The Distribution and Properties of Transhydrogenase from Animal Tissues

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In 1952 Colowick, Kaplan, Neufeld & Ciotti described a new enzyme which they named pyridine nucleotide transhydrogenase. This enzyme, which was extracted from *Pseudomonas fluorescens*, catalyses the following reaction:

where TPN^+ and TPNH represent the oxidized and reduced forms of triphosphopyridine nucleotide and DPN^+ and DPNH represent the corresponding forms of diphosphopyridine nucleotide. Further studies by Kaplan, Colowick & Neufeld (1952, 1953) showed that the reactions were brought about by hydrogen, not electron, transfer and that the enzyme occurred in some animal tissues.

The purpose of the present study was to examine more closely the distribution in animal tissues and also to obtain some information on the nature of the enzyme.

EXPERIMENTAL

Materials

Animals. Earthworms, crabs and opossums were collected from their natural environment and killed at the laboratory. Rats and rabbits were obtained from laboratory animal houses. Slaughterhouse material was used for studies of chickens, pigs, oxen, sheep and horses; tissues from these animals were brought to the laboratory in ice-filled Dewar flasks, the transport time being always less than 2 hr.

Chemicals. DPN^+ and DPNH were purchased from Boehringer and Sons, Mannheim; purities were determined by alcohol dehydrogenase, DPN^+ assaying 95–100% and DPNH 54–67%. TPN^+ (72%) was kindly given by Professor F. Dickens, and some samples (78–80% pure) were purchased from Sigma Chemicals, Inc., U.S.A. (assayed by isocitric dehydrogenase). DL-isocitric lactone was purchased from L. Light and Co., and converted into isocitrate by the method of Krebs & Eggleston (1944). Flavine mononucleotide (FMN) was presented by Sigma Chemicals and flavin-adenine dinucleotide by Dr L. G. Whitby, Cambridge. Digitonin was purchased from L. Light and Co., and British Drug Houses Ltd.

Enzymes. isocitric dehydrogenase was prepared by the method of Grafflin & Ochoa (1950). Crystalline alcohol dehydrogenase was purchased from Boehringer and Sons or isolated according to Racker (1950).

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Methods

All manipulations were conducted at 0–2°.

Particles. The 'KCl particles' of Kaplan *et al.* (1953) were prepared by blending tissue with 10 vol. of 0.9% KCl in 0.01 M- NaHCO_3 for 2 min., centrifuging for 5 min. at 400 g and decanting the supernatant through two layers of coarse muslin. This fluid was then centrifuged for 20 min. at 20000 g. The sedimented particles were suspended in 1 ml. of 0.9% KCl for each g. of tissue originally blended.

Mitochondria. These were isolated by grinding tissue in 5 vol. of 0.25 M sucrose in a Potter-Elvehjem homogenizer. The suspension was poured through muslin and centrifuged for 10 min. at 700 g. The residue (nuclear fraction) was washed with 5 vol. of sucrose and the combined supernatants were centrifuged for 20 min. at 5000 g. The mitochondrial fraction was washed once with 5 vol. of sucrose and suspended in 1 ml. of 0.9% KCl for each g. of tissue.

Digitonin-calcium phosphate extract. This was prepared according to Kaplan *et al.* (1953) with minor modifications. The digitonin-particle suspension was centrifuged for 20 min. at 25 000 g and the digitonin-calcium phosphate mixture for 15 min. at the same speed. The gel from 20 g. of tissue was washed with 4 ml. portions of M- K_2HPO_4 and eluted with 3 ml. portions of M- KH_2PO_4 .

Ammonium sulphate fractionation. The solid salt was added to give the required concentration and M- NaHCO_3 was used to maintain the pH.

Assays. These were all conducted at room temperature (18–23°). The experiments on distribution were carried out according to Kaplan *et al.* (1953) by following the reaction $\text{TPNH} + \text{DPN}^+ \rightarrow \text{TPN}^+ + \text{DPNH}$ by means of the increase in $E_{340 \text{ m}\mu}$. Each 1 cm. cell contained 0.25 ml. of TPN^+ (1 mg./ml.), 0.1 ml. of KCN (2 mg./ml.), 0.1 ml. of nicotinamide (73 mg./ml.), 1.5 ml. of 0.16 M aminotrihydroxymethylmethane (tris) buffer, pH 7.5, and 0.2 ml. of isocitric dehydrogenase (sufficient to give instantaneous reduction of the TPN^+); the blank received 0.6 ml. of water and the experimental cell 0.6 ml. of isocitrate (1.5 mg./ml.). To both cells was then added 0.2 ml. of the preparation to be tested and when the $E_{340 \text{ m}\mu}$ became constant (1–5 min.) 0.2 ml. of DPN^+ (2 mg./ml.) was added. With active preparations the $E_{340 \text{ m}\mu}$ increased because of the accumulation of DPNH by transhydrogenase action and the instantaneous regeneration of TPNH by the isocitric system. Nicotinamide inhibits nucleotidases and KCN prevents reoxidation of coenzymes by the cytochrome system.

When using KCl particles one of the units defined by Kaplan *et al.* (1953) was chosen, i.e. 100 times the change in $E_{340 \text{ m}\mu}$ (between 1 and 7 min. after DPN^+ addition)/g. of tissue. It was not feasible to use this unit in studies with mitochondria and purified extracts and in these cases the

unit was taken as $\mu\text{g.}$ of DPNH formed/mg. of enzyme protein/hr. It is these units which are referred to as activity. The amount of enzyme was given by the amount of co-enzyme which could be oxidized in 1 hr. by the whole fraction. Protein in enzyme preparations was estimated by observing the $E_{280\text{ m}\mu}$ after solution in 0.1 N-NaOH. It was arbitrarily assumed that the enzyme had the same absorption characteristics as bovine serum albumin, i.e. that a solution containing 1.5 mg./ml. gave an $E_{280\text{ m}\mu}$ of 1.0 in a 1 cm. cell.

The method given above was not suitable for studying inhibitors because these compounds often acted on the isocitric dehydrogenase, thus obscuring their effect on transhydrogenase. Therefore the reaction

was studied. To conserve some reagents, 0.2 cm. cells were used and each contained 0.1 ml. of enzyme in M phosphate buffer, pH 6.3, and 0.05 ml. of TPN^+ (1 mg./ml.); the blank received 0.3 ml. of water, the control 0.2 ml. of water plus 0.1 ml. of DPNH (0.3 mg./ml.), and the experimental cell 0.2 ml. of inhibitor (adjusted to pH 6.3) plus 0.1 ml. of DPNH. The reaction was allowed to proceed for 6 min. and then 0.025 ml. of tris [saturated solution in 25% (v/v) ethanol] was added. This not only stopped the reaction but provided a suitable pH (10) and substrate (ethanol) for the action of alcohol dehydrogenase (0.06 ml. of 0.5 mg./ml.) which was next added. The increase in $E_{340\text{ m}\mu}$ was noted until it became constant (1–3 min.); this gave the amount of DPN^+ previously formed. Finally 0.01 ml. of DPN^+ (2 mg./ml.) was added to check the efficiency of the DPN^+ determination, particularly with regard to the possible action of an inhibitor on alcohol dehydrogenase. Where less than 90% recovery of the DPN^+ was obtained, the experiment was rejected. The only inhibitors used which affected alcohol dehydrogenase were those which reacted with sulphhydryl groups. In these cases, an excess of glutathione was added immediately after stopping the transhydrogenase reaction. Because the DPNH samples sometimes contained up to 10% of DPN^+ , it was necessary to apply a small correction for the amount actually found by analysis.

RESULTS

Distribution

Table 1 shows the distribution of transhydrogenase in different tissues of several animals as exemplified by the activity of KCl particles. Muscle, and in particular, heart muscle, was the only type of tissue found to give high activity. In some cases (rat kidney and liver) there was a decrease in $E_{340\text{ m}\mu}$ after the addition of DPN^+ , leading to negative values for the activity. This was probably due to the presence of systems capable of oxidizing the reduced coenzymes without the mediation of the cytochrome system. Such results are to be expected when crude preparations are used. Therefore, the distribution studies were extended to determine if activity were localized in some subcellular unit. Table 2 shows that it is the mitochondria which contain the transhydrogenase, and Table 3 compares the activity of mitochondria from different tissues. Again heart

muscle was most active and tissues which previously gave negative values (rat kidney and liver) now showed moderate activities. All the results given below were obtained with rat heart; similar results were obtained with ox heart.

Table 1. Activity of KCl particles

The values are means. Units of activity are $100 \times$ the change in $E_{340\text{ m}\mu}/6\text{ min.}/\text{g.}$ of tissue.

Tissue	Activity	No. of observations
Rat brain	4	5
Rat heart	87	14
Rat kidney	Negative	2
Rat liver	Negative	3
Rat muscle	28	2
Rat spleen	4	3
Rat testis	9	2
Pig heart	36	3
Ox heart	50	4
Sheep heart	22	1
Horse heart	20	2
Opossum heart	67	1
Chicken heart	125	1
Chicken liver	4	1
Crab muscle	5	2
Earthworm muscle	7	1

Zero values were given by rat prostate and seminal vesicle, pig brain, calf brain, ox brain, sheep brain, and rabbit brain and liver.

Table 2. Subcellular distribution in rat tissues

Values are $\mu\text{g.}$ of DPN^+ /mg. of protein/hr.

	Nuclei	Mitochondria	Soluble fraction
Brain	0	2	0
Heart	5	47	4
Kidney	6	27	0
Liver	2	13	0
Spleen	2	8	3

Table 3. Activity of mitochondria

Values are means as $\mu\text{g.}$ of DPN^+ /mg. of protein/hr.

Tissue	Activity	No. of experiments
Rat brain	4	2
Rat heart	68	3
Rat kidney	16	3
Rat liver	13	1
Rat muscle	11	1
Rat spleen	4	2
Rat testis	4	1
Rabbit heart	33	2
Rabbit kidney	5	1
Pig heart	15	3
Horse heart	21	1

Purification

Transhydrogenase could be extracted from fresh KCl particles or mitochondria by grinding with glass powder and 0.9% KCl containing 0.005M

phosphate buffer, pH 6.9. There was only a slight increase in activity. If frozen-dried KCl particles or mitochondria were stirred with KCl-phosphate the resulting extract showed about double the activity of the original material and these extracts could be fractionated with ammonium sulphate (Table 4), giving, between 20 and 35% saturation, good yield and increased activity. The 20–35% fraction could be reprecipitated with little loss of material and a further gain in activity, e.g. a 20–35% fraction of 318 units of activity ($\mu\text{g. of DPN}^+/\text{mg. of protein/hr.}$) on reprecipitation between 20 and 35% saturation had an activity of 524 units with 81% recovery of the original amount of enzyme.

All these preparations were unstable, losing 25–30% of their activity overnight at 0° or -10° . Re-activation could not be obtained by adding glutathione, flavine mononucleotide or flavine adenine dinucleotide; nor could activity be preserved by storage in 1% albumin, one-tenth saturated ammonium sulphate or 0.0013M glutathione. The effect of pH on the activity of transhydrogenase was tested by replacing the tris buffer with phosphate (final concentration 0.06M). Fig. 1 shows the curve obtained, the pH values plotted being those determined at the end of the experiment; the pH optimum was 6.3 and this was used in subsequent experiments. The same optimum was found for the reaction $\text{DPNH} + \text{TPN}^+ \rightarrow \text{DPN}^+ + \text{TPNH}$ (Fig. 1).

Kaplan *et al.* (1953) extracted transhydrogenase from KCl particles by adding digitonin and purified the extract by adsorption on calcium phosphate gel and ten elutions with $\text{M-KH}_2\text{PO}_4$. Table 5 shows that most of the enzyme could be obtained with only six elutions and that on storage overnight there were losses of up to 30% in activity. In this experiment 30% of the amount of enzyme in the digitonin extract was not adsorbed by the gel, and of the amount adsorbed 19% was not recovered in the seven eluates. In a further experiment, six active eluates were combined and had a pH of 6.6. Overnight at 0° there was 45% loss of activity; at -10° with no addition there was 56% loss, in 1% albumin 45%, in one-tenth saturated ammonium sulphate 80%, and in 0.0013M glutathione 50%. The effect of pH on storage was investigated by combining six active eluates and adjusting samples to different pH values. After storage overnight the samples were re-adjusted to pH 6.3, centrifuged and activities determined (Table 6). It can be seen that 6.3 was the optimum pH for storage, the increase in activity probably being due to removal of inert proteins. Further, such solutions could be fractionated with ammonium sulphate between 20 and 35% saturation to give increases in activity of up to 50%. Also the activity could be increased by prolonged centrifuging at high speeds, e.g. 3 hr. at 160 000 g;

Table 4. *Fractionation with ammonium sulphate*

Units are $\mu\text{g. of DPN}^+/\text{mg. of protein/hr.}$ The mitochondria had an activity of 70 units. They gave a KCl-phosphate extract equal to 125 units with an amount of enzyme equivalent to 4.88 mg. of $\text{DPN}^+/\text{hr.}$

Fraction (saturation with $(\text{NH}_4)_2\text{SO}_4$, %)	Activity ($\mu\text{g. of DPN}^+/\text{mg. of protein/hr.}$)	Amount of enzyme (mg. of $\text{DPN}^+/\text{hr.}$)
0–10	483	0.41
10–20	248	0.18
20–30	381	1.78
30–35	248	1.13
35–40	16	0.03
	Sum	3.53
		(72% recovery)

Fig. 1. Effect of pH on transhydrogenase activity of rat heart. ●, $\text{TPNH} + \text{DPN}^+ \rightarrow \text{TPN}^+ + \text{DPNH}$; x, $\text{DPNH} + \text{TPN}^+ \rightarrow \text{DPN}^+ + \text{TPNH}$. Experimental conditions were as described in the text under Methods. It should be noted that the conditions are not directly comparable.

Table 5. *Activity of eluates from calcium phosphate gel*

The eluates were stored at -10° for 16 hr.

Eluate no.	Activity ($\mu\text{g. of DPN}^+/\text{mg. of protein/hr.}$)	Amount of enzyme (mg. of $\text{DPN}^+/\text{hr.}$)	Activity after storage ($\mu\text{g. of DPN}^+/\text{mg. of protein/hr.}$)
1	148	6.9	124
2	360	14.4	298
3	551	14.4	427
4	1030	12.2	710
5	1070	8.9	759
6	1120	4.1	888
7	250	1.5	186

the eluates were usually light golden in colour but after such centrifuging the contents of the tube could be drawn off in several fractions (Table 7) ranging from colourless to brown. The increased activity of the top and intermediate fractions was probably due to removal of the coloured impurities; the extent of this increase in different experiments varied between 100 and 500 %.

Inhibitors

Studies with inhibitors were made both with combined eluates and also with the fractions pre-

pared by high-speed centrifuging of these eluates. The results in Table 8 show that sulphhydryl reagents were powerful inhibitors.

Michaelis constants

These were determined for the reaction

by the assay method used for studying inhibitors. The values found were $1.4 \times 10^{-5} \text{ M}$ DPNH and $15.7 \times 10^{-5} \text{ M}$ TPN⁺.

Absorption spectra

All preparations showed a maximum at 275 m μ . and unpurified preparations also had a small peak at 407 m μ . which was probably due to contaminating methaemoglobin.

DISCUSSION

The distribution reported is similar to that observed by Kaplan *et al.* (1953), i.e. heart being most active and some tissues entirely inactive, but these workers found higher activities than those reported here. The localization of transhydrogenase in mitochondria is in keeping with the importance of these particles in metabolism.

Most of the enzyme preparations had a faint yellow colour and some green fluorescence indicative of flavines. However, since no flavine bands were present in the absorption spectra and since colourless, active solutions could be obtained by means of high-speed centrifuging, the enzyme is probably not a flavoprotein. Further, aged preparations could not be re-activated by adding flavine mono- or dinucleotide. Also, attempts to split off a prosthetic group by means of ammonium sulphate at pH 3 and 0° were unsuccessful; the supernatants obtained were clear, colourless and non-fluorescent. The marked inhibition brought about by various sulphhydryl reagents shows that some intact sulphhydryl groups are necessary for activity. The lack of inhibition by metal-combining reagents suggests that the enzyme is not a metalloprotein.

The pH optimum of 6.3 agrees well with the 6.2 found by Kaplan *et al.* (1952). An attractive hypothesis for the function of transhydrogenase would be that its presence allows cells to make greater use of systems requiring triphosphopyridine nucleotide. Most tissues have much less TPN⁺ + TPNH than DPN⁺ + DPNH (Glock & McLean, 1955), and in many cases the amount of TPN⁺ + TPNH is so small that it is questionable whether systems requiring triphosphopyridine nucleotide can play a quantitatively significant role. If it could be shown that tissues rich in transhydrogenase had a high TPN⁺/TPNH ratio, and that tissues poor in transhydrogenase had a low value,

Table 6. *Effect of pH and storage on activity*

Values give the activity compared to the original combined eluates taken as 100.

pH	Storage temp.	
	0°	-10°
5.8	75	66
6.3	132	108
7.1	60	45
8.0	0	0
8.3	12	24

Table 7. *Separation of transhydrogenase by centrifuging*

Combined eluates (30 ml.; activity, 209 units) were centrifuged for 3 hr. at 160 000 g.

Fraction	Volume (ml.)	Colour	Activity ($\mu\text{g. of DPN}^+/\text{mg. of protein/hr.}$)
1	2.4	Colourless	358
2	2.4	Colourless	560
3	5.0	Colourless	489
4	5.1	Faint yellow	692
5	5.2	Faint yellow	319
6	7.5	Yellow	124
7	1.9	Yellow-brown	89
Residue	—	Brown	—

Table 8. *Effect of inhibitors on transhydrogenase*

Figures give the percentage of the control activity.

Inhibitor	Concn. (mm)	Activity (% of control)
Fluoride	10	99
Pyrophosphate	2	91
Pyrophosphate	10	53
Azide	9	105
<i>o</i> -Phenanthroline	5	96
Diethyldithiocarbamate	9	105
Glycine	9	94
Oxalate	9	96
Cyanide	1	100
<i>p</i> -Chloromercuribenzoate	2.2	68
<i>p</i> -Chloromercuribenzoate	4.5	14
Iodosobenzoate	0.17	79
Phenyl mercuriacetate	0.03	33
HgCl ₂	0.05	65
Arsenite	1.0	75

then the role of transhydrogenase would be clearly indicated. However, the evidence for this is not definite. Rat heart muscle, kidney and liver, which all have high transhydrogenase activities, contain 3–6 $\mu\text{g.}$ of TPN^+ /g. but the concentration of TPNH is 33–205 $\mu\text{g.}/\text{g.}$, the TPN^+/TPNH ratio being 0.03–0.13 (Glock & McLean, 1955). Rat prostate, seminal vesicle, spleen and testis, which have negligible transhydrogenase activity, contain negligible quantities of TPN^+ (<2 $\mu\text{g.}/\text{g.}$) and only 8–12 $\mu\text{g.}$ of $\text{TPNH}/\text{g.}$ It could be argued that transhydrogenase occurs along with the higher concentrations of $\text{TPN}^+ + \text{TPNH}$; on the other hand, it would be expected that the presence of transhydrogenase would lead to a value for TPN^+/TPNH higher than that observed (0.03–0.13), especially since all tissues so far tested contain more DPN^+ than DPNH , e.g. Glock & McLean (1955) give DPN^+/DPNH for rat heart as 1.6 and Jedeikin & Weinhouse (1955) give 5.5 for the same tissue, the total concentrations given being respectively 483 and 563 $\mu\text{g.}/\text{g.}$

SUMMARY

1. Transhydrogenase occurs in animal tissues, particularly in heart muscle and to a smaller extent in skeletal muscle, kidney and liver. Other tissues such as brain, prostate, seminal vesicle, spleen and testis show small or negligible activities.
2. Transhydrogenase is localized in mitochondria.
3. Transhydrogenase can be purified by precipitation with ammonium sulphate in the range 20–35 % saturation.
4. Inert material can be removed from solutions

of transhydrogenase and the activity thus increased, by centrifuging for 3 hr. at 160 000 g.

5. The pH optimum for each of the reactions

is 6.3.

6. Michaelis constants for the reaction

are $1.4 \times 10^{-5} \text{ M}$ DPNH and $15.7 \times 10^{-5} \text{ M}$ TPN^+ .

7. The action of transhydrogenase is strongly inhibited by sulphhydryl reagents.

8. Solutions of transhydrogenase show maximum light absorption at 275 m μ .

9. There is no evidence at present for the presence of any flavine or metal prosthetic group in the enzyme.

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REFERENCES

- Colowick, S. P., Kaplan, N. O., Neufeld, E. F. & Ciotti, M. N. (1952). *J. biol. Chem.* **195**, 95.
 Glock, G. E. & McLean, P. (1955). *Biochem. J.* **61**, 388.
 Grafflin, A. L. & Ochoa, S. (1950). *Biochim. biophys. Acta*, **4**, 205.
 Jedeikin, L. A. & Weinhouse, S. (1955). *J. biol. Chem.* **213**, 271.
 Kaplan, N. O., Colowick, S. P. & Neufeld, E. F. (1952). *J. biol. Chem.* **195**, 107.
 Kaplan, N. O., Colowick, S. P. & Neufeld, E. F. (1953). *J. biol. Chem.* **205**, 1.
 Krebs, H. A. & Eggleston, L. V. (1944). *Biochem. J.* **38**, 426.
 Racker, E. (1950). *J. biol. Chem.* **184**, 313.

The Utilization of Inorganic Sulphate by Granulation Tissue

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In a preceding paper (Kodicek & Loewi, 1955), the uptake of $^{35}\text{SO}_4^{2-}$ by granulation tissue *in vivo* and *in vitro* was described. It was found that a sulphated mucopolysaccharide akin to chondroitin sulphate was formed, the formation depending on enzymic activity of the tissue.

The present communication is concerned with studies of the relationship between the respiratory activity of granulation tissue at various stages of maturation and the uptake of $^{35}\text{SO}_4^{2-}$. The effect of various added substances has also been investigated. We have examined the inhibitory effects on

the system of various phenols, some sulphated substances and fluoroacetate. Addition of glucose to respiring sections of cartilage (Boyd & Neuman, 1954; Boström & Jorpes, 1954) resulted in increased incorporation of $^{35}\text{SO}_4^{2-}$, and Boström, Roden & Vestermark (1955) reported that glutamine acted as an accelerator of chondroitin sulphate synthesis in cartilage.

Since granulation tissue possesses a system for the synthesis of sulphated mucopolysaccharides, we thought it of interest to determine whether this tissue would also sulphate phenols. Such activity