Studies on the Hydrogen Transfer Mediated by the DPN-Linked Isocitric Dehydrogenase of Heart*

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Threo-D_s-Isocitrate-α-T was enzymically synthesized with α-ketoglutarate, CO_2, TPNT, and TPN-linked isocitric dehydrogenase. When this isocitrate was oxidized using purified DPN-linked isocitric dehydrogenase from bovine heart, the tritium was transferred to DPN^+ without loss into the medium. The DPNT produced was found to have the tritium atom on the α-side of the nicotinamide ring by testing in the lactic and glutamic dehydrogenase systems. These results show that both isocitric dehydrogenases utilize the same hydrogen of isocitrate and the same side of the pyridine ring. *Threo*-D_s-isocitrate-β-T was converted to labeled α-ketoglutarate by both isocitric dehydrogenases, without label appearing in TPNH or DPNH; hence the *enol* form of oxalosuccinate is not likely to be an intermediate in the oxidation of isocitrate.

Dehydrogenation of isocitrate, a key step in the Krebs cycle, can be catalyzed by two separate enzymes, one specific for TPN[1] and the other for DPN. DPN-linked isocitric dehydrogenase, first described by Kornberg and Pricer (1951) in yeast, was later demonstrated to occur in animal tissue by Plaut and Sung (1954). The DPN-linked enzyme was separated from the TPN-linked dehydrogenase of extracts of pigeon breast muscle and of heart. It was purified from extracts of mitochondria of bovine heart.

Marked differences in properties of the two isocitric dehydrogenases have been observed. Thus, DPN-specific isocitric dehydrogenase, in contrast to the TPN-linked enzyme, neither decarboxylates nor reduces oxalosuccinate and cannot be shown to form isocitrate by reductive carboxylation of α-ketoglutarate (Plaut and Sung, 1954; Kornberg and Pricer, 1951). DPN-linked isocitric dehydrogenase from yeast and from *Aspergillus niger* requires AMP for activity (Kornberg and Pricer, 1951; Ramakrishnan and Martin, 1955), while the activity of the enzyme from bovine heart is enhanced by ADP and is inhibited by certain other nucleotides (Chen and Plaut, 1962). A number of other differences between the two dehydrogenases have been reviewed (Plaut, 1963).

In view of the indicated marked variation in the mechanism of these enzymes it became of interest to compare the mode of hydrogen transfer mediated by the DPN- and TPN-linked isocitric dehydrogenases. It has been shown in studies using deuterium as the tracer (Englard and Colowick, 1957; Englard, 1960a) that TPN-specific isocitric dehydrogenase from porcine heart transfers the α-hydrogen atom (but not the β-hydrogen atom) of *threo*-D_s-isocitrate directly to TPN^+. Evidence is now presented that a direct transfer of hydrogen from isocitrate to DPN^+ is catalyzed by DPN-linked isocitric dehydrogenase from bovine heart and that the hydrogen atom removed from the substrate is the same one as that involved in the TPN-specific reaction. Furthermore, it is shown that this hydrogen goes onto the α-side of the nicotinamide ring of DPN^+ just as it does in the analogous situation with the TPN-linked enzyme (Nakamoto and Vennesland, 1960).

Materials and Methods

TPN-linked isocitric dehydrogenase was prepared by chromatography on carboxymethylcellulose (Rose, 1960) of the 0.6 to 0.8 saturated ammonium sulfate fraction of a phosphate buffer extract of whole bovine heart acetone powder (Siebert *et al.*, 1957a). The specific activity of the enzyme employed was 1900 units/mg. One unit is defined as the amount of enzyme causing an OD change of 0.01 per minute at 340 mμ under standard assay conditions (Siebert *et al.*, 1957b). DPN-specific isocitric dehydrogenase activity was absent.

DPN-linked isocitric dehydrogenase with a specific activity of 800–1000 units/mg was prepared from beef heart mitochondrial acetone powder as previously reported (Plaut and Sung, 1954) and stabilized with 10^{-4} M ADP (Chen and Plaut, 1962). The enzyme solution employed was also 30% saturated with respect to ammonium sulfate. TPN-linked isocitric dehydrogenase activity was absent. A unit of enzyme caused an OD change of 0.01 per minute at 340 mμ under the following assay conditions at 25°: In a cuvet with a 1.0-cm light path are placed 1.0 ml of 0.10 M Tris acetate buffer at *p*H 7.2, 0.2 ml of 0.02 M $MnCl_2$, 0.2 ml of 0.01 M ADP, 0.1 ml of 0.01 M DPN^+, 0.2 ml of 0.08 M *threo*-D_sL_s-isocitrate, enzyme, and water to a final volume of 3.0 ml.

DPN^+, TPN^+, and ADP were obtained from Sigma Chemical Company and Pabst Laboratories and used without further purification.

Tritiated water, 100 mc/ml, was obtained from New England Nuclear Corp. and distilled before use.

Norit A (acid washed) from Pfanstiehl Laboratories, Inc., was washed with ethylenediaminetetraacetate at *p*H 6.2 for 48 hours before use.

Crystalline liver L-glutamic dehydrogenase (ammonium sulfate suspension), crystalline rabbit muscle lactic dehydrogenase (Type I), and yeast glucose-6-phosphate dehydrogenase (Type V) were purchased

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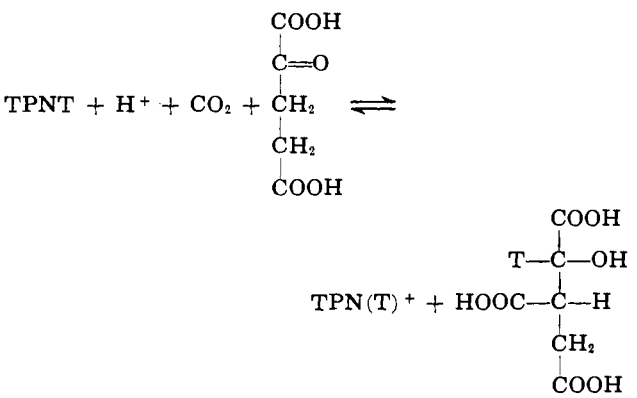
[1] Abbreviations: TPN^+ and DPN^+ are the oxidized forms of the nicotinamide adenine nucleotides. $TPN(T)^+$ and $DPN(T)^+$ represent oxidized forms of the nucleotides with tritium in the *para* position of the nicotinamide ring. TPNH, DPNH, TPNT, and DPNT are the corresponding reduced nucleotides. The prefixes α- and β- refer to the side of the nicotinamide ring to which tritium is attached, taking α-DPNT as the form which would lose its tritium atom to acetaldehyde in the alcohol dehydrogenase system (Westheimer *et al.*, 1951). AMP and ADP are adenosine-5′-phosphate and adenosine-5′-diphosphate. DEAE-cellulose is diethylaminoethyl-cellulose. T_2O is tritiated water. Tris is tris(hydroxymethyl)aminomethane.

from Sigma Chemical Company. A unit of glucose-6-phosphate dehydrogenase reduces 1.0 μmole of TPN^+ per minute under standard assay conditions (Kornberg and Horecker, 1955).

α-Ketoglutaric acid was obtained from the Matheson, Coleman and Bell Company and from the Aldrich Chemical Company and purified by recrystallization from ether and benzene. The barium salt of glucose-6-phosphate heptahydrate was purchased from the Sigma Chemical Company and converted to a solution of the potassium salt before use. Crystalline lithium lactate was synthesized by the method of Hillig (1937). Sodium pyruvate was obtained from Nutritional Biochemicals Corporation. *Threo*-D_sL_s-Isocitric acid lactone from California Corporation for Biochemical Research was hydrolyzed by heating at 95° at *p*H 10 for 20 minutes, and neutralized with 2 N HCl. DEAE-cellulose from the Brown Co., Berlin, N.H., 0.9 meq/g, was washed by the procedure of Kaziro *et al.* (1961) before equilibration with buffer and use. All DEAE-cellulose column separations were performed at 25°. Carboxymethylcellulose, with a capacity of 0.7 meq/g, was obtained from the Brown Co. The resin was washed with HCl and NaOH before use.

The method of preparing TPNT was a modification of that used by Popják *et al.* (1961). To 0.30 ml of T_2O containing 52 mc/ml were added 3.9 mg $NaHCO_3$, 5.05 mg TPN^+ (6.0 μmoles), and 3.60 mg $Na_2S_2O_4$. The mixture stood at 25° for 45 minutes and then was lyophilized. The residue was taken up in 0.4 ml of water and the nucleotide was precipitated with 12 ml of redistilled acetone at −15°. After 45 minutes, the TPNT was centrifuged down, taken up with 10 ml of 0.01 M $NaHCO_3$, and placed on an 0.5 × 5.5 cm water-washed DEAE-cellulose column (Pastore and Friedkin, 1961). The column was washed with 30 ml of 0.005 M potassium phosphate buffer, *p*H 7.2. Unreacted nucleotide was eluted with 10 ml of 0.10 M NaCl in the same buffer, and TPNT was eluted with 0.25 M NaCl in buffer. The yield of TPNT was 60–75%, and it contained 19,000 cpm/μmole.

Tritiated isocitrate was prepared enzymically by reversal of the TPN-dependent reaction (Ochoa, 1948; Siebert *et al.*, 1957b) (Reaction 1). Glucose-6-phos-



Reaction 1

phate and glucose-6-phosphate dehydrogenase were also present to recycle the $TPN(T)^+$ and shift the equilibrium toward isocitrate production (Ochoa, 1948). In a test tube were placed 2.0 ml of 2.0 M Tris acetate buffer, *p*H 7.5, 8.0 μmoles of $MnSO_4$, 6.0 μmoles of TPNT, 60 μmoles of α-ketoglutarate, 40 μmoles of glucose-6-phosphate, 4.0 ml of 0.10 M $NaHCO_3$ saturated with CO_2, and 400 units of TPN-linked isocitric dehydrogenase; final volume, 13.2 ml. The mixture was incubated for 45 minutes at 25°. Then 2.4 units of glucose-6-phosphate dehydrogenase were added, and the reaction was allowed to continue 15 minutes more. The contents were cooled to 2°, and 7.0 ml of 2% (w/v) $NaHSO_3$ was added, followed in 5 minutes by 1.9 ml of concentrated H_2SO_4 and 14.4 g of $MgSO_4 \cdot 7H_2O$. The slightly turbid solution was extracted continuously with ether for 3 days by the method of Wood *et al.* (1942). The ether layer was evaporated to dryness, taken up in 4.0 ml of water, and kept at *p*H 9.0 with KOH while heating at 90° for 10 minutes. After cooling, the solution was neutralized with HCl, diluted to 50 ml, and applied to a water-washed 1.0 × 10 cm DEAE-cellulose column. Linear gradient elution was employed, with 100 ml of water in the mixing chamber and 100 ml of 0.20 M NaCl in 0.005 M potassium phosphate buffer at *p*H 7.2 in the reservoir. The purified tritiated isocitrate appeared between 80 and 100 ml of effluent. To concentrate the isocitrate, the pooled fractions were diluted and put through a 0.3 × 3.0 cm DEAE-cellulose column. The isocitrate was then eluted with 0.3 M NaCl. Isocitrate was assayed enzymically with TPN-specific isocitric dehydrogenase, and it was found that 4.87 μmoles of isocitrate were recovered with 6250 cpm/μmole. When stored at *p*H 6.0 in the frozen state, isocitrate lost negligible amounts of radioactivity into water within 4 weeks.

α-Ketoglutarate was determined by the method of Koepsell and Sharpe (1952), lactate by the method of Barker and Summerson (1941), and glutamate by the ninhydrin method (Moore and Stein, 1954).

Radioactivity was determined in a Packard scintillation counter with the ternary system water-ethanol-toluene used in the proportion 1:14:35. All counts were corrected for background. Counting time was at least 5 minutes, so that the standard error in counting was ±3% or less.

Results and Discussion

It has been shown recently that naturally occurring isocitrate has the *threo*-D_s configuration (Kaneko and Katsura, 1960; Gawron *et al.*, 1961; Patterson *et al.*, 1962; Vickery, 1962). Englard and Colowick (1957) have presented evidence that the α-hydrogen of isocitrate is transferred by TPN-linked isocitric dehydrogenase directly to TPN^+. By reductive carboxylation of α-ketoglutarate with TPNT, therefore, *threo*-D_s-isocitrate-α-T is produced. In the present experiments, the use of tritium as label, rather than deuterium, allowed the use of quite small amounts of material. The extent of the reductive carboxylation of α-ketoglutarate was enhanced by driving the reaction with the glucose-6-phosphate dehydrogenase system, which served to regenerate TPNH. Although the *specific* radioactivity of the isocitrate produced is diminished by production of more unlabeled TPNH, the *total* label introduced into isocitrate should be increased for the following reasons: Since randomly labeled TPNT was employed in the enzymic synthesis, reduction of α-ketoglutarate leads to labeled isocitrate plus $TPN(T)^+$. When $TPN(T)^+$ is reduced by glucose-6-phosphate dehydrogenase, a β-specific enzyme (Stern and Vennesland, 1960), α-TPNT is produced. This in turn would given labeled isocitrate and unlabeled TPN^+ through the action of TPN-linked isocitric dehydrogenase, an enzyme with α-specificity (Nakamoto and Vennesland, 1960). Thus, label from both sides of the randomly labeled nicotinamide ring of TPNT finds its way into isocitrate.

Once *threo*-D_s-isocitrate-α-T had been produced, a direct transfer of the tritium to DPN^+ by DPN-specific isocitric dehydrogenase could be demonstrated.

Experiment 1 of Table I shows that the specific radioactivity of the original isocitrate and the DPNT isolated from DEAE-cellulose were about the same. Of the total radioactivity in isocitrate, the recovery in DPNT was about 75%, not inconsistent with the loss of DPNT during the chromatographic reisolation and washing procedures. It therefore seemed unlikely that any extensive loss of tritium into the medium could have occurred. However, in this experiment the hydrogen acceptor, DPN^+, was present in excess, and it seemed possible that the enzyme might catalyze a relatively slow exchange of the tritium of isocitrate with water when DPN^+ was absent. In Table II are the results of experiments testing this possibility. In view of the fact that the very small number of counts in the water in experiments 1 and 3 is about the same as that obtained without enzyme, no enzymic labilization seems to occur. This was confirmed in experiment 4, where enzymic incorporation of tritium from T_2O into isocitrate could not be demonstrated.

In view of the specificity of TPN-linked isocitric dehydrogenase for the α side of TPN (Nakamoto and Vennesland, 1960), it was of interest to investigate and essentially complete oxidation of DPNT had occurred. The separation of glutamate from DPN^+ was accomplished as in experiment 2. After the filtrate was made alkaline with saturated K_2CO_3, ammonia was removed by aeration and the glutamate determined. The filtrate contained less than 0.004 μmole of DPN^+. The DPN^+ was eluted from Norit with 10% isoamyl alcohol as in experiment 2, except that a contact time of 4 hours was used, resulting in a much higher recovery of DPN^+.

TABLE I

TRANSFER OF LABEL FROM ISOCITRATE-α-T TO DPN^+

Compound	μmoles	Total cpm	cpm/μmole
Experiment 1: Isocitrate-α-T + DPN^+ → DPNT + H^+ + α-ketoglutarate + CO_2			
Initial isocitrate	2.00	12,500	6250
Isocitrate consumed	1.81	11,300[a]	6250
Recovered reduced DPN	1.45	8,440	5820
Experiment 2: DPNT + H^+ + pyruvate → labeled lactate + DPN^+			
Initial DPNT (from experiment 1)	0.116	675	5820
Recovered lactate	0.107	630	5890
Recovered oxidized DPN	0.040	0	0
Experiment 3: DPNT + H^+ + α-ketoglutarate + NH_3 → glutamate + DPN(T)$^+$			
Initial DPNT (from experiment 1)	0.109	635	5820
Recovered glutamate	0.087	12	140
Recovered oxidized DPN	0.096	538	5620

Experiment 1: The following mixture was incubated at 25° for 74 minutes: 0.05 ml of 2.0 M Tris acetate buffer, *p*H 7.5, 4.0 μmoles of $MnCl_2$, 2.03 μmoles of labeled isocitrate, 10.0 μmoles of DPN^+, 2.0 μmoles of ADP, and DPN-linked isocitric dehydrogenase (40 units); final volume, 3.0 ml. According to the change in optical density at 340 mμ, 89% of the isocitrate was oxidized in this time. The mixture was then heated at 90° for 1 minute, diluted with water to 50 ml, and passed through a 1 × 8 cm cellulose column which had been equilibrated with 0.005 M potassium phosphate buffer, *p*H 7.25. Linear gradient elution was accomplished with 150 ml of buffer in the mixing chamber and 150 ml of buffer plus 0.20 M NaCl in the reservoir. The order of elution was DPN^+, α-ketoglutarate, DPNH, and isocitrate. With a flow rate of 1.0 ml/minute, collections of 5 ml per tube yielded only slight overlap between DPNH and any isocitrate which was still unoxidized. The DPNH-containing fractions were pooled, diluted, and rechromatographed on an 0.5 × 5.5 cm DEAE-cellulose column with similar gradient conditions in order to concentrate the nucleotide and free it from the remaining isocitrate.

Experiment 2: An aliquot of DPNT produced in experiment 1 was oxidized as follows: In a cuvet were placed 0.05 ml of 2.0 M Tris acetate buffer, *p*H 7.5, 0.80 μmole of sodium pyruvate, 0.12 μmole of DPNT, and 0.01 mg of lactic dehydrogenase; final volume, 3.0 ml. By observation at 340 mμ, it was determined that 0.116 μmole of DPNT was oxidized within 15 seconds. The solution was then heated for 1.5 minutes at 90° and cooled to room temperature. 7 mg of Norit A was added and the mixture stirred for 20 minutes. The charcoal was filtered off on a layer of Celite in a Buchner funnel. The Norit-Celite mixture was stirred with 3.0 ml of 10% isoamyl alcohol for 20 minutes (Kornberg and Lindberg, 1948). The filtrate of this mixture was extracted with ether and the aqueous layer taken to dryness under reduced pressure. The residue was taken up with water in a final volume of 6.5 ml. 3.0 ml was used for radioactivity determination, and another 3.0 ml was placed in a cuvet and assayed for DPN^+ by a modification of the method of LePage (1957). The filtrate left after Norit treatment was assayed for lactate and counted. The filtrate had no detectable DPN^+; conversely, control experiments showed no lactate in the eluate from Norit after ether extraction.

Experiment 3: Another aliquot of DPNT from Experiment 1 was oxidized as follows: In a cuvet were placed 0.05 ml of 2.0 M Tris acetate buffer, *p*H 7.5, 94 μmoles of α-ketoglutarate, 15 μmoles of NH_4Cl, 0.11 μmoles of DPNT, and 1.0 mg of glutamic dehydrogenase in ammonium sulfate suspension; final volume 3.0. After 15 minutes,

TABLE II

STUDY OF THE EXCHANGE REACTION BETWEEN ISOCITRATE AND WATER

Experiment 1: The following mixture was incubated at 25° for 60 minutes: 0.05 ml of 2.0 M Tris acetate buffer, *p*H 7.5, 4.0 μmoles of $MnCl_2$, 0.2 μmole of tritiated isocitrate with 1400 cpm/μmole, 0.2 μmole of ADP and 40 units of DPN-linked isocitric dehydrogenase. Final volume, 0.34 ml. Afterwards, the reaction mixture was lyophilized and the radioactivity of the water determined. The radioactivity of the residue could not be determined directly because the large amount of solutes resulted in phase separation in the counting vials.

Experiment 2: Same as experiment 1 except that no DPN-specific isocitric dehydrogenase was added; final volume was 0.29 ml.

Experiment 3: Same as experiment 1 but with 0.1 μmole of DPNH added; final volume was 0.36 ml.

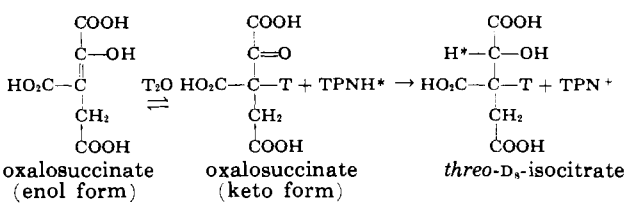
Experiment 4: The following mixture was lyophilized: 0.05 ml of 2.0 M Tris acetate buffer, *p*H 7.5, 0.02 ml of 0.2 M $MnCl_2$, 0.2 ml of unlabeled *threo*-D_5L_5-isocitrate, and 40 units of DPN-specific isocitric dehydrogenase. To the residue was added 0.196 g of T_2O with a specific radioactivity of 100 mc/ml. The solution was left at 25° for 2 hours and lyophilized. Enzyme activity was destroyed with perchloric acid. The isocitrate was reisolated and washed on DEAE-cellulose as described under Materials and Methods.

Experiment	Content of Incubation Mixture	Radioactivity (cpm)[a] Water Recovered	Isocitrate Recovered	Present Initially in Isocitrate
1	Enzyme, Mn^{++}, labeled isocitrate	26	—	280
2	Same as 1, but no enzyme	33	—	280
3	Same as 1, plus DPNH	25	—	280
4	Enzyme, Mn^{++}, unlabeled isocitrate, T_2O	4.0×10^{9}[b]	0	—

[a] Total counts added or recovered per experiment. [b] Added.

compare the stereospecificity for DPN of the DPN-enzyme. This was done by subjecting the DPNT recovered in experiment 1 of Table I to oxidation by muscle lactic dehydrogenase and liver glutamic dehydrogenase (experiments 2 and 3 of Table I). In experiment 2, excellent agreement was obtained between the specific radioactivities of the initial DPNT and the lactate which was produced by reduction of pyruvate, and the recovery of radioactivity in lactate was 94%. Since lactic dehydrogenase is α-specific (Loewus *et al.*, 1953), the fact that all the radioactivity appears in lactate rather than in DPN^+ shows that DPN-dependent isocitric dehydrogenase must have α-specificity. This conclusion was confirmed by utilizing the glutamic dehydrogenase reaction, which is β-specific (Levy and Vennesland, 1957). In experiment 3, the specific radioactivity of the $DPN(T)^+$ recovered was in good agreement with that of the DPNT initially present, and the yield of radioactivity in the oxidized nucleotide was 87%. This indicates that the hydrogen put onto DPN^+ by DPN-linked isocitric dehydrogenase is not removed by glutamic dehydrogenase.

Direct evidence has been obtained that the β-hydrogen of isocitrate is not lost in *either* isocitric dehydrogenase reaction. *Threo*-D_s-isocitrate-β-T has been synthesized by reduction of oxalosuccinate in T_2O with TPNH and TPN-linked isocitric dehydrogenase.



On oxidation of this isocitrate with either the DPN- or the TPN-isocitric dehydrogenase, α-ketoglutarate was obtained which contained essentially all of the radioactivity, while the reduced pyridine nucleotides formed were unlabeled (Table III). The demonstration of the transfer of label from the β-hydrogen of isocitrate to α-ketoglutarate by TPN-linked isocitric dehydrogenase would give direct support to the observations of Rose *et al.* (1962) who had oxidized citrate-α-T in the presence of aconitase and a large amount of TPN-specific isocitric dehydrogenase to α-ketoglutarate containing about 30% of the label. Presumably, aconitase formed isocitrate-β-T, which was then oxidized to α-ketoglutarate without loss of label. The label which *was* lost apparently went into the medium during the aconitase reaction. Direct evidence has been provided in the present experiments that the β-hydrogen of isocitrate is retained in α-ketoglutarate in *either* isocitric dehydrogenase reaction. Hence, the *enol* form of oxalosuccinate is not likely to be an intermediate in the oxidation of iscoitrate by either enzyme.

These results show that DPN-linked isocitric dehydrogenase from bovine heart catalyzes the removal of the α-hydrogen atom of isocitrate and transfers it directly to the α-side of the nicotinamide ring of DPN^+. Except for the difference in coenzymes, this is also what occurs in the case of TPN-linked isocitric dehydrogenase from the same source. Experiments which employ TPN-specific isocitric dehydrogenase for studies on the stereochemistry of the Krebs cycle hydrogen transfers (for review, see Englard, 1960b) tacitly imply that it is the TPN-enzyme which is chiefly responsible for isocitrate oxidation in this cycle. However, such experiments would have yielded the same results if the DPN-linked isocitric dehydrogenase had been used, as is now apparent from the similarity of the substrate-to-coenzyme hydrogen transfer properties of the two enzymes.

TABLE III

OXIDATION OF *threo*-D_s-ISOCITRATE-β-T

To 0.30 ml of T_2O (50 mc/ml) was added 0.20 ml of 0.016 M oxalosuccinate, *p*H 7.0. After 10 minutes at 25°, the following were added: 0.015 ml of 2.0 M imidazole buffer, *p*H 5.8; 0.03 ml of 0.02 M $MnCl_2$; 0.05 ml of 0.005 M TPNH; and 0.01 ml of TPN-linked isocitric dehydrogenase solution containing 2 units. After standing for 2.5 hours, the reaction mixture was lyophilized. To the residue were added 0.5 ml of 1.0 M H_2SO_4 and 1.0 ml of 0.001 M $Al_2(SO_4)_3$, followed by 50 ml of water and 8 μmoles of *threo*-D_sL_s-isocitrate carrier. Labeled isocitrate was obtained by resolution of DEAE-cellulose as described under Materials and Methods.

The labeled isocitrate was oxidized in both the DPN- and TPN-specific isocitric dehydrogenase systems, and the pyridine nucleotides were separated by Norit treatment as in Table I. The filtrates were extracted with ether. The residues after evaporation of the ether were taken up in a small amount of water for radioactivity determination. The prolonged ether extraction probably resulted in some loss of label from α-ketoglutarate into water by enolization.

Substance	Isocitric Dehydrogenase Reaction	μmoles	Total cpm	cpm/μmole
Initial isocitrate-β-T[a]	DPN or TPN	0.111	711	6460
Recovered α-ketoglutarate	DPN	0.084	418	4980
Recovered reduced DPN	DPN	0.052	0	0
Recovered α-ketoglutarate	TPN	0.097	438	4520
Recovered reduced TPN	TPN	0.078	8	100

[a] Determined as *threo*-D_s-isocitrate with TPN-linked isocitric dehydrogenase.

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Studies on the Electron Transfer System. LIV. Isolation of the Unit of Electron Transfer*

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The procedure described for the fractionation of mitochondria leads to the isolation of a particle, which uniquely contains all of the fixed components of electron transfer; the enzymic activity increases concomitantly with the purification of the particle. The rates of oxidation of DPNH and of succinate have been increased by a factor of 2.5 and the concentration of cytochrome *a* by a factor of 2.2. The specific concentrations of the other components have been increased to about the same degree. The isolated particle (designated the elementary particle) appears to be a physical and functional aggregate of the four complexes known collectively to constitute the electron transfer system; the theoretical molecular weight (on a protein basis) is 1.4×10^6; that of the particle described was calculated to be 2.1×10^6. Further purification (by removal of structural protein, etc.) led to an estimated minimal molecular weight of 1.4×10^6 but also decreased the enzymic activity.

It has been appreciated for some time that the essential features of mitochondrial function are retained by submitochondrial particulate preparations—features such as electron transfer from substrate to oxygen and oxidative phosphorylation. The digitonin particle of Cooper and Lehninger (1956), the electron transfer particle described by Crane, Glenn, and Green (1956), and the particles of the Keilin-Hartree preparation (1947) are well-known examples of submitochondrial particles that retain many of the attributes of the intact mitochondrion. The problem has been not whether a functional unit smaller than the mitochondrion exists but rather what are the dimensions of this subunit and how is the mitochondrion built from such subunits.

When mitochondria are subjected to sonic irradiation they undergo a limited disruption as organized units and are fragmented into particles of much smaller dimensions. During the fragmentation of beef heart mitochondria some 20% of the total protein is released into the medium in soluble form whereas the remaining 80% is found associated with the water-insoluble particulate fragments (Linnane and Ziegler, 1958). The complete electron transfer chain and the apparatus for coupled phosphorylation are localized in the particles; in the soluble fraction are found auxiliary enzymes and enzyme complexes that are concerned with the citric acid cycle oxidation, fatty acid oxidation, and, in general, with the release of electrons from substrates for entry into the electron transfer chain (although significant amounts of the enzyme complexes remain with the particulate fraction). The fragmentation of mitochondria by sonic irradiation into a particulate structured fraction and a soluble fraction makes it clear that there cannot be a single mitochondrial subunit which is all-embracing, but rather that several fractions or subunits exist, each designed for a specific function. The particular subunit carrying out the special function with which we are concerned in the present communication is the one that contains the complete electron transfer chain (or chains)—the apparatus for the transfer of electrons from succinate and DPNH to molecular oxygen.

The electron transfer chain contains eleven known oxidation-reduction components, arranged in an orderly sequence based upon drops in potential; all these components participate in the transfer of electrons along the chain. If we assume that these components are present in repeating electron transfer chains and that all chains are identical single-branched chains each containing only one molecule (or a multiple thereof) of each component, it is possible to calculate the molecular or particle weight of a complete electron transfer chain. The flavoprotein associated with succinic–coenzyme *Q* reductase activity is an essential component of the electron transfer chain; there are many lines of evidence, which will be pointed out later, to support the thesis that there is only one molecule of this type of flavoprotein per chain. In the electron transfer parti-

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