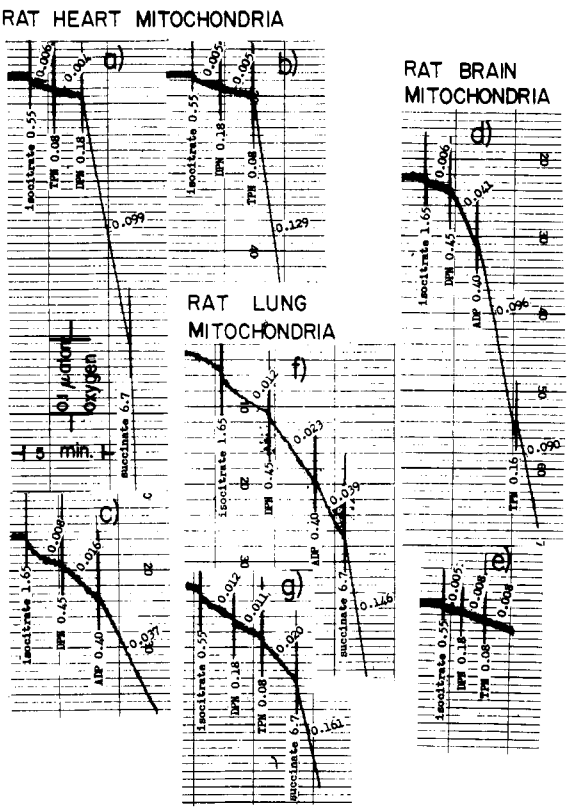

FIGURE 4: Oxygen uptake by pyridine nucleotide depleted mitochondria of various rat organs. (traces a–c) heart, 2.9 mg of biuret protein is added. (traces d and e) brain, 9.0 mg of protein is added. (traces f and 5), lung, 2.0 mg of protein is added. See legend to Figure 3.

centrations of DNP do not stimulate respiration (not shown), as would be expected from the uncoupled state of the aged mitochondria. It should be noted that the uncoupled state of the mitochondria renders unlikely the possibility that the ADP stimulation of the DNP-inhibited respiration is concerned with phosphorylation at the electron transport level. The modulation of respiration by isocitrate, DPN^+, ADP, and DNP resembles closely the effect of these compounds on the soluble ascites enzyme (Stein *et al.*, 1967). Effects of DNP, such as shown in Figure 3b have also been demonstrated with phosphate-aged preparations of heart, liver, and brain mitochondria. The traces in Figure 3c, d show a small stimulation of respiration by TPN^+ at low DPN^+ and isocitrate concentrations, consistent with the finding of transhydrogenase activity by the pyridine nucleotide analog assay (Tables I and II).

Respiration of rat brain mitochondria (Figure 4d,e) shows an essentially complete dependence on the DICDH pathway. Figure 4a,b shows that in the presence of TPN^+, rat heart mitochondria readily oxidize isocitrate at low isocitrate and DPN^+ concentrations. Unlike the ascites or brain mitochondria, the rate of oxygen uptake under conditions favoring the DICDH pathway (Figure 4c) is low relative to the TPN^+-dependent rate. The effect of pyridine nucleotide concentration on respiration in rat heart mitochondria (shown in Figure 8) supports this conclusion.

Figure 5 shows the respiration of rat liver mitochondria under conditions favoring the TPN^+-dependent pathway (a, b) or the DICDH pathway (c). Addition of TPN^+ to the mitochondria respiring in the reaction mixture favoring the DICDH pathway (Figure 5c) increases the rate of oxygen uptake, in contrast to the effect noted with ascites mitochondria. A similar observation has been made by Hawtrey (1962), who suggested operation of both pathways in liver. Failure of TPN^+ to inhibit respiration would be expected, since accumulation of TPNH is prevented by the operation of the transhydrogenase pathway. Unpublished studies with extracts of rat liver mito-

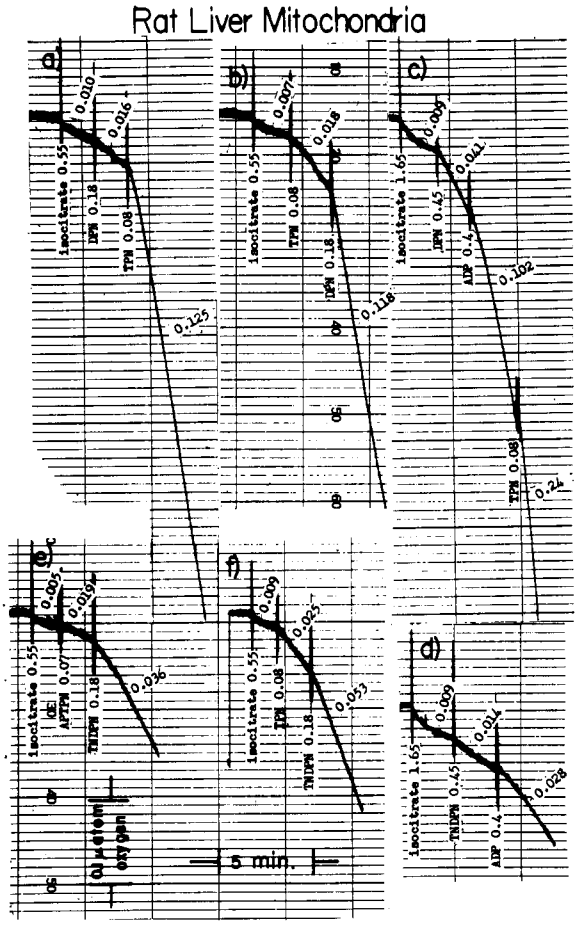

FIGURE 5: Oxygen uptake by pyridine nucleotide depleted mitochondria of rat liver. To each reaction mixture 15 mg of biuret protein is added.

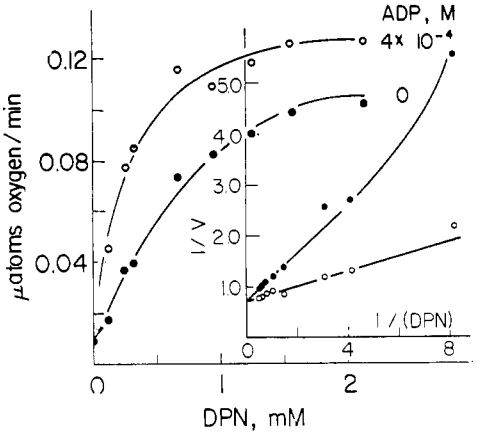

FIGURE 6: The effect of DPN^+ concentration and ADP on respiration by pyridine nucleotide depleted mitochondria of the Ehrlich ascites carcinoma. To each reaction mixture is added 1.65 μmoles of isocitrate/ml and 5.3 mg of biuret protein. Oxygen uptake is given for the entire reaction mixture.

chondria have demonstrated the inhibition of liver DICDH by TPNH, much as in the case of the soluble ascites enzyme. The response of respiration of liver mitochondria to pyridine nucleotide concentration is shown in Figure 9. Figure 5e,f,d shows additional experiments designed to test the two pathways in liver. Since TNDPN is an effective acceptor in the mammalian unsymmetrical pyridine nucleotide transhydrogenase reaction (unpublished experiments) and has low activity in the DICDH reaction (Chen and Plaut, 1963), it was expected that this analog would be effective only under conditions of the transhydrogenase reaction. Figure 5d shows that TNDPN is more active in respiration under conditions favoring the DICDH pathway than would be expected on the basis of its activity with the soluble enzyme (Stein *et al.*, 1967). In the absence of data on the relative activity of TNDPNH with DPNH oxidase, these data are difficult to interpret, although they demonstrate the operation of the pyridine nucleotide analogs in the isocitrate-linked respiration of this system.

Part 1 of Table III shows additional experiments on the effect of TPN^+ and DNP on the DICDH reaction in respiration of ascites mitochondria. The oxygen uptake data are obtained from experiments similar to those shown in Figures 3–5. Added in sufficient amount, ADP is shown to reverse completely inhibition by TPN^+ and DNP. Inhibition of the transhydrogenase pathway in respiration of mouse liver mitochondria by high concentration of DNP is shown in part 2 of Table III. ADP has no effect on this inhibition. In preliminary experiments, thyroxin has been found to inhibit respiration in mouse liver mitochondria in the DICDH pathway to a small extent. As in the case of DNP, this inhibition is reversed by ADP. However, the inhibition of the transhydrogenase-mediated respiration by thyroxin (Ball and Cooper, 1957) is not reversed by ADP.

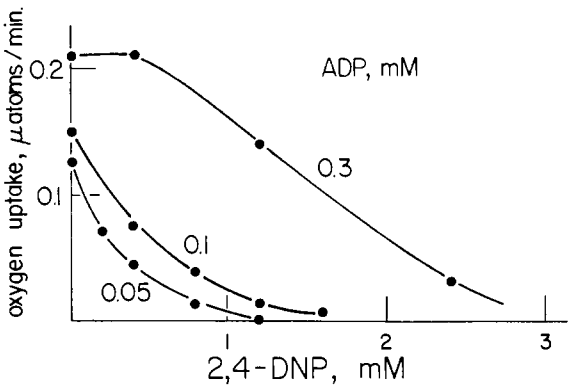

FIGURE 7: The effect of DNP and ADP concentrations on respiration by pyridine nucleotide depleted mitochondria of the Ehrlich ascites carcinoma. To each reaction mixture are added 2 μmoles of isocitrate and 0.26 μmole of DPN/ml and 2.3 mg of biuret protein. Oxygen uptake is given for the entire reaction mixture.

TABLE III: Effects of TPN^+ and DNP on Respiration of Pyridine Nucleotide Depleted Mitochondria of Ehrlich Ascites Carcinoma and Mouse Liver.

Additions	μmoles/ml	μatoms of O_2/ min[a]	Additions	μmoles/ml	μatoms of O_2/ min[a]
		1. Ascites Mitochondria[b]			
Isocitrate	1.35	0.03	Isocitrate	1.35	0.03
DPN^+	0.43	0.16	DPN^+	0.27	0.17
TPN^+	0.09	0.10	TPN^+	0.27	0.11
TPN^+	0.43	0.08	ADP	0.60	0.20
Isocitrate	1.35	0.03	Isocitrate	1.35	0.03
DPN^+	0.27	0.13	DPN^+	0.27	0.16
ADP	0.20	0.21	ADP	0.60	0.21
DNP	1.30	0.02			
ADP	1.00	0.18			
		2. Mouse Liver Mitochondria[c]			
Isocitrate	1.10	0.02	Isocitrate	2.70	0.02
DPN^+	0.20	0.02	ADP	0.67	0.02
TPN^+	0.20	0.11	DPN^+	0.57	0.08
DNP	2.00	0.06	DNP	2.00	0.02
ADP	0.80	0.06			

[a] Oxygen uptake for total reaction mixture; cumulative values. [b] To each reaction mixture 2.8 mg of biuret protein is added. [c] To each reaction mixture 7.0 mg of biuret protein is added.

Discussion

Evidence has been presented for the widespread distribution in mammalian mitochondria of the two pyridine nucleotide linked isocitric dehydrogenases (Figure 2; Plaut and Sung, 1954; Goebell and Klingenberg, 1964) and of pyridine nucleotide transhydrogenase (Stein *et al.*, 1959). These findings suggest alternate pathways of isocitrate oxidation in respiring mitochondria: either by direct reduction of DPN^+ mediated by the DPN^+-specific dehydrogenase or, indirectly, by the action of the TPN^+-specific dehydrogenase followed by transhydrogenase (Kaplan *et al.*, 1956).

The mode of oxidation of isocitric acid in mammalian mitochondria has been the subject of controversy, with the roles of the two pathways variously brought into question in reports from several laboratories. A pertinent discussion of this problem is given by Plaut (1963) who suggests that the discrepancy in results obtained by different workers might have arisen from differences in techniques employed. The data reported here indicate that the oxidation of isocitrate in respiring mitochondria depleted of pyridine nucleotides may show, depending on tissue and addition to the reaction medium, characteristics reasonably attributable to components of either pathway. Thus, in the absence of added TPN^+, the oxidation of isocitrate is favored by ADP and high concentrations of DPN^+ and isocitrate and is inhibited by DNP and TPNH, properties which indicate operation of the DICDH route. Conversely, the response of respiration to small additions of TPN^+ to reaction mixtures low in DPN^+ and isocitrate concentration is interpreted as involving the TPN^+-dependent transhydrogenase pathway.

The possibility that TPN^+, rather than fuctioning as an electron carrier in the TICDH–transhydrogenase

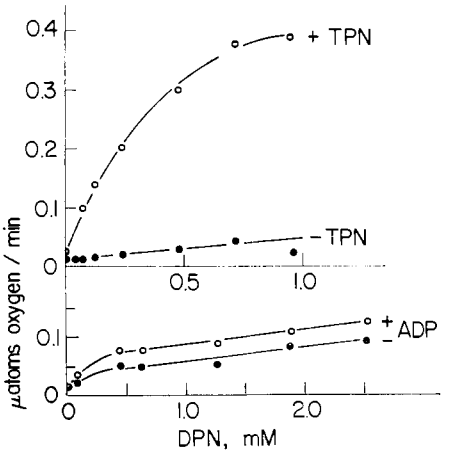

FIGURE 8: The effect of DPN^+ concentration, TPN^+, and ADP on respiration by pyridine nucleotide depleted mitochondria of rat heart. To each reaction mixture is added 3.4 mg of biuret protein. Additions per milliliter: upper curves, 0.55 μmole of isocitrate and 0.05 μmoles of TPN^+, as indicated; lower curves, 1.65 μmoles of isocitrate and 0.6 μmole of ADP, as indicated. Oxygen uptake is given for entire reaction mixture.

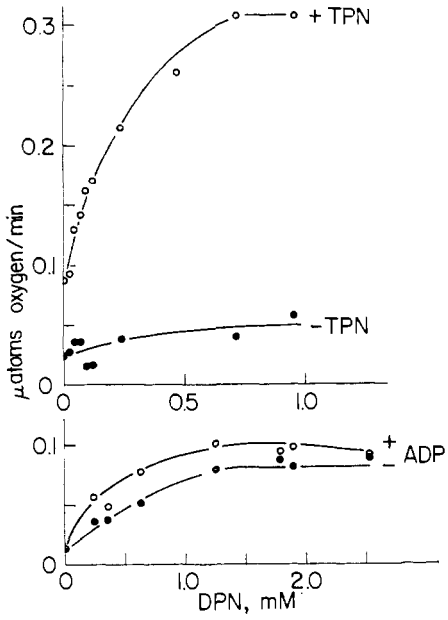

FIGURE 9: The effect of DPN^+ concentration, TPN^+, and ADP on respiration by pyridine nucleotide depleted mitochondria of rat liver. To each reaction mixture are added 10.3 mg of biuret protein. Additions per milliliter: upper curves, 0.55 μmole of isocitrate and 0.05 μmole of TPN^+, as indicated; lower curves, 1.65 μmoles of isocitrate and 0.4 μmole of ADP, as indicated. Oxygen uptake is given for entire reaction mixture.

system, serves as a positive effector for DICDH in the mitochondrion appears unlikely. Chen and Plaut (1963) found no interaction between TPN^+ and the purified beef heart DICDH. Further, this assumption would complicate the interpretation of the inhibition by TPN^+ of the isocitrate-coupled respiration in ascites mitochondria (Figure 3a).

It is also possible that ADP stimulates respiration in the TPN^+-dependent pathway by phosphorylation of DPN *via* adenylate and DPN^+ kinases. This interpretation appears unlikely by the observation that ADP stimulates respiration maximally within a few seconds of addition; on addition of TPN^+ to liver mitochondria respiring in the presence of high DPN^+ concentration plus ADP (Figure 5c), the rate of oxygen uptake increases by an amount corresponding to that obtained under conditions which demonstrate the TPN^+-dependent reaction (Figure 5a,b). Further, ADP markedly stimulates respiration in ascites and brain mitochondria where little or no TPN^+ stimulation is obtained.

The oxygen uptake rates determined here may be regarded as indicating the potential respiration by the two pathways; the contribution by the DICDH pathway will probably reflect the intramitochondrial concentrations of isocitrate, pyridine nucleotides, adenylates, divalent metals, and pH. The present finding of close agreement between the catalytic properties of DICDH in solution and the respiratory properties of mitochondria supports this contention and lends weight to suggestions by others for a function of DICDH in regulation of respiration.

The occurrence in respiration of an active pathway in parallel with the regulatory DICDH is of interest. In addition to an obvious role as an intramitochondrial system generating TPNH for reductive biosynthesis, the TPN^+-specific dehydrogenase appears to be capable of significant activity in the respiration of heart and liver mitochondria. Goebell and Klingenberg (1964) note that the partition of isocitrate oxidation between the two pathways would be influenced by the state of the DPN^+ enzyme as well as that of the TPNH–TPN^+ couple. However, by providing a potentially continuous bypass around DICDH, the introduction of the TICDH–transhydrogenase reaction would tend to nullify the controlling effect of this enzyme. A solution to this problem might be given by the separation of TPN^+ and DPN^+ into intramitochondrial compartments which exchange reducing equivalents only under defined conditions, a proposal equivalent to postulating a regulatory function for transhydrogenase. One possible mode of function is that transhydrogenase, which might be assumed to be inactive under maintenance conditions, would become operative under conditions of high energy demand to permit rapid oxidation of isocitrate by supplementing or overriding the DICDH control point. The utility of this system in a tissue such as heart is apparent.

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Reactions of Nucleosides on Polymer Supports. Synthesis of Thymidylylthymidylylthymidine*

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ABSTRACT: A procedure is described for synthesizing thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine on an insoluble polymer support. The support is a carboxylated styrene popcorn polymer. In the acid chloride form it reacts with 5′-*O*-monomethoxytritylthymidine, forming an ester link at the 3′-*O* position of the nucleoside. Subsequent cleavage of the monomethoxytrityl group by acid and condensation of the liberated 5′-hydroxyl group with 5′-*O*-monomethoxytritylthymidine 3′-phosphate afford a dinucleoside phosphate derivative on the support. On repetition of these steps and treatment with alkali to break the ester link holding the nucleotide product to the support, thymidylyl-(3′–5′)-thymidylyl-(3′–5′)-thymidine is obtained in 51% yield based on thymidine originally bound to the support.

Since introduction of the technique of synthesizing oligonucleotides on polymer supports (Letsinger and Mahadevan, 1965, 1966) several laboratories have reported work in the area. A major advantage of the support technique is that the products in a multistep synthesis may be separated easily from excess reagents and soluble by-products at each step. In the original procedure 5′-*O*-trityldeoxycytidine was joined to an insoluble polystyrene-type popcorn polymer by reaction of the 4-amino group with an acid chloride function on the support. Nucleotide units were added by successive phosphorylation of the 3′-hydroxyl group with β-cyanoethyl phosphate and dicyclohexylcarbodiimide, activation of the phosphate with mesitylenesulfonyl chloride, and condensation of the active phosphate with a nucleoside at the 5′-oxygen position.

Recently Hayatsu and Khorana (1966) and Cramer *et al.* (1966) described a synthetic procedure in which the initial nucleoside was joined through the 5′-hydroxyl group to a triarylmethyl derivative of a polymer that was soluble in pyridine. The chain was lengthened from the 3′ position by condensation with

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