

STUDIES OF OXIDATIVE PHOSPHORYLATION  
AND RELATED PHENOMENA IN  
EPHRLICH ASCITES TUMOUR CELL MITOCHONDRIA

By

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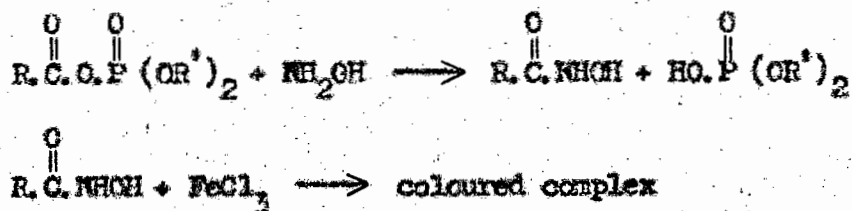
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(ii) Paper Electrophoresis

This was carried out according to Gurin and co-workers (7), on Whatman No. 1 paper with 0.05 M phosphate buffer (pH 6.8) at 2°C. for 18 - 20 hours.

(iii) Reactive Acyl Linkage Determination

Mixed anhydrides were determined by the method of Tuttle and Lipmann (1), as described by Stadtman (4), employing as standards succinic anhydride and acetic anhydride. The method is based on the fact that acyl phosphates react rapidly with hydroxylamine at pH 6.5 to 7.0 to form hydroxamic acids, which in the presence of ferric salts produce red complexes which can be estimated colorimetrically. The reaction is shown below:



Reagents

- (a) Hydroxylamine reagent - This is prepared just before use by mixing equal volumes of 4.0 N  $\text{NH}_2\text{OH.HCl}$  and 3.5 M NaOH.
- (b) Ferric chloride solution - Prepared by mixing equal volumes of 5%  $\text{FeCl}_3$  (in 0.1 N HCl), 12% trichloroacetic acid, and 3 N HCl.
- (c) Hydroxamic acid standard - Prepared from succinic anhydride as follows: Succinic anhydride (0.1 g.) was dissolved in 4.0 ml. of the hydroxylamine reagent and then diluted to 10.0 ml. with water. When 1.0 ml. of this solution was diluted to 40 ml. with water, 1.0 ml. of the resulting solution was equivalent to 2.0 micromoles of succinohydroxamate.

### Assay

To 0.1 ml. of the unknown solution plus 1.9 ml. of water, was added 1.0 ml. of the neutralised hydroxylamine reagent. After being allowed to stand for 10 minutes at room temperature, 3.0 ml. of the ferric chloride reagent was added and the resultant colour read at 540 m $\mu$  in a Beckman spectrophotometer.

### (iv) Phosphorus

An aliquot portion of the aqueous extract was digested with sulphuric acid and hydrogen peroxide (5), and the liberated inorganic phosphate determined by the method of Fiske and Subbarow (2).

### (v) Ribose

This was determined by the orcinol procedure (3) using D-ribose as standard. Optical densities at 660 m $\mu$  were read in a Beckman spectrophotometer.

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Aliquots (0.15 ml.) of the mitochondrial suspension in 0.25 M sucrose (containing 4 - 6 mg. protein/ml.) were added to 2.85 ml. of the medium in which swelling was to be determined. After thorough mixing, the change in optical density at 520 mμ was read in a Model DU 2400 Beckman Spectrophotometer against a blank containing the medium under examination plus 0.25 M sucrose in place of the mitochondrial suspension. Values are expressed as Specific Extinction coefficients ( $E_{1\%}^{1\text{cm}}$ ) for suspensions containing 1% of mitochondrial protein.

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## SECTION C

### STUDIES ON ISOCITRATE OXIDATION IN ENRICH ASCITES CELL MITOCHONDRIA

#### 1 MATERIALS

Both the source and method of assay of the following substances have been described in Section B: hexokinase, cytochrome c, ADP, AMP, ATP and diphosphopyridine nucleotide.

##### 1.1 Nicotinamide

This compound was supplied by Lorek and Co., Darmstadt, Germany.

##### 1.2 Isocitrate

This was prepared from DL-isocitric acid lactone (obtained from General Biochemicals Inc., U.S.A.) as follows: DL-isocitric acid lactone (17.4 mg.) in 0.6 ml. of 0.5 N KOH was heated on a boiling water bath for 10 minutes, cooled, neutralised with dilute HCl (pH 7.4), and adjusted to a final volume of 1.0 ml. with distilled water. Solutions were stored at  $-30^{\circ}\text{C}$ . and used within seven days.

##### 1.3 Amytal (5-ethyl-5'-isocamyl-barbituric acid)

This was used as the sodium salt, supplied by E. Lilly and Co., U.S.A. Solutions were prepared in water.

##### 1.4 Dioxane (diethylene dioxide)

Dioxane was obtained from British Drug Houses (England), and was purified according to Vogel (1), as follows: Dioxane (100 ml.) was refluxed over an excess of clean sodium for 12 hours, and then distilled through a small fractionating column giving a clear liquid

which was stored in a well stoppered dark brown bottle at  $-30^{\circ}\text{C}$ . Whether purified or not, dioxane failed to effect reduction of 2,6-dichlorophenol-indophenol.

#### 1.5 2,6-Dichlorophenol-indophenol

This compound was obtained from Light and Co., England, and was used as such. Solutions were prepared in distilled water and stored at  $2^{\circ}\text{C}$ .

#### 1.6 Ethanol

Reagent grade ethanol (96%) was refluxed for 2 - 3 hours over KOH pellets and twice redistilled. Schiff's and Brady's tests for carbonyl groups were both negative.

#### 1.7 Ovalbumin

This material was a twice recrystallized and lyophilized preparation supplied by Light and Co., England.

#### 1.8 Alcohol Dehydrogenase

The enzyme was supplied by General Biochemicals Inc., U.S.A. It was assayed by the method of Hacker (8).

#### 1.9 D-Glucose-6-phosphate Dehydrogenase

The enzyme was supplied by General Biochemicals Inc., U.S.A.

#### 1.10 D-Glucose-6-phosphate

The barium salt was supplied by General Biochemicals Inc., U.S.A. It was converted to the sodium salt by the method of De Meess (2), as follows: To a solution of barium glucose-6-phosphate (52 mg.) in 0.25 ml. of N/1 HCl was added 0.4 ml. of saturated  $\text{Na}_2\text{SO}_4$ . After the mixture had stood for five minutes, precipitated  $\text{BaSO}_4$  was brought down by centrifugation (ten minutes at 2500 r.p.m.),

## 2.2 Pyridine Nucleotide Depleted Mitochondria

A mitochondrial suspension as prepared above was depleted in one of two ways.

- (i) Depletion by treatment with 0.25 M sucrose plus 0.03 M phosphate buffer (pH 7.4). To 1.6 ml. of mitochondrial suspension in 0.25 M sucrose was added 1.6 ml. of 0.25 M sucrose plus 0.06 M phosphate buffer (pH 7.4). Gentle homogenization with 5 strokes of the tight plunger in a small Dounce homogeniser resulted in a uniform suspension of mitochondria, which was incubated in a Carbury bath at 30° for 35 - 40 minutes aerobically. Following incubation, the mitochondrial suspension was immediately stored in ice.
- (ii) Depletion by ageing a mitochondrial suspension in 0.25 M sucrose for 5 - 6 hours at 0°.

## 2.3 Digitonin Treated Mitochondria

To 0.6 ml. of pyridine nucleotide depleted mitochondria (in 0.25 M sucrose plus 0.03 M phosphate, pH 7.4), was added 0.6 ml. of a freshly prepared 1% digitonin solution (6). After thorough mixing the digitonin preparation was allowed to stand 20 minutes at 0° with occasional stirring, and then immediately assayed.

## 3 REACTION MEDIA

### 3.1 Medium for Spectrophotometric Assays with 2,6-dichlorophenol-indophenol and Cytochrome c as Electron Acceptors

The medium used is based upon that of Kaplan (3).

The following were present in a final volume of 3.0 ml:

K<sub>2</sub>Na phosphate buffer (pH 7.4), 0.10 M; KCN, 0.001 M; MgCl<sub>2</sub>, 0.003 M; DL-isocitrate (K<sup>+</sup> salt), 0.0023 M; nicotinamide, 0.02 M; 2,6-dichlorophenol-indophenol, 50 µg. The concentrations of DHI, TPN, TPNH, DPNH and other components are given for each particular experiment.

Solutions of 2,6-dichlorophenol-indophenol contained 50 µg. of the dye per 0.2 ml. This, when present in a final volume of 3.0 ml., gave an initial optical density reading of approximately 0.90 at 600 mµ. Stock solutions of the dye were kept at 4°C. and used within one week of preparation.

Cytochrome c was used as a 1% solution in isotonic saline. The concentration was 2 mg. ( $1.174 \times 10^{-7}$  moles) per 0.2 ml.

Mitochondrial protein contents are given for each experiment.

Nicotinamide at a final concentration of 0.02 M was always present to prevent breakdown of DPH by DPH-ase action. In cases where L-glutamate or α-ketoglutarate were used as substrate, 0.07 ml. of 0.1 M L-glutamate or 0.07 ml. of 0.2 M α-ketoglutarate was added to the Beckman cuvette.

### 3.2 Generation of DPH

In experiments where DPH was required as substrate, the reduced pyridine nucleotide was generated in situ through use of alcohol dehydrogenase, ethanol and DPH. To a Beckman cuvette containing all the normal additions, was added 0.04 ml. of ethanol (96%) and 0.07 ml. of a solution containing the alcohol dehydrogenase. The reaction was started by the addition of 400 - 1500 µg. of DPH to both cuvettes. After rapid mixing, the change in optical density was followed either at 600 mµ (2,6-dichlorophenol-indophenol) or at 550 mµ (cytochrome c).

Alcohol dehydrogenase solution - This was prepared by adding 0.06 ml. of a stock alcohol dehydrogenase solution (contains 25.0 mg. enzyme per ml.) to 0.14 ml. of 0.01 M phosphate buffer (pH 7.4) plus 0.1% v/v ovalbumin. After gentle mixing the solution was stored in ice. As described above, 0.07 ml. of this solution was used per cuvette.

### 3.3 Generation of TPNH

In experiments where TPNH was required as substrate, it was generated in situ by the action of glucose-6-phosphate dehydrogenase on D-glucose-6-phosphate and TPN. The reaction medium is shown below:

2.00 ml. of 0.15 M K<sub>2</sub>H<sub>2</sub> phosphate buffer (pH 7.4)  
 0.30 ml. of 0.01 M KCN  
 0.03 ml. of 0.30 M MgCl<sub>2</sub>  
 0.10 ml. of TPN (400 - 1000 µg.)  
 0.10 ml. of 0.6 M nicotinamide  
 0.05 ml. of 0.1 M D-glucose-6-phosphate (Na<sup>+</sup> salt, pH 7.4)  
 0.20 ml. of 2,6-dichlorophenol-indophenol (50 µg.) or cytochrome c (2 mg.)  
 0.12 ml. of D-glucose-6-phosphate dehydrogenase (1 mg.)  
 0.10 ml. of water, or other substance under test

3.00 ml. (final volume)

The high concentration of phosphate used in these experiments did not appear to inhibit the action of the dehydrogenase (5).

### 3.4 Medium for the Determination of Isocitric Dehydrogenase Activity at Different pH Values

| <u>Components</u>                                                  | <u>Final Molarity</u> |
|--------------------------------------------------------------------|-----------------------|
| 1.20 ml. of 0.25 M tris-acetate <sup>2</sup> buffer (various pH's) | 0.100                 |
| 0.30 ml. of 0.01 M KCN . . . . .                                   | 0.001                 |
| 0.03 ml. of 0.30 M MgCl <sub>2</sub> . . . . .                     | 0.003                 |
| 0.10 ml. of DPN, TPN etc. (400 - 1500 µg.) . . . . .               | -                     |
| 0.07 ml. of 0.10 M DL-isocitrate (pH 7.4) . . . . .                | 0.0023                |
| 0.10 ml. of 0.60 M nicotinamide . . . . .                          | 0.020                 |
| 0.20 ml. of dye or cytochrome c . . . . .                          | -                     |
| 0.10 ml. of depleted mitochondria (0.25 M sucrose) . . . . .       | -                     |
| 0.90 of water or other substances under test . . . . .             | -                     |

3.00 ml. (final volume)

<sup>2</sup>Tris-acetate buffers were prepared by titration of the free base with 0.25 M acetic acid.

### 3.5 Medium for Measurement of Oxidative Phosphorylation

This was as described in Section B of the Experimental Section. Phosphate was assayed according to Fiske and Subbarow (7). Mitochondrial protein determined by method of Cleland and Slater (4).

## 4 SPECTROPHOTOMETRIC TECHNIQUES

### 4.1 2,6-Dichlorophenol-indophenol as Terminal Electron Acceptor

In studies with this electron acceptor, absorption measurements were made at 600 m $\mu$ . The blank cuvette contained all additions, with the exception of the electron acceptor. Reactions were initiated by the addition of substrate (isocitrate), and after rapid mixing, readings were taken at various time intervals. It is important to note that when 2,6-dichlorophenol-indophenol is reduced, the optical density at 600 m $\mu$  decreases, thus giving a - OD value. The results were, however, plotted as positive OD values, in conformity with other workers.

### 4.2 Cytochrome c as Terminal Electron Acceptor

With this electron acceptor, absorption measurements were made at 550 m $\mu$ . The blank contained all additions, with the exception of substrate. Reactions were initiated by addition of substrate to the test cuvette, and the increase in optical density recorded at one minute intervals.

Spectrophotometric assays were carried out with a Beckman Spectrophotometer Model DU, using 1 cm. cells. Cuvettes were always examined visually at the completion of each reaction for agglutination or precipitation of mitochondria.

5. TITRATION OF TRIS (HYDROXYETHYL)-  
AMINOETHANE WITH ACETIC ACID

A standard curve was prepared by titration of 0.25 M tris (free base) with 0.25 M acetic acid. Measurements of pH were made with a Cambridge pH meter previously standardised against the following buffers.

- (1) 1/20 Potassium Fthalate  
pH = 4.00 at 15°C.  
$$\text{pH}(t) = 4.00 + \frac{1}{2} \frac{(t-15)^2}{100}$$
- (11) 1/20 Borate Buffer  
pH = 9.27 at 15°C.  
$$\text{pH}(t) = 9.27 - 0.0085 (t-15)$$
- (111) A buffer of pH 7.0 was also employed.

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## SECTION D

### STUDIES ON FATTY ACID OXIDATION

#### 1 MATERIALS

##### 1.1 n-Octanoic Acid

This was prepared from n-octanol by chromic acid oxidation under mild conditions. The product was purified by twice recrystallising from hot water, and distillation under reduced pressure. Determination of the equivalent weight by alkali titration gave a value of 143.7 (theoretical, 144.0).

##### 1.2 Pyridine

Pyridine (Analar Grade) was treated with KOH pellets for 24 hours and then twice redistilled.

##### 1.3 Succinic Anhydride

This compound was prepared by treatment of succinic acid with acetic anhydride, and after purification was stored in vacuum over  $\text{CaCl}_2$ .

##### 1.4 Adenosine-5'-phosphate

The nucleotide was obtained from General Biochemicals Inc., Ohio, U.S.A.

##### 1.5 N,N'-Dicyclohexylcarbodiimide

This material was supplied by the Aldrich Chemical Co., U.S.A.

#### 2 METHOD

##### 2.1 Preparation of n-Octanoyl-Menylate

To 10 mg. (0.03 m.mole) of adenosine-5-phosphate

dissolved in a mixture of 0.25 ml. of distilled water and 0.45 ml. of anhydrous pyridine, was added 29 mg. (0.02 m.mole) of n-octanoic acid. The mixture was cooled in an ice-bath (0°C.) and then added to a solution of 0.10 g. of N,N'-dicyclohexylcarbodiimide dissolved in 0.30 ml. of pyridine.

The resultant two-phase mixture was then stirred (mechanically) for 40 minutes at room temperature. A single homogeneous phase developed after 4 minutes, and then at 8 minutes, crystallisation of N,N'-dicyclohexylurea began. The reaction was stopped by the addition of 1.0 ml. of ice-cold distilled water. The precipitate of N,N'-dicyclohexylurea was removed by filtration and washed twice with distilled water (0°C.). The washings and clear filtrate were then combined and extracted with diethyl ether (4 x 2.0 ml.) to remove unreacted n-octanoic acid and carbodiimide. After removal of traces of ether (air gassing) the aqueous solution was stored at -30°C.

In some experiments the nucleotides in the aqueous extract were precipitated by addition of cold acetone, and then isolated by centrifugation. However, owing to the instability of n-octanoyl-adenylate, it was best stored in aqueous solution at -30°C.

## 2.2 Analysis

### (1) Paper Chromatography

Aliquot portions of the aqueous solution were chromatographed on Whatman No. 1 paper using the solvent system, isopropanol: water (7:3 v/v), by the descending method at 2°C. for 18 - 24 hours. The chromatograms were air-dried and examined with an ultra-violet lamp (Black Ray Master, wave length of 260 mμ). Morrison's spray reagent (6) for reactive acyl linkages was also employed.

Standard (Klett) 173.0 = 0.025 mg.  $P_i$

Dilution factor = 25

$P_i$  value in Klett units for 25 minutes incubation = 22.5

Therefore

$P_i$  value in Klett units for 15 minutes incubation = 13.5

$P_i$  in mg. (15 minutes) =  $\frac{(13.5 \times .025 \times 25)}{173.0}$

$P_i$  in  $\mu$ g. moles =  $\frac{(13.5 \times .025 \times 25 \times 10^3)}{173.0 \times 31} = 1.573$

Therefore 0.01 mg. hexokinase phosphorylates 1.573  $\mu$  moles

D-glucose/15 minutes.

Therefore 1.0 mg. hexokinase phosphorylates 157.3  $\mu$  moles

D-glucose/15 minutes.

Hence the specific activity of the hexokinase preparation is 157.3.

#### 4.6 Diphosphopyridine Nucleotide

This was obtained from General Biochemicals Inc., and was assayed either by a modification of the method of Brodie (10), or enzymatically with alcohol dehydrogenase (12). Assay by the former method showed the DEN to be 85% pure.

##### Modified assay method of Brodie

Initially, DEN was assayed by the method of Lo-Fage (11). This consists of reducing the DEN by means of sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) in a dilute bicarbonate buffer. Low and erratic results were consistently obtained with this procedure. A number of experiments were therefore carried out, as a result of which the following conclusions were drawn.

(1) During reduction, the pH must be kept slightly alkaline (pH 7.4), and care must be taken to ensure sufficient buffering capacity in the solution.

(11) The sodium dithionite solution must be freshly prepared.

#### Sodium dithionite solution

Sodium dithionite (0.12 g.) is weighed out in a 20 ml. test tube, to which is carefully added 3.0 ml. of 0.2 M  $\text{Na}_2\text{HPO}_4$ . The contents are gently mixed (gas evolution is kept at a minimum if the procedure is carried out as described), and the pH checked (usually  $\pm$  6.5). A further 5 - 6 ml. of 0.2 M  $\text{Na}_2\text{HPO}_4$  is then added, and the contents mixed (pH 7.4).

#### Reduction of DPN

Diphosphopyridine-nucleotide (0.007 g.) is accurately weighed into a pointed 10.0 ml. centrifuge tube. One ml. of dithionite-phosphate buffer solution is added, and the tube after rapid mixing is immersed in a boiling water bath for exactly one minute. After cooling in crushed ice for 5 minutes, an aliquot portion (0.4 ml.) of the cooled solution is pipetted into another conical centrifuge tube and immediately diluted accurately to 10.0 ml. with 0.2 M  $\text{Na}_2\text{HPO}_4$ . Following thorough mixing the sample is well gassed with oxygen for 10 - 12 minutes (using compressed  $\text{O}_2$  through a saline wash).

A blank consisting of dithionite in 0.2 M phosphate buffer is run simultaneously, and gassed as above. The samples are then read in a Beckman spectrophotometer at 340 m $\mu$ . (An absorption curve of a sample of DPN reduced by this modification showed a symmetrical peak at 340 m $\mu$ .)

Results of DEN Assay

| Cell No. | Sample               | Log I/I<br>(340 mμ) | % DENH after<br>reduction | % DENH before<br>reduction |
|----------|----------------------|---------------------|---------------------------|----------------------------|
| 4189     | Blank                | -                   | -                         | -                          |
| 4190     | DEN                  | 0.547               | 82.50                     | -                          |
| 4190     | DEN<br>(not reduced) | 0.018               | -                         | 2.10                       |

$$\% \text{ DENH} = \frac{(\text{Reading in mμ} \times 118)}{(\text{Vt DEN in mg./0.1 ml. soln})}$$

For calculation, the molar extinction coefficient used, is that of Horecker and Kornberg ( $6.22 \times 10^6 \text{ cm}^2$  per mole DEN) (9).

Enzymatic assay

Estimation of DEN by the use of alcohol dehydrogenase and ethanol, was carried out by the method of Recker (12), the reduced DEN being estimated spectrophotometrically at 340 mμ.

## 2 METHODS

### 2.1 Isolation of Ehrlich Ascites Tumour Cell Mitochondria

All operations were carried out at 0 - 4°C. Mice were killed by cervical fracture and ascites fluid was withdrawn from the peritoneal cavity with a sterile syringe. The fluid was mixed immediately with sodium citrate solution [1 ml. of 3.8% (w/v) sodium citrate/10.0 ml.] and stored in ice. The cells were collected by centrifuging at 1800 g for 10 minutes and washed once or twice by resuspending in iso-osmotic NaCl solution. They were then dispersed in hypo-osmotic ( $\frac{1}{4}$  strength) Ringer-phosphate solution and allowed to stand 15 - 20 minutes in the cold with occasional stirring. Phase-contrast microscopy at this stage showed the ascites cells to be swollen but not lysed. Contaminating

erythrocytes were completely lysed. The suspension was then centrifuged at 1800 g for 10 minutes and the supernatant discarded. The cells (6 - 8 g. wet wt) were immediately resuspended in ice-cold water (60 ml.) for exactly 3.0 minutes by using a Dounce homogeniser with loose plunger (clearance approximately 0.001 in.) as supplied by Blaessig Glass Specialities, Rochester, N.Y., U.S.A. [To ensure complete lysis and removal of adhering cytoplasm from the nuclei, it is important to use 8 - 10 vol. of water/g. (wet wt) of cells.] Concentrated sucrose solution was then quickly added to give the usual 0.25 M sucrose suspension, which was further homogenised with 6 - 8 passes of the loose plunger, followed by 15 passes with the tight plunger (clearance approximately 0.0005 in.). Phase-contrast microscopy at this stage revealed a 90 - 95% homogenate in which the nuclei were free of cytoplasmic material. The pH of the homogenate measured at 25° with the glass electrode was 7.1.

Nuclei and cell debris were removed by centrifuging at 600 - 700 g for 20 minutes. The supernatant was carefully withdrawn and centrifuged 10 minutes at 12,000 - 13,000 g in the high-speed head of the M.S.E. Major Refrigerated Centrifuge. The sediment from this centrifuging consisted of a firmly packed mitochondrial pellet with a so-called 'fluffy' layer loosely packed above it. The mitochondrial pellet was resuspended in 0.25 M sucrose by using the homogeniser and again centrifuged for 10 minutes at 12,000 g. Two such washings effectively removed the 'fluffy' layer and gave a uniform mitochondrial pellet which was finally resuspended in 0.25 M sucrose [approximately 5 - 6 ml./g. (wet wt)] equivalent to a concentration of 3 - 11 mg. of mitochondrial protein/ml. The pH of the final suspension measured with the glass electrode was 6.9. (In certain cases the mitochondrial suspension after the first washing was recentrifuged at 600 - 700 g for 10 minutes to ensure complete removal of nuclei.)

## 2.2 Measurement of Oxidative Phosphorylation

The following medium in a volume of 2.36 ml. was placed in the main compartment of each Warburg flask (molarities refer to the final volume of 3.0 ml. after addition of the side-arm contents and mitochondrial suspension): 0.045 M-sodium potassium phosphate buffer, pH 7.4; 15 mM-KP; 8 mM-MgCl<sub>2</sub>; 25 mM-adenosine diphosphate (ADP); mM-adenosine 5'-phosphate (AMP); 2 mM-ethylenediaminetetraacetic acid (EDTA); 34 mM-D-glucose; 0.01 M-KCl; 62 mM-sucrose; 0.24 mM-diphosphopyridine nucleotide (DPN); 7 mM-substrate; 9.8 µM-cytochrome c. (In measurement of α-oxoglutarate oxidation 0.02 M-malonate was added as an inhibitor. AMP, ADP and all substrates were neutralised to pH 7.4 with KOH and stored at -30° before use.) The side arms of each flask contained 70% of the total D-glucose, together with 1 - 3 mg. of crystalline hexokinase, in a volume of 0.24 ml.

To each Warburg vessel was added 0.4 ml. of a mitochondrial suspension in 0.25 M sucrose, giving a concentration of 0.4 - 1.5 mg. of protein/ml. of incubation medium after addition of the side-arm contents. The centre well of each flask contained 0.2 ml. of 40% (w/v) KOH for CO<sub>2</sub> absorption, and the gas phase was air. Oxygen uptake was measured for 25 - 30 minutes after 13 minutes equilibration at 30°. Phosphate uptake was determined by the method of Fiske and Subbarow (5), on cold trichloroacetic acid filtrates. The oxygen uptake of the mitochondria ( $Q_{O_2}$ ) was expressed as µl. of O<sub>2</sub>/mg. of protein/hr according to Slater and Holton (13).

## 2.3 Mitochondrial Protein Content

This was determined according to the biuret method of Cleland and Slater (8), using an ovalbumin standard, previously analysed by the Kjeldahl procedure. Estimations were carried out as follows: Mitochondrial protein was precipitated with cold

trichloroacetic acid (final concentration of 5%), and the precipitated proteins washed with 50% aqueous ethanol (containing a few drops of TCA), and 95% ethanol respectively. The protein pellets were then allowed to dissolve in known volumes of biuret reagent and water. To ensure complete solubilisation and reaction of protein with the reagent, samples were allowed to stand overnight at room temperature. They were then freed from a small amount of insoluble material by centrifugation and the optical densities read in a Beckman spectrophotometer at 510 mμ. A blank and standard were run with each determination.

It was noticed that in all samples of Ehrlich ascites mitochondria examined, solution in the biuret reagent occurred within a few hours.

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## EXPERIMENTAL

## SECTION A

### FRACTIONATION OF CELL EXTRACT

#### EXTRACT IN ISOTONIC SALINE FLUID

#### BY SHORTER GRAVITY CENTRIFUGATION

##### 1. PREPARATION OF FUCOSE GRADIENT

A suitable sucrose gradient was prepared in a large centrifuge tube (17 x 3 cm. diameter). After first filling with 0.07 M potassium-codium phosphate buffer of pH 7.6, a layer of 50% w/v sucrose in phosphate buffer was introduced into the bottom 2 cm. of the tube using a long Pasteur pipette with a small upturned tip. Successive 1 cm. layers of sucrose solutions in phosphate buffer, each solution 5% w/v less in concentration than the previous one, were then introduced by upward displacement through the Pasteur pipette until a concentration gradient of 10 - 50% w/v of sucrose was obtained. After standing 15 - 30 minutes a glass rod was moved gently up and down through the solution to ensure an even gradient. Mouse ascites fluid (10 ml.) (which is stored in crushed ice) containing sodium citrate as an anticoagulant (1 ml. of 3.0% Na-citrate per 10.0 ml. ascites fluid), was centrifuged at 2500 r.p.m. for 10 minutes to sediment the cells. The cell mass (2 - 3g. wet wt) was twice washed in cold isotonic saline (5°C.), and finally resuspended in 10 ml. of isotonic saline. A portion of the resulting cell suspension (1.5 - 2.0 ml.) was carefully layered on top of the sucrose gradient, and the tube centrifuged at 250 - 300 r.p.m. for 30 minutes at 10 - 15°C.

## SECTION B

### ISOLATION OF EHRLICH ASCITES TUMOUR CELL MITOCHONDRIA AND STUDIES OF OXIDATIVE PHOSPHORYLATION WITH SUCCINATE, L-ASCORBATE, $\alpha$ -KETOGUtarate, L-GLUTAMATE AND IREI AS SUBSTRATES

#### 1 MATERIALS

##### 1.1 Ehrlich Ascites Tumour

This tumour was kindly supplied by Dr. K. Sugiyama of the Sloan-Kettering Institute, New York, U.S.A., and has been maintained by serial transplantation in Swiss albino mice. Ascites fluid was used between the ninth and tenth day after inoculation.

##### 1.2 Adenosine-5-phosphate and Adenosine-diphosphate

These were used as supplied by General Biochemicals Inc., and by the H.E. Chemical Company of Los Angeles, California, U.S.A., respectively. Paper chromatography in the solvent system, isobutyric acid-water-ammonia (66:31:1) of Krebs (1), and examination of the chromatograms by means of an ultra-violet lamp (Black Ray Ester; light of 260 m $\mu$  wave length), revealed the ADP to be slightly contaminated with AMP, while the AMP sample travelled as a single spot.

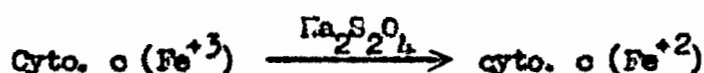
##### 1.3 Adenosine-triphosphate

This was used as the tetra-sodium salt supplied by General Biochemicals Inc. It was assayed by the method of Crane and Sols (2), and shown to be 95% pure. The method of analysis depends upon measurement of acid labile phosphate liberated during short periods of hydrolysis. A similar approach is used in the "Hexokinase" assay, which is described in detail on page 105.

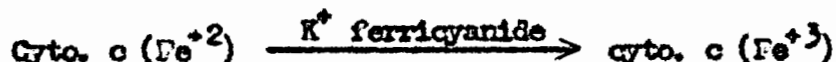
Paper chromatography in the solvent system of Krebs (1) and examination of the chromatograms under ultra-violet light, revealed the ATP sample to be contaminated with small amounts of ADP and AMP.

#### 1.4 Cytochrome c

This was used as a 1% w/v solution in isotonic saline as supplied by General Biochemicals Inc. It was assayed by the method of Umbriet, Burris and Stauffer (3) and found to contain  $5.87 \times 10^{-7}$  moles cytochrome c per ml. The assay consists of reduction of cytochrome c by sodium dithionite (hydrosulphite), and measurement of the optical density at 550 mμ (c-band of reduced cytochrome c).



Excess dithionite is destroyed by gassing, and the reduced cytochrome c reoxidised with potassium ferricyanide as follows:



Absorption measurements are made again at 550 mμ.

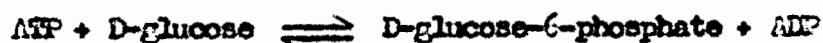
#### 1.5 Hexokinase

Crystalline material was supplied by General Biochemicals Inc., Chio, U.S.A. The enzyme was assayed by measurement of hexose disappearance according to Sogoyi (4), and by measurement of the disappearance of acid-labile phosphorus according to Crane and Sols (2). Preparations were found to have an activity of 150 - 200 units/mg.

#### Hexokinase assay by the method of Crane and Sols

##### Principle

The hexokinase reaction is shown by the following equation:



to obtain 5 - 35 units of enzyme per ml. The enzyme solution is stored in ice until used.

The assay system is set up as shown in the following table. Tube 1, is a zero (time) control; tubes 2 and 3, contain enzyme; and tube 4, is a blank without enzyme.

|                        | Tube 1 | Tube 2 | Tube 3 | Tube 4 |
|------------------------|--------|--------|--------|--------|
| ATP-Mg mixture         | 0.2    | 0.2    | 0.2    | 0.2    |
| Buffer solution        | 0.2    | 0.2    | 0.2    | 0.2    |
| D-Glucose solution     | 0.2    | 0.2    | 0.2    | 0.2    |
| Water                  | 0.3    | 0.3    | 0.3    | 0.4    |
| Dilute enzyme solution | 0.1    | 0.1    | 0.1    | -      |
| Final volume (ml.)     | 1.0    | 1.0    | 1.0    | 1.0    |
| 1.25 N - HCl at (min.) | Zero   | 15     | 25     | 15     |

The above components are mixed together, warmed to 30°C., and 0.1 ml. of the diluted enzyme added. The reactions are then allowed to proceed for various lengths of time (usually 15 - 25 minutes) and are stopped by the addition of 4.0 ml. of 1.25 N HCl to individual tubes. The tubes are transferred to a water bath (100°C.) for exactly 7 minutes, cooled, and centrifuged for 10 minutes at 2500 r.p.m. Aliquot portions are then removed for phosphate analysis by the method of Flake and Subbarow (5).

#### Inorganic phosphate analysis

| <u>Tube</u> | <u>Klett readings</u> |
|-------------|-----------------------|
| Standard    | 173.0                 |
| 1           | 229.3                 |
| 2           | 215.6                 |
| 3           | 206.8                 |
| 4           | 231.8                 |

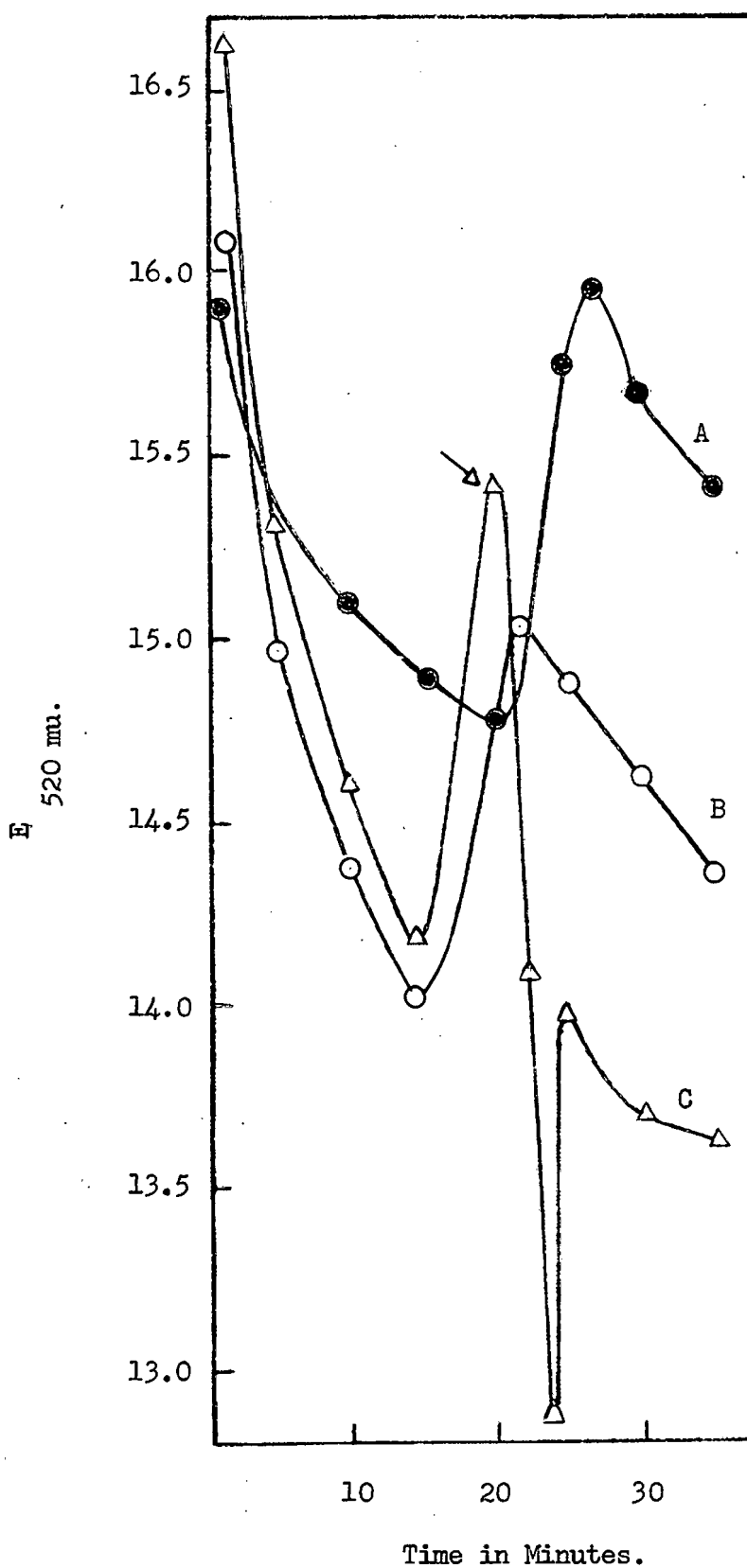


Fig. 9. Effect of thyroxine on swelling of respiring mitochondria in the phosphorylating system of Table 2 with succinate as substrate. A,  $3 \times 10^{-5}$  M thyroxine present initially; B, no thyroxine; C,  $3 \times 10^{-5}$  M thyroxine added as indicated ( ).

#### 4 DISCUSSION

Swelling of non-respiring Ehrlich tumour mitochondria was minimal in water and in 0.25 M sucrose used for the isolation procedure. The method of isolation involved brief contact of the mitochondria with water over a period of 3.0 minutes during initial lysis of the ascites cells, but it has been shown that such treatment does not impair their capacity for oxidative phosphorylation (Table 3, Chapter III). Concentrated sucrose solution is added to the homogenate immediately after the 3.0 minutes lysis period and this, in all probability, completely reverses any swelling which may occur during contact with the water [Tedeschi and Harris (2)].

The observation that initial  $E_{520\text{ m}\mu}$  values for a single batch of mitochondria are different in sucrose solutions of varying osmolarity (cf. Figs 1 and 2) is attributable to differences in refractive index of the suspending media [Tedeschi and Harris (2)]. The same divergence of initial  $E_{520\text{ m}\mu}$  values in varying sucrose concentrations was found by Nutolo and Abrignani (4). In a later paper by Nutolo and Abrignani (5) curves are shown for tumour mitochondria which have the same initial  $E_{520\text{ m}\mu}$  value for a single sucrose concentration. Our results also show that in a single medium the swelling curves for Ehrlich mitochondria tend towards a common origin.

The observation that non-respiring tumour mitochondria show an increased degree of swelling with increasing sucrose molarity in buffered or unbuffered solution, is in contrast to the finding of Lehninger (6) that rat liver mitochondria exhibit least swelling in hypertonic sucrose. The apparent increase of swelling with increasing solution osmolarity is in itself not easily explicable, but may be due to a gradual change in internal refractive index of the mitochondrial particles [Tedeschi and Harris (2), and Barer, Ross and Tkaczyk (7)].

Failure of EDTA to prevent the swelling of non-respiring Ehrlich mitochondria in unbuffered sucrose is in keeping with the results of Mutolo and Abrignani (4), who found that it tended to promote the swelling of Walker 256 carcinosarcoma mitochondria. EDTA effectively prevents the swelling of rat liver mitochondria (Mutolo and Abrignani), and as shown by Cleland and Slater (8), also stabilises the oxidative activity of heart muscle sarcosomes.

The increase in swelling of non-respiring Ehrlich mitochondria in 0.25 M sucrose at successively higher pH values, parallels the results of Witter and Cottone (9) using rat liver mitochondria. Liver and Ehrlich tumour mitochondria show least swelling at pH 6.0, and below pH 6.0 rapid agglutination of both types occurs [Gamble (10)].

The swelling of non-respiring Ehrlich mitochondria in 0.25 M sucrose buffered to pH 7.4 was not reversed on addition of ATP, ADP, AMP, DPN or ATP + DPN to the medium. DPN and to a lesser extent ADP were, however, effective in decreasing the rate of swelling, although the protective action of DPN was nullified when ATP was also present in solution. By contrast Fomescu and Davies (11) and Dickens and Salmony (12) found the swelling of liver mitochondria to be reversed by addition of ATP. A similar effect was shown by Chappell and Ferry (13) using heart muscle sarcosomes. Failure of ATP and DPN to reverse the swelling of Ehrlich ascites cell mitochondria, may be due to the high ATP-ase and DPN-ase activities of tumour mitochondria as distinct from those of normal cells. No fluoride or nicotinamide were added to inhibit these actions in the media used to examine the Ehrlich mitochondria.

The uncoupling agent 2:4-dinitrophenol (DNP) was without effect on the swelling of non-respiring Ehrlich mitochondria in 0.25 M sucrose buffered to pH 7.4. On the other hand, Lehninger (6) observed that DNP effectively prevented the swelling of rat liver

mitochondria. Thyroxine, another uncoupling agent, caused a rapid increase in the swelling of non-respiring Ehrlich mitochondria which parallels the findings of Dickens and Salmons (12) and Lehninger (6) with non-respiring rat liver mitochondria, and is in contrast to the findings of Emmelot and Bos (14) with rat hepatoma mitochondria. However, the effect of thyroxine on normal cell mitochondria is apparently variable. Tapley and Cooper (15) found that rat liver and kidney mitochondria underwent swelling in the presence of thyroxine, whereas those of the heart, testis and spleen did not.

Rapid swelling of non-respiring Ehrlich tumour mitochondria was observed in the presence of oxidisable substrate (succinate) and inorganic phosphate. This is analogous to the behaviour of rat liver mitochondria reported by Chappell and Greville (16), and is in keeping with the statement of Witter and Cottons (9) that succinate and phosphate cause a breakdown of organised mitochondrial structure. In a fortified phosphate medium but without substrate, Ehrlich mitochondria underwent the same degree of swelling as observed in 0.25 M sucrose buffered to pH 7.4.

The swelling of respiring Ehrlich mitochondria was studied in the same fortified incubation medium with a variety of substrates. Uninterrupted swelling was observed with L-glutamate and  $\beta$ -hydroxybutyrate, possibly due to the low quantity of DPN present, but with succinate and ascorbate an apparent reversal of swelling was observed. Moreover, this reversal occurred only in the presence of added cytochrome c and was unaffected by removal of the hexokinase trapping system. The possibility exists that two phenomena are involved simultaneously in the observation of swelling reversal at 520 m $\mu$ : (a) actual physical shrinkage of the mitochondria in the presence of cytochrome c, and (b) reduction of the external cytochrome c which could act as an electron acceptor if the conditions in the Beckman cuvette were anaerobic at the time when the reversal of swelling

was observed. Part of the increased adsorption at 520 m $\mu$  could therefore be due to the  $\beta$ -band of reduced cytochrome c. Blank experiments in which solid sodium dithionite was added to the cuvette at the start of the observed shrinkage, showed only 30% of the increase in optical density usually found with succinate as substrate. Thus 30% of the increase in optical density observed after 15 minutes with succinate and ascorbate may be due to actual shrinkage. Holton (17) and Chance (18) have shown that reduction of cytochromes in a mitochondrial suspension can be followed in the Beckman spectrophotometer.

Shrinkage of respiring mitochondria in the presence of succinate and cytochrome c was found to take place more rapidly at successively higher values of pH. Agglutination of the mitochondria occurred below pH 6.0 and above pH 8.0 heavy precipitates formed in the solution.

If added initially, thyroxine decreased the usual rate of swelling of respiring Ehrlich mitochondria, whereas the opposite effect was observed with non-respiring mitochondria. However, if thyroxine was added when maximum shrinkage of respiring mitochondria had taken place, an immediate swelling of the particulates was observed. This was followed first by rapid shrinkage and then by a more gradual rate of swelling.

The physical characteristics of Ehrlich ascites cell mitochondria therefore appear to be similar to those reported for other tumour mitochondria, but differ in a number of respects from those of normal cell mitochondria. However, on the basis of the comparisons drawn it would not be possible to decide whether the observed differences might be generally characteristic of tumour cell mitochondria.

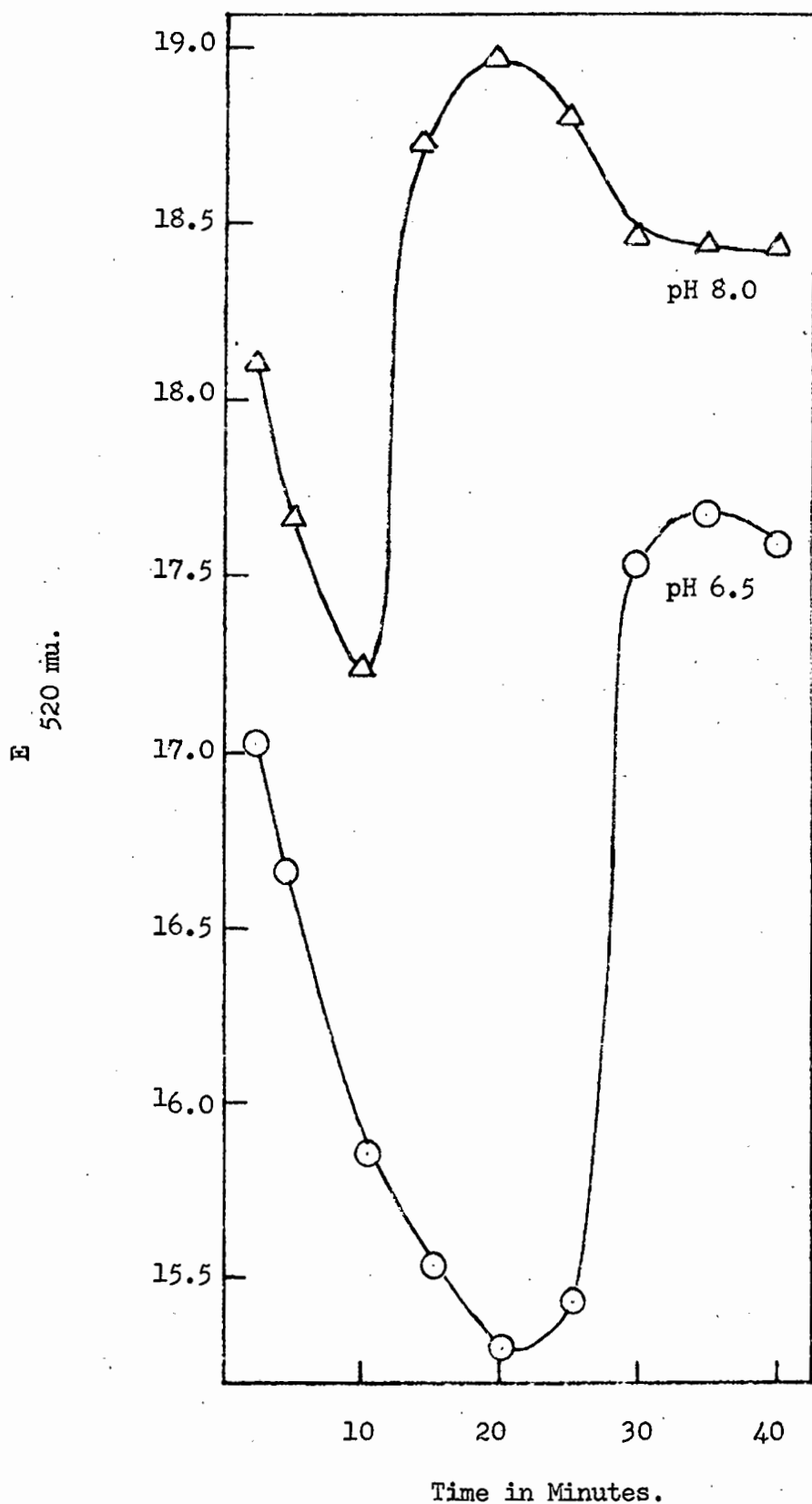


Fig. 8. Swelling of respiring mitochondria in phosphorylating media at various pH values. Succinate as substrate. pH 8.0: Medium as in Table 2 but with (0.005 M K,Na phosphate buffer + 0.040 M tris-acetate buffer) in place of the 0.045 M K,Na phosphate buffer specified. pH 6.5: Medium as specified for pH 8.0. Agglutination of mitochondria occurred below pH 6.0, and heavy precipitates formed in the medium above pH 8.0.

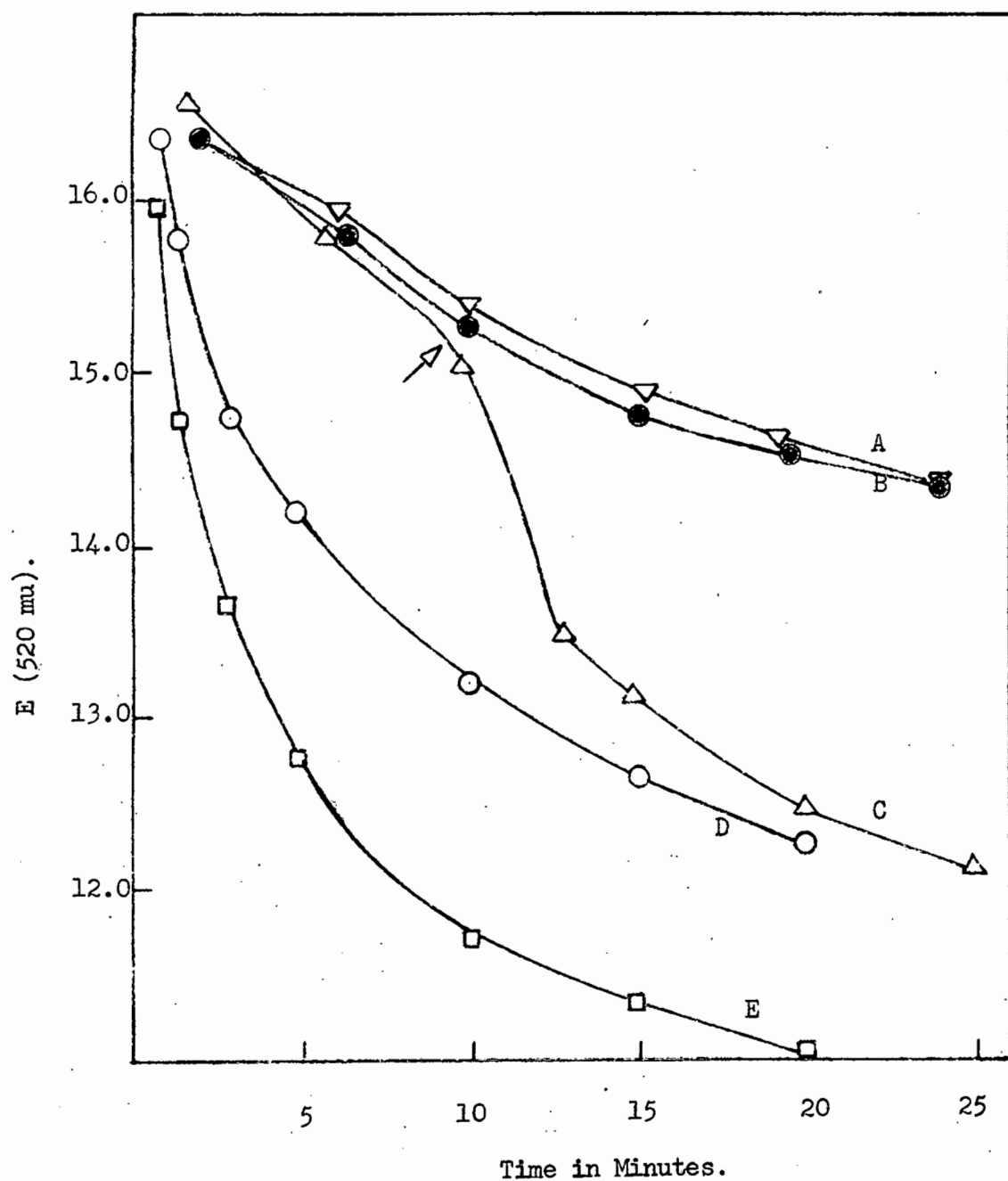


Fig. 5. Swelling of non-respiring mitochondria in 0.25 M sucrose solutions buffered with 0.05 M tris-acetate (pH 7.4) and containing various additions:- A,  $1.2 \times 10^{-4}$  M 2:4 - dinitrophenol (DNP); B, control; C,  $3 \times 10^{-5}$  M L-thyroxine added as indicated ( ); D,  $3 \times 10^{-5}$  M L-thyroxine; E, 0.001 M K,Na phosphate + 0.001 M potassium succinate

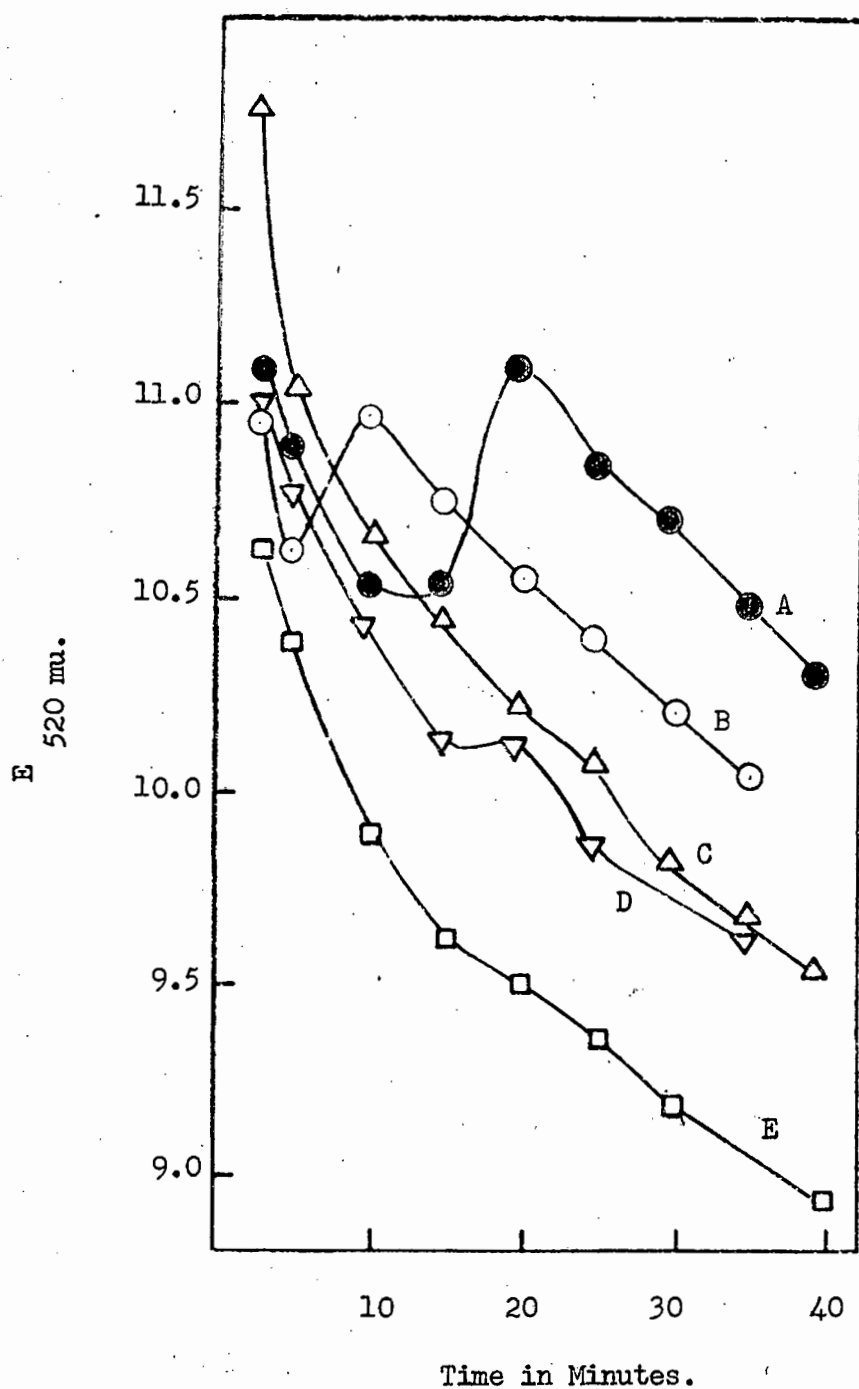


Fig. 6. The swelling of mitochondria respiring with various substrates. The complete phosphorylating system as in Tables 1, 2 and 3 was used in all experiments:- A, 0.01 M succinate; B, 0.0066 M ascorbate; C, 0.01 M DL - - hydroxybutyrate; D, 0.0066 M L-glutamate; E, no substrate.

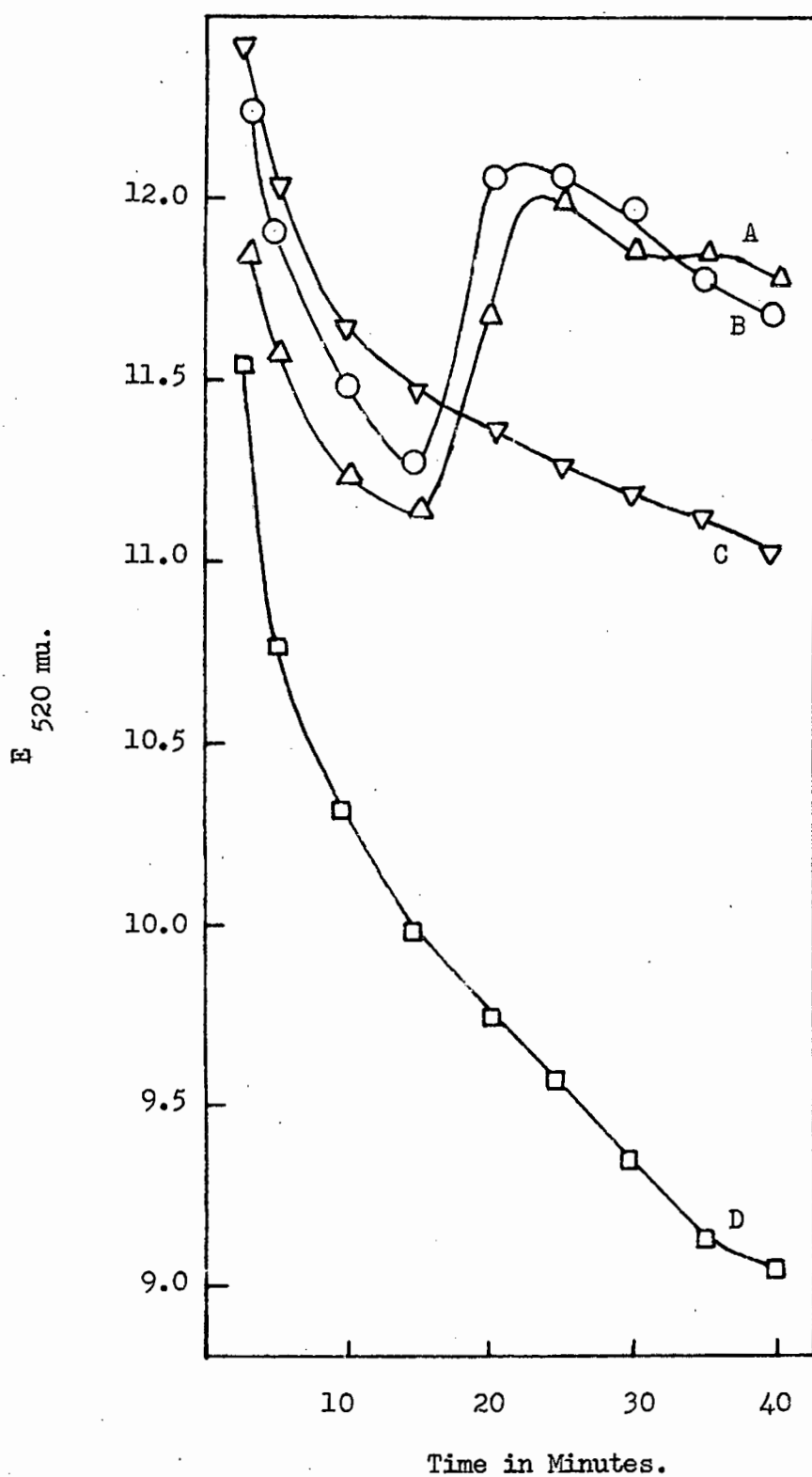


Fig. 7. Swelling of respiring mitochondria in the phosphorylating medium specified in Table 2 with succinate as substrate. Effect of removing co-factors from the medium. A, no hexokinase; B, control; C, no cytochrome c or hexokinase; D, no cytochrome c.

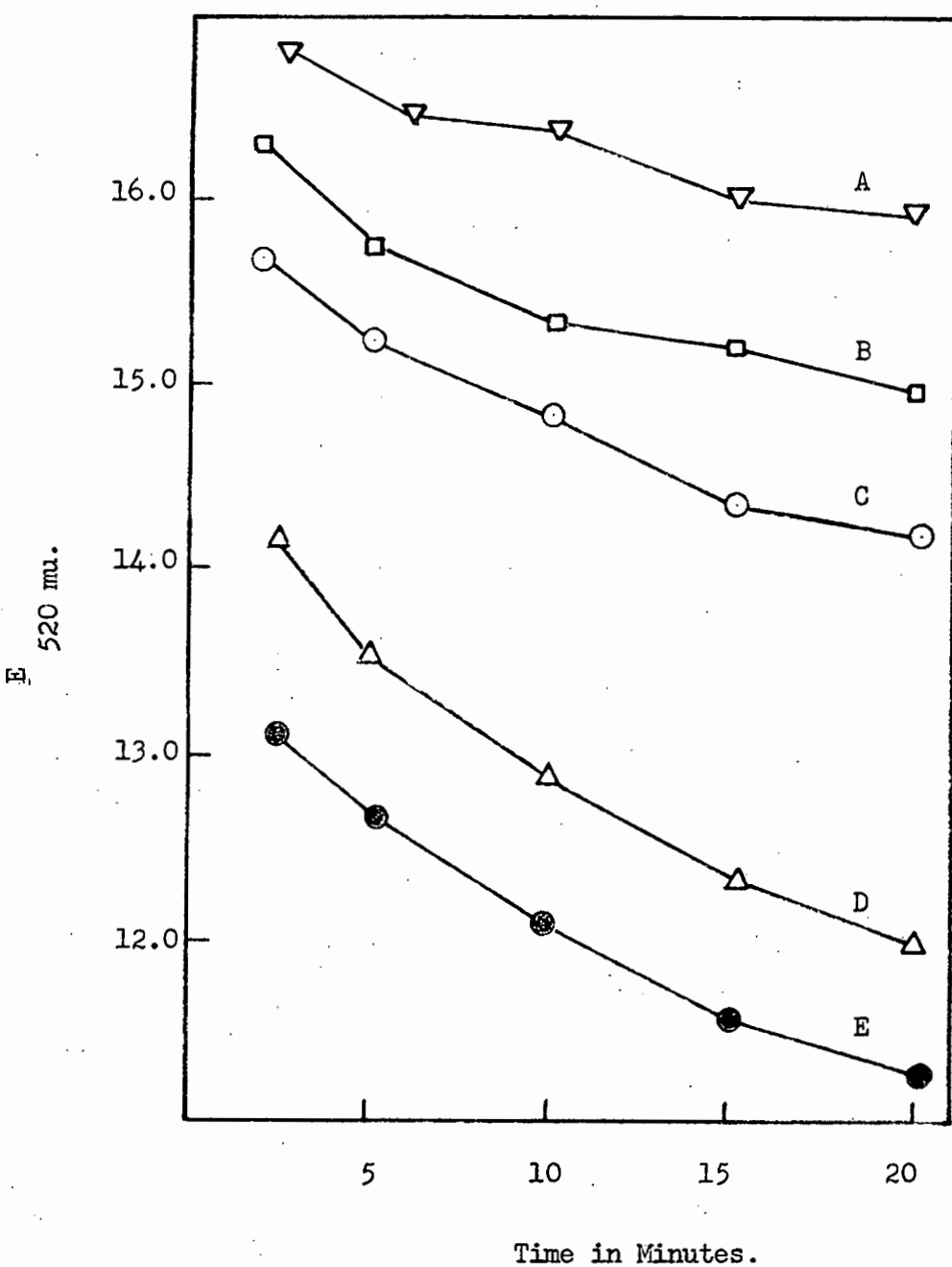


Fig. 2. Swelling of non-respiring mitochondria in sucrose solutions buffered to pH 7.4 by addition of 0.05 M tris (hydroxymethyl) - amino - methane - acetate. A, 0.25 M sucrose + 0.01 M EDTA; B, 0.25 M sucrose; C, 0.44 M sucrose; D, 0.60 M sucrose; E, 0.80 M sucrose.

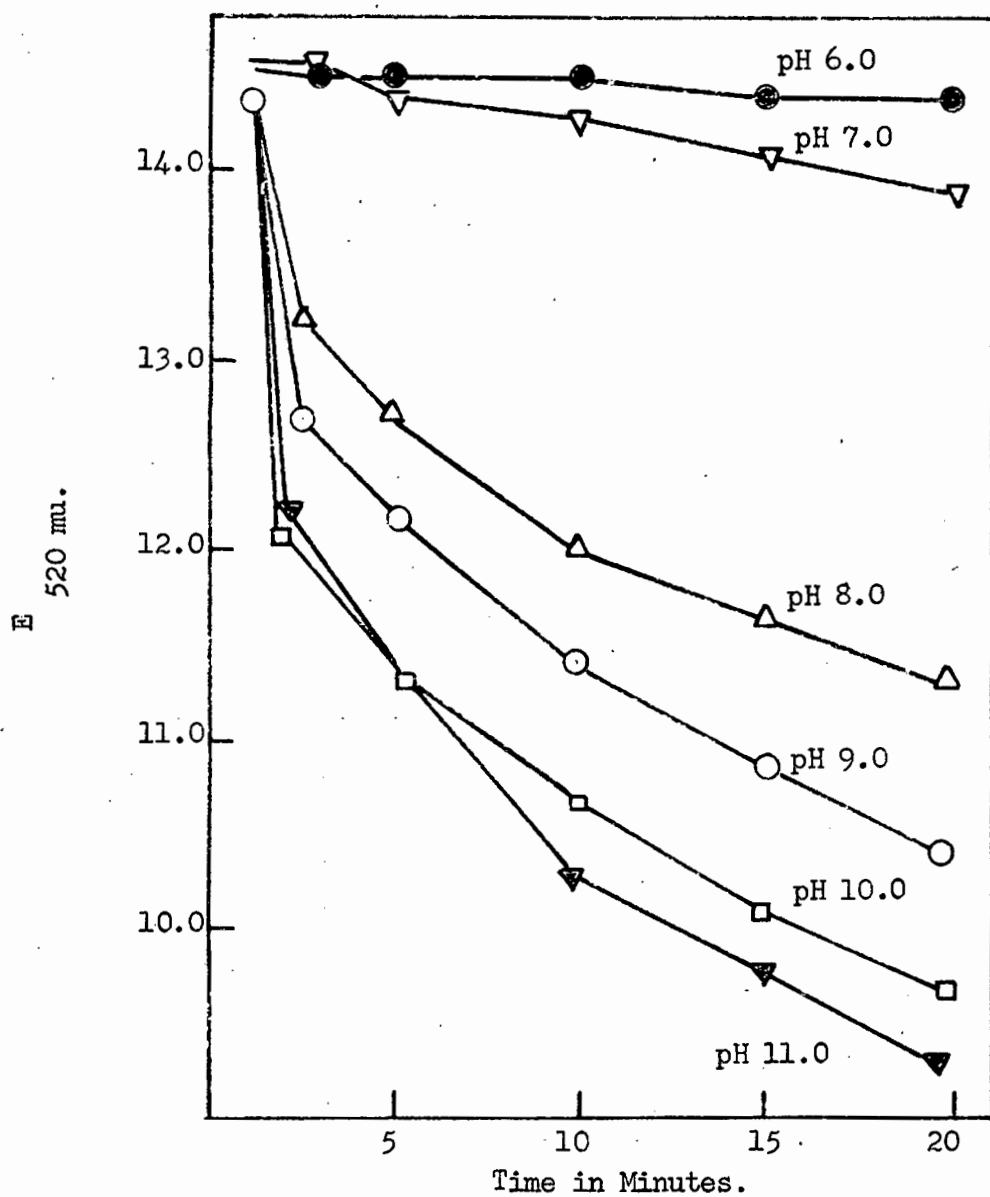


Fig. 3. The effect of pH on the swelling of non-respiring mitochondria. Mitochondrial suspension in 0.25 M sucrose (0.15 ml.) was added to 2.85 ml. of 0.25 M sucrose buffered with 0.05 M tris-acetate to the pH indicated for each curve. At pH values below 6.0, agglutination of the mitochondria occurred.

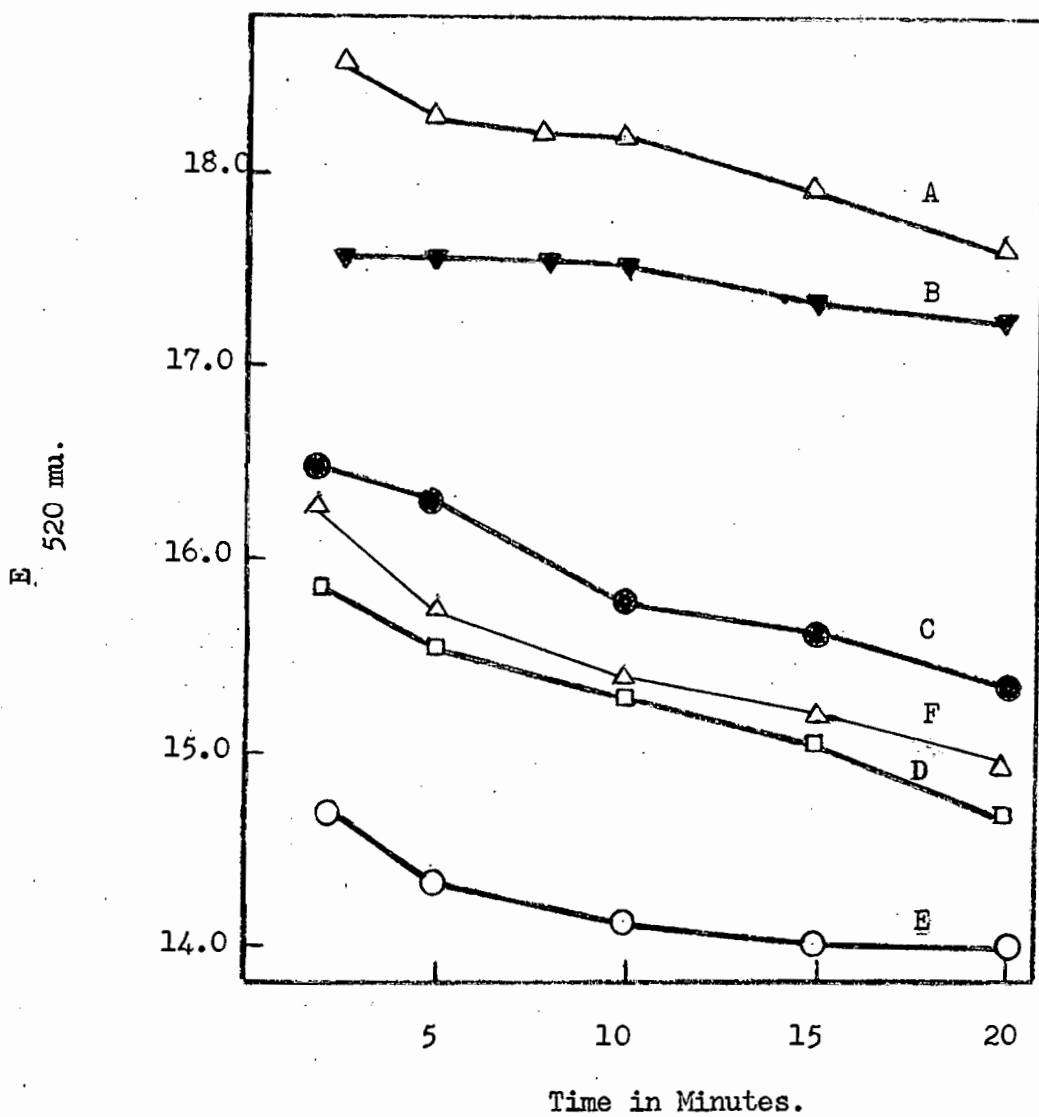
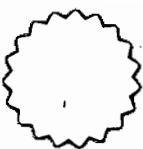


Fig. 4. Swelling of non-respiring mitochondria in 0.25 M sucrose solutions buffered with 0.05 M tris-acetate (pH 7.4) and containing added co-factors:- A, 0.001 M ATP; B, 0.001 M DPN; C, 0.001 M AMP; D, 0.001 M ATP + 0.001 M DPN; E, 0.001 M ADP; F, control.

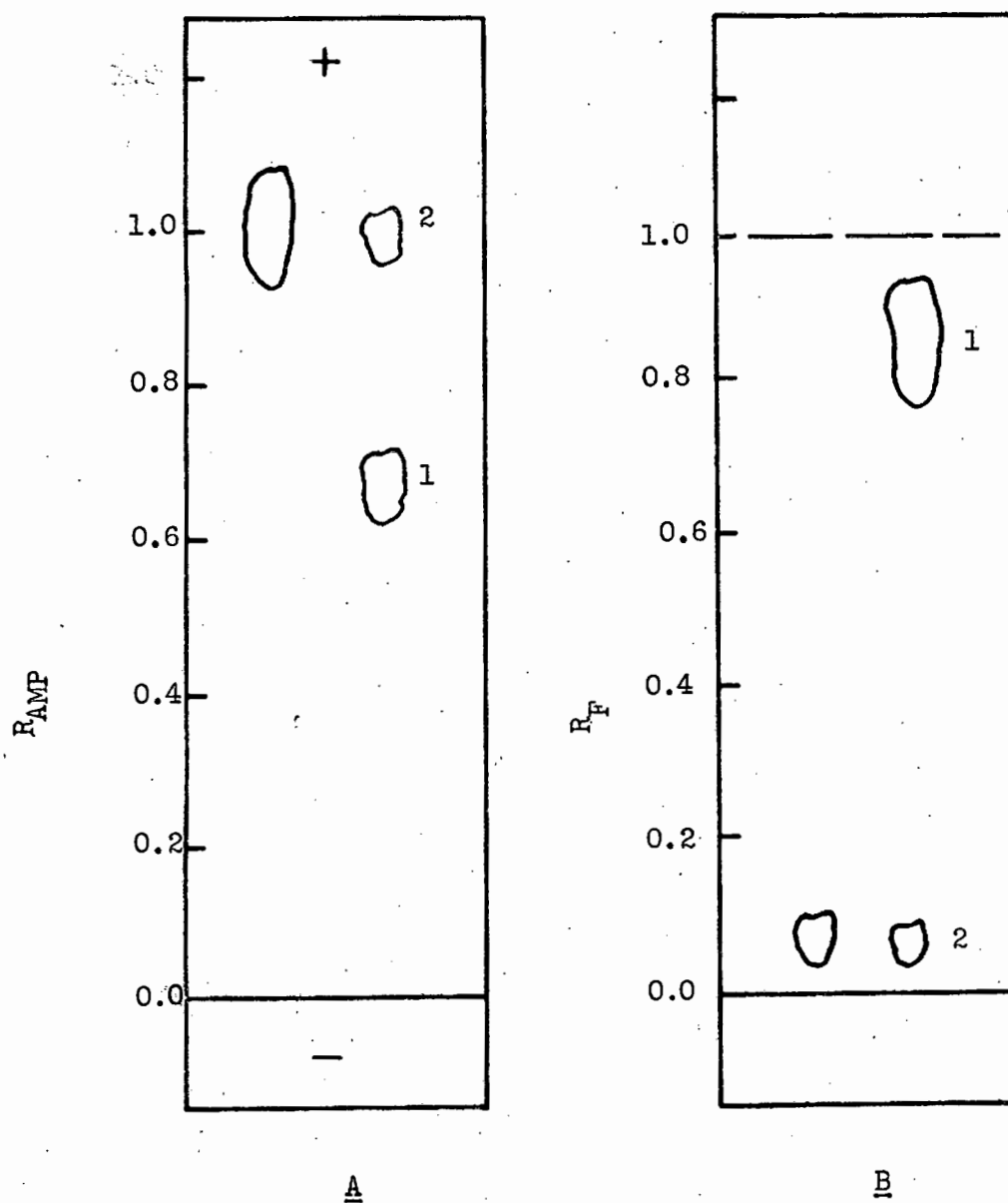


Fig. 1. (A) Paper Chromatography, and (B) Electrophoresis  
of n-Octanoyl-AMP.

Paper chromatography was carried out in isopropanol :  $H_2O$ .  
(70 : 30 v/v), while electrophoresis was done in 0.05 M  
phosphate buffer, pH 6.8. Octanoyl-AMP (1); AMP (2).

AMP, 1.00. These results agreed with those reported by Gurin (3) who synthesised n-octanoyl-AMP by the anhydride method.

Large scale paper chromatography was carried out on sheets of Whatman No. 1 paper (40 x 60 cm.) employing the above solvent system. The fast moving ultra-violet zone ( $R_f$  0.86) was cut out and eluted with 2% w/v potassium carbonate. The resulting eluate served for the analysis of total phosphate, ribose, mixed anhydride and ultra-violet absorption spectra.

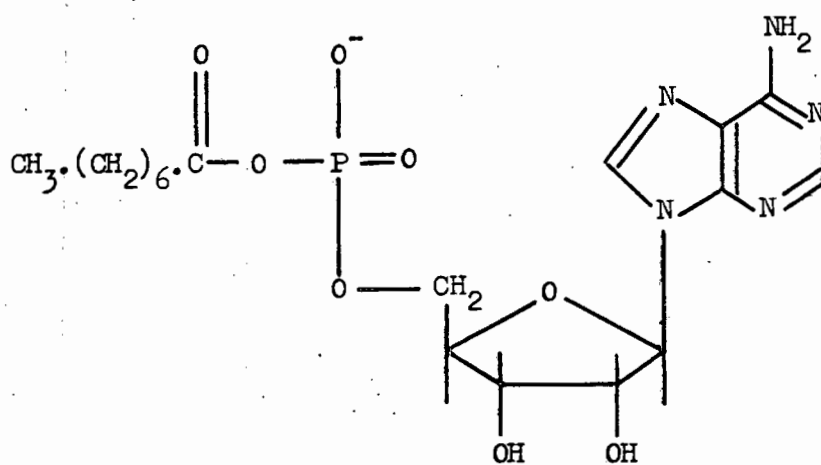
The ratio of the assays, ribose/phosphate/mixed anhydride, was 1.05/0.90/0.84 respectively. Theoretical ratios are 1.0/1.0/1.0. Percentage yield as calculated by the above analysis was of the order of 50 - 60%.

The ultra-violet absorption spectrum of an aqueous sample of n-octanoyl-AMP at pH 6.8 revealed a peak in the region 258 - 260 m $\mu$ .

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n-Octanoyl Adenylate



CHAPTER VI

SWELLING CHARACTERISTICS OF

EHRLICH ASCITES CELL MITOCHONDRIA

## CHAPTER VI

### SWELLING CHARACTERISTICS OF EHRLICH ASCITES CELL MITOCHONDRIA

#### 1 INTRODUCTION

The contention of Warburg (1) that tumour cell mitochondria are irreparably damaged, has prompted numerous comparisons between the properties of normal and tumour cell mitochondria. The isolation of Ehrlich ascites tumour mitochondria and studies of their oxidative phosphorylation with a variety of substrates has been previously reported in this thesis. The present results concern further studies, but of a physical nature - vide the swelling characteristics of both respiring and non-respiring Ehrlich ascites mitochondria in a variety of media. The characteristics have been compared with those of mitochondria from normal cells and from other tumour types under similar conditions. A comparison between the swelling characteristics of normal and tumour cell mitochondria provides information concerning the influence of intracellular and extra cellular fluid environment on mitochondrial functioning.

Such physical studies are of importance because of the possible influence of swelling and shrinkage on the structural arrangement of enzymes necessary for chemical functioning of the mitochondria (2).

#### 2 STUDIES ON THE SWELLING OF EHRLICH ASCITES CELL MITOCHONDRIA

##### 2.1 Method

Mitochondria were isolated in 0.25 M sucrose as described in the Experimental Section.

Media for determination of mitochondrial swelling

- (a) Non-respiring mitochondria - The medium is indicated for each experiment.
- (b) Respiring mitochondria - The following medium was used: 0.045 M sodium potassium phosphate buffer (pH 7.4); 0.015 M KF; 0.008 M  $\text{NaCl}_2$ ; 0.0025 M ADP; 0.001 M AMP; 0.002 M EDTA (ethylene diamine tetra acetic acid); 0.01 M d-glucose; 0.032 M sucrose;  $2.4 \times 10^{-4}$  M DPN;  $9.8 \times 10^{-6}$  M cytochrome c; 1 - 3 mg. hexokinase (crystalline); 0.007 M substrate. (Media with  $\alpha$ -ketoglutarate as substrate contained 0.02 M malonate in addition. AMP, ADP and all substrates were neutralised to pH 7.4 with KOH and stored at  $-30^\circ\text{C}$ . before use.)

For experiments in which co-factors or the glucose-hexokinase phosphate acceptor system were omitted from the medium, 0.25 M sucrose was used to make up the necessary volume.

Measurement of mitochondrial swelling

The change in optical density of a dilute suspension of mitochondria was measured according to the method of Cleland (3). Measured volumes (0.15 ml.) of the mitochondrial suspension in 0.25 M sucrose (containing 4 - 6 mg. protein/ml.) were added to 2.85 ml. of the medium in which swelling was to be determined. After thorough mixing, the change in optical density at 520 m $\mu$  was read in a Beckman Spectrophotometer Model DU 2400 against a blank containing the medium under examination plus 0.25 M sucrose in place of the mitochondrial suspension. Values are expressed as Specific Extinction coefficients ( $E_{1\%}^{1\text{cm}}$ ) for suspensions containing 1% of mitochondrial protein.

Non-respiring mitochondria did not swell in sucrose solution buffered to pH 6.0 with tris-acetate, but swelling was observed at pH 7.0 and was found to be increased at successively higher pH values (Fig. 3). Agglutination of the mitochondria occurred below pH 6.0.

The swelling of non-respiring mitochondria in 0.25 M sucrose buffered to pH 7.4 with tris-acetate was considerably decreased by addition of 0.001 M DEPN to the medium (Fig. 4). Addition of ADP caused a slight inhibition of swelling, while AMP and ATP were without effect. The protective action of DEPN was nullified when ATP was also present in solution (Fig. 4).

The swelling of non-respiring mitochondria in 0.25 M sucrose buffered to pH 7.4 with tris-acetate was not affected by addition of the uncoupling agent DNP (2,4-dinitrophenol). On the other hand addition of thyroxine ab initio or at a subsequent stage in the experiment, caused a marked increase in swelling (Fig. 5) but only with 6 out of 8 mitochondrial preparations examined. Rapid swelling of non-respiring mitochondria in buffered sucrose was observed on addition of succinate and phosphate to the medium (Fig. 5).

### 3.3 Swelling of Respiring Mitochondria

Mitochondria in the phosphorylating medium without added substrate, underwent the same degree of swelling as observed for non-respiring mitochondria in 0.25 M sucrose. However, in the presence of certain added substrates shrinkage occurred followed by a gradual degree of swelling (Fig. 6). Succinate caused the maximum shrinkage of swollen mitochondria, while ascorbate was practically as effective though more rapid in its action. Addition of L-glutamate caused only mild shrinkage of the mitochondria while addition of  $\beta$ -hydroxybutyrate was without effect on the swelling (Fig. 6).

The shrinkage of swollen mitochondria in phosphorylating medium with succinate as substrate, was unchanged by removal of the hexokinase-phosphate esterifying system from the medium (Fig. 7). On the other hand removal of cytochrome c from the medium resulted in rapid swelling and complete failure of the mitochondria to undergo shrinkage. Removal of cytochrome c and hexokinase together also resulted in failure to undergo shrinkage, but the degree of initial mitochondrial swelling was less than in the case where only cytochrome c was removed from the medium (Fig. 7).

The effect of medium pH on the shrinkage of swollen mitochondria in phosphorylating medium with succinate as substrate, was studied in a series of experiments. Shrinkage took place after 10 minutes at pH 8.0, after 12 minutes at pH 7.4 but only after 22 minutes at pH 6.0. Agglutination of mitochondria occurred below pH 6.0, while heavy precipitates formed in the medium above pH 8.0 (Fig. 8).

Shrinkage of swollen mitochondria in phosphorylating medium with succinate as substrate, was also observed in the presence of  $3 \times 10^{-5}$  M thyroxine, although the initial swelling was less than in a medium without thyroxine. Addition of  $3 \times 10^{-5}$  M thyroxine to the medium immediately after shrinkage of the respiring mitochondria had taken place, caused instantaneous and marked swelling followed by rapid shrinkage and a subsequently more gradual rate of swelling (Fig. 9).

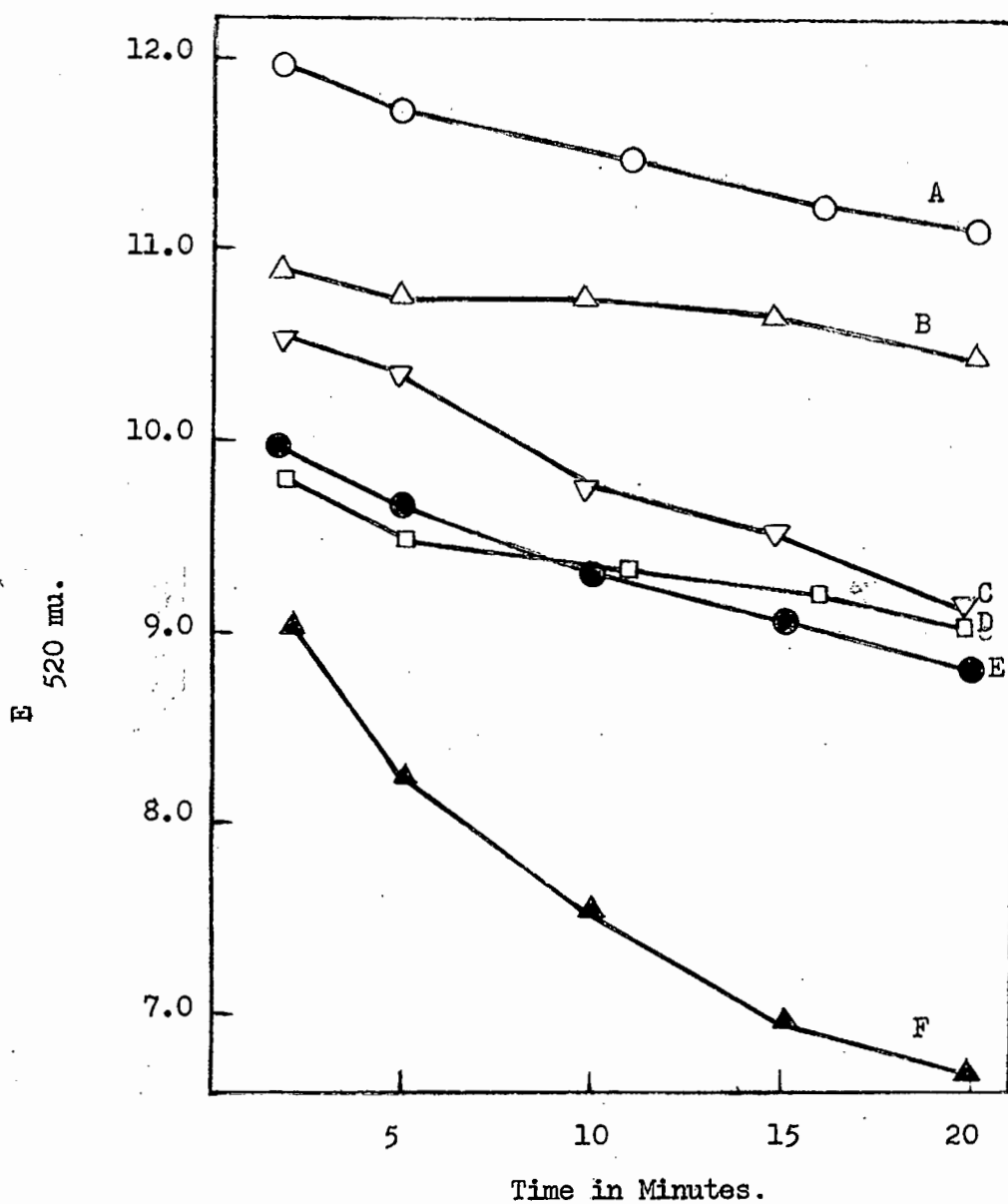
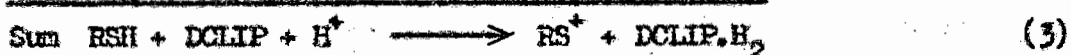
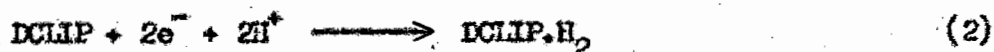


Fig. 1. Swelling of non-respiring mitochondria in unbuffered sucrose solutions. A, 0.25 M sucrose + 0.01 M EDTA (pH 7.4); B, 0.25 M sucrose; C, 0.44 M sucrose; D, distilled water; E, 0.80 M sucrose; F, 0.60 M sucrose.

16 NOTE ON THE CHEMISTRY OF  
2,6-DICHLOROPHENOL-  
INDOPHENOL REDUCTION

Basford and Huernekens (59) have made a detailed spectrophotometric study of the oxidation of thiol groups by 2,6-dichlorophenol-indophenol. The reaction rate depends upon the structure of the particular thiol under test. It is, either relatively fast approaching a final stoichiometric oxidant-reductant ratio of 1:1, or else slow, approaching a stoichiometric ratio of 1:2. Compounds containing a free thiol (-SH) group are able to reduce 2,6-dichlorophenol-indophenol non-enzymatically.

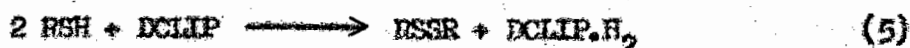
The reaction rate is directly proportional to the concentration of both oxidant and reductant and hydrogen ion concentration. Metals such as  $\text{Cu}^{++}$ ,  $\text{Ni}^{++}$ ,  $\text{Fe}^{++}$  depress the rate, while versene (EDTA) reverses this effect. Acyl thiols, disulphides and certain other sulphur-containing compounds do not react in this system. Potential thiols such as thiazolidines, thiazolines and thiazoles react with DCLIP, but at a much slower rate. Basford and Huennekens have postulated the following reaction scheme:



In these reactions, RSH = thiol, and  $\text{RS}^+$  is its oxidation product at the level of a sulphenic acid. If the thiol is of such a structure that it is easily oxidised, and the resulting ion  $\text{RS}^+$  is stabilised by resonance, the reaction will proceed as above. Furthermore, the non-reactivity of disulphides toward indophenol would indicate that the  $\text{RS}^+$  ion may be achieved by a two-electron loss from RSH, and not by a one-electron loss from  $\text{RSSR}$ . However, if the thiol is of such a nature that reaction (1) is slow, then it may be followed by,



so that the overall reaction (5) can be represented by the sum of (1), (2) and (4).



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## CHAPTER V

### STUDIES ON FATTY ACID OXIDATION

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#### 1 BIOLOGICAL OXIDATION OF FATTY ACIDS

##### 1.1 Introduction

In normal cells such as those of liver and kidney, oxidation of fatty acids occurs via a complicated series of enzymes located in the mitochondria. The process involves a number of successive enzymatic cleavage reactions, each resulting in the formation of the two carbon fragment, acetyl-S-CoA. The latter compound enters the citric acid cycle at the level of the condensing enzyme to react with oxaloacetate and form citrate. Fatty acids therefore provide an important source of fuel for oxidation in certain cells.

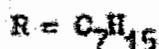
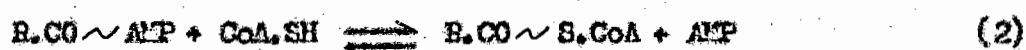
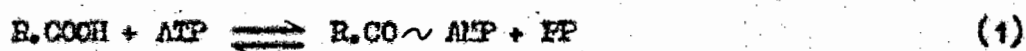
Ehrlich ascites tumour cells are known to have extremely low levels of glucose, fructose, galactose and glycogen; substances which are required for the operation of the Embden-Meyerhof glycolytic pathway. However, these cells are capable of respiring at fairly rapid rates for a number of hours in the absence of added substrate, with a respiratory quotient (RQ) usually less than one. These facts suggest the possibility of fatty acids serving as substrates for oxidation in ascites cells. In this connection it is of interest that homogenates of the Ehrlich ascites cell contain large numbers of lipid granules, which are easily seen by phase-contrast microscopy and which stain with sudan black.

Kun, Telalay and Williams-Ashman(1) have studied the oxidation of *n*-octanoate by Ehrlich ascites cell mitochondria. In a medium fortified with DPN, ATP and cytochrome c, they were

unable to demonstrate oxidation of this substrate. Moreover the rate of succinate oxidation was depressed approximately 75%.

However, Weinhouse and his co-workers (2) have shown that if rat serum containing palmitate-1-C<sup>14</sup> as a component of the lipid fraction is used as substrate, fairly rapid oxidation occurs.

Fatty acid oxidation in normal cells takes place as follows:

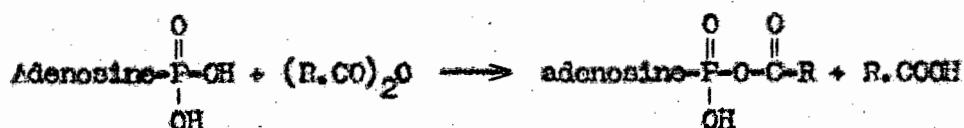


In tumour mitochondria, reaction (1) could be impaired to some extent, owing to the ATP-ase activity of these particulates. By circumventing the initial carboxyl activation step it might be possible to demonstrate fatty acid oxidation by tumour mitochondria either at the level of the acylindonylate ( $R.CO \sim AMP$ ) or acyl-coenzyme A derivative ( $R.CO \sim S.CoA$ ) as in reactions (2) and (3). An attempt was therefore made to by-pass the preliminary ATP activation step of reaction (1) through the use of  $R.CO \sim AMP$  synthesised by organic chemical methods.

## 1.2 Method

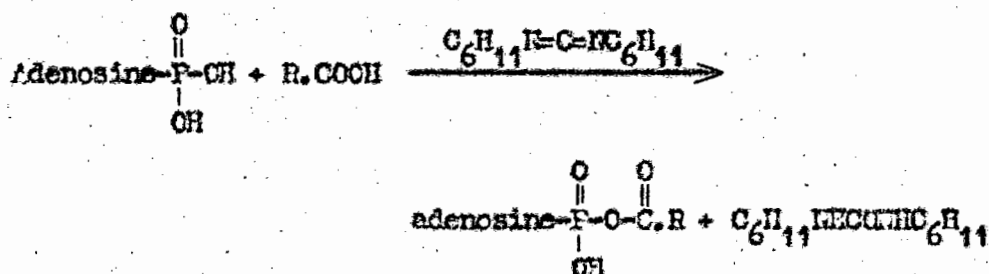
Acyladenrylates ( $R.CO \sim ATP$ ) may be synthesised by the following methods:

(a) Reaction of adenosine-5-phosphate and a carboxylic acid anhydride in aqueous pyridine at low temperature (3, 4).

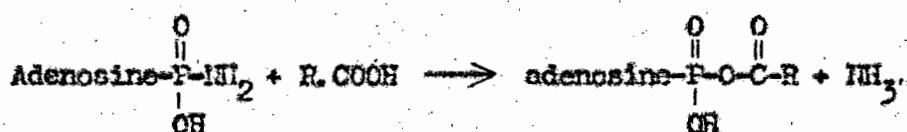


(b) Reaction of the silver salt of adenosine-5-phosphate with a carboxylic acid chloride (5, 6).

(c) Condensation between adenosine-5-phosphate and a carboxylic acid in the presence of  $N,N'$ -dicyclohexylcarbodiimide (7, 8).



(d) Reaction between adenosine-5-phosphoramidate and a carboxylic acid



Approach (d) has apparently not been tried as yet. The nucleoside phosphoramidate ( $\text{AMP-NH}_2$ ) has been synthesised by Chambers, Khorana and Loffat (9,10) who have condensed it with riboflavin monophosphate under relatively mild conditions to achieve a very successful synthesis of flavin adenine dinucleotide (10). The carbodiimide approach [reaction (c)] to the synthesis of mixed anhydrides (carboxy-phosphates) suffers from a lack of specificity as regards condensation, whereas the phosphoramidate method should be highly specific.

### 1.3 Synthesis of n-Octanoyl-Adenylate

n-Octanoyl-adenylate (octanoyl-AMP) was synthesised by the carbodiimide procedure as follows:

To an aqueous-pyridine solution containing AMP and n-octanoic acid, was added a solution of  $N,N'$ -dicyclohexylcarbodiimide in pyridine. After thorough mixing, the two-phase reaction mixture was stirred mechanically for forty minutes at room temperature. During this time, the two-phase system became homogenous and crystallisation of  $N,N'$ -dicyclohexylurea occurred. The reaction was stopped by the addition of ice-cold water, and the insoluble  $N,N'$ -dicyclohexylurea was removed by filtration.

The clear filtrate containing the *n*-octanoyl-adenylate was then extracted with diethyl ether to remove unreacted octanoic acid and carbodiimide and the last traces of ether were removed by aeration. The remaining aqueous solution containing the octanoyl-adenylate was stored at  $-30^{\circ}\text{C}$ .

Analysis was carried out by the following methods:

Paper chromatography, paper electrophoresis, hydroxamate assay for reactive acyl content, phosphorous and ribose determinations (7), and ultra-violet absorption spectrophotometry.

(a) Paper Chromatography

Aliquot portions of the aqueous octanoyl-adenylate solution were spotted on Whatman No. 1 filter paper and chromatographed in the solvent system, isopropanol:water (7:3 v/v) at  $2^{\circ}\text{C}$ . for 18 - 24 hours using the descending method. The chromatograms were air-dried and examined by means of an ultra-violet lamp (260 mμ wave length). Absorbing spots were marked in with a pencil, and the chromatograms were then sprayed for reactive acyl linkages by the method of Morrison (11). Reactive acyls produce an orange to red coloration with this reagent. Results are shown in Fig. 1.  $R_F$  values: *n*-octanoyl-AMP, 0.86; AMP, 0.08. *n*-Butyryl-AMP has an  $R_F$  of 0.68 in the above system.

(b) Paper Electrophoresis

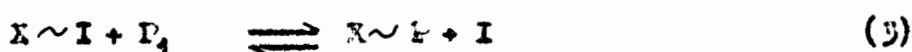
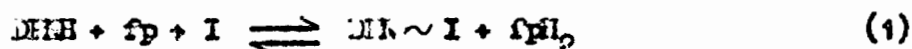
This was carried out according to Gurin and co-workers (3) on Whatman No. 1 paper with 0.05 M phosphate buffer (pH 6.8) at  $2^{\circ}\text{C}$ . for 18 - 20 hours. After air drying, papers were examined under ultra-violet light and the absorbing spots marked in. Morrison's spray reagent for reactive acyls was also employed. Results of the electrophoresis experiments are shown in Fig. 1. Electrophoretic mobilities relative to AMP ( $R_{AMP}$ ) were: octanoyl-AMP, 0.66;

Antimycin A (5  $\mu$ g.), inhibited  $\text{DH} + \text{isocitrate-cytochrome } c$  reduction to a considerable extent. However, after two minutes the rate of cytochrome  $c$  reduction increased again, and the change in optical density at 550 m $\mu$  approached the control value after fifteen minutes. This result indicates an initial inhibition of electron transfer, which is subsequently overcome as the reaction proceeds. The reaction with  $\text{DH}$  as co-factor was not examined.

### 13.3 Discussion

The results in Table 7 are of a preliminary nature and require further investigation. Rates of cytochrome  $c$  reduction were found to be linear during the first two minutes of each reaction.

Amytal ( $\text{Na}^+$  salt of 5-ethyl-5'-isocamylbarbituric acid), at a concentration of  $2 \times 10^{-3}$  M, lowered the rates of both  $\text{DH}$  and  $\text{DH}$ -linked isocitrate-cytochrome  $c$  reductase activity by approximately 30%. Amytal at this concentration completely blocks oxidations linked with diphosphopyridine nucleotide in rat liver mitochondria; acting between flavoprotein and  $\text{DH}$  (11, 53, 54). Slater and co-workers (11, 55) have postulated the following theoretical respiratory-chain phosphorylation scheme:

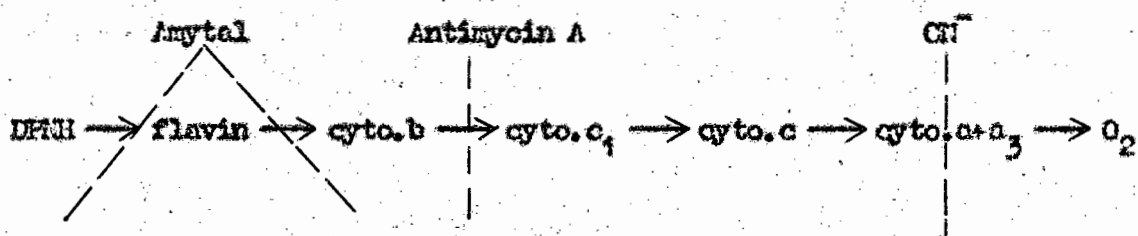


Abbreviations used:  $\text{P}_i$ , inorganic phosphate; fp, oxidised flavoprotein; I and X, unknown intermediates postulated to occur in oxidative phosphorylation;  $\text{DH} \sim \text{I}$ , energy rich diphosphopyridine nucleotide intermediate.

Hurvis (11) working in Slater's laboratory, has postulated that the primary site of amytal action is at reaction (2). This means that

presence of antimycin A. Baltscheffsky and Baltscheffsky (58) have described the isolation of sub-mitochondrial DPNH-oxidase fractions from both liver and heart muscle mitochondria of the rat and guinea-pig. They reported that the respiratory-chain inhibitors, antimycin A, amytal and 2-n-heptyl-4-hydroxyquinoline-N-oxide, blocked electron transport in the heart muscle preparations, but not in the liver preparations.

A diagram illustrating the probable sites of inhibitory action on electron transfer in mitochondria is shown below:



#### 14 THE EFFECT OF MANGANESE ION ON ISOCITRATE OXIDATION

Manganese ( $Mn^{+2}$ ) is an essential co-factor of the FPN-linked isocitric dehydrogenase in mammalian tissues. This ion was accordingly tested for its effect on DPN + isocitrate-diaphorase activity in Ehrlich ascites tumour cell mitochondria.

##### 14.1 Results

At concentrations of  $1.5 \times 10^{-4}$  M and  $2.0 \times 10^{-4}$  M, manganese ion had an almost negligible but slightly inhibitory effect on the diaphorase activity associated with isocitrate oxidation when DPN was used as co-factor (Table 8). Magnesium ( $Mg^{+2}$ ) ions normally present in the reaction medium may, however, have supplanted the manganese ions as co-factors, thereby masking any possible effect of the latter.

TABLE 8

Effect of Manganese on the Rate of Dye Reduction  
in the Diphosphopyridine Nucleotide  
plus Isocitrate-diaphorase Reaction

The reaction medium in a volume of 3.0 ml. was as described in Table 7, with the exception that DPN was present at a concentration of 1,500  $\mu\text{g.}$  per cuvette. Depleted mitochondria (0.31 mg. protein), 2,6-dichlorophenol-indophenol (50  $\mu\text{g.}$ ),  $\text{Mn}^{+2}$  concentrations given in the table below.

| Reaction studied                                          | Electron acceptor<br>2,6-dichlorophenol-indophenol   |
|-----------------------------------------------------------|------------------------------------------------------|
|                                                           | $\Delta \text{OD}_{600 \text{ m}\mu}$ per 10 minutes |
| Isocitrate + DPN                                          | 0.572                                                |
| Isocitrate + DPN + $2 \times 10^{-4} \text{ M Mn}^{+2}$   | 0.503                                                |
| Isocitrate + DPN + $1.5 \times 10^{-4} \text{ M Mn}^{+2}$ | 0.511                                                |

## 15 L-GLUTAMATE OXIDATION

The oxidation of L-glutamate by depleted Ehrlich ascites cell mitochondria was studied for purposes of control, during the work on isocitrate oxidation.

### 15.1 L-Glutamate Oxidation with 2,6-dichlorophenol-indophenol as Electron Acceptor

Mitochondria completely depleted of pyridine nucleotides, oxidised L-glutamate satisfactorily with DPN as co-factor and 2,6-dichlorophenol-indophenol as electron acceptor (Table 9). Amytal ( $2 \times 10^{-3}$  M), inhibited this reaction by approximately 12%.

### 15.2 L-Glutamate Oxidation with Cytochrome c as Electron Acceptor

With L-glutamate as substrate, rapid reduction of cytochrome c occurred in mitochondria which had been depleted of pyridine nucleotides by ageing for 5 - 6 hours at 0°C. However, no reduction occurred under similar conditions with mitochondria which had been completely depleted of pyridine nucleotides by incubation with sucrose-phosphate buffer at 30°C.

### 15.3 Discussion

It may be concluded that when Ehrlich ascites cell mitochondria are completely depleted of pyridine nucleotides, damage or removal of an essential component of the electron transfer chain associated with L-glutamate oxidation takes place. This is evidenced by the failure to detect cytochrome c reduction with L-glutamate as substrate. Isocitrate oxidation is not, however, impaired in mitochondria completely depleted of pyridine nucleotides.

TABLE 9

L-Glutamate Oxidation in Depleted  
Ehrlich Tumour Mitochondria with  
2,6-dichlorophenol-indophenol as Electron Acceptor

Cuvettes contained the following in a final volume of 3.0 ml: 0.10 M K,Na-phosphate buffer (pH 7.4), 0.001 M KCN, 0.003 M  $\text{HgCl}_2$ , 0.02 M nicotinamide, 2,6-dichlorophenol-indophenol (50  $\mu\text{g.}$ ), DPN (1,500  $\mu\text{g.}$ ), 0.0023 M L-glutamate, depleted mitochondria (0.22 mg. protein). Temp.  $30^\circ$ .

| Reaction                                  | Electron acceptor<br>2,6-dichlorophenol-indophenol   |
|-------------------------------------------|------------------------------------------------------|
|                                           | $\Delta \text{OD}_{600 \text{ m}\mu}$ per 10 minutes |
| L-Glutamate                               | 0.402                                                |
| L-Glutamate + $2 \times 10^{-3}$ M amytal | 0.355                                                |

OXYGEN UPTAKE IN  $\mu$ L. AT 30°C.

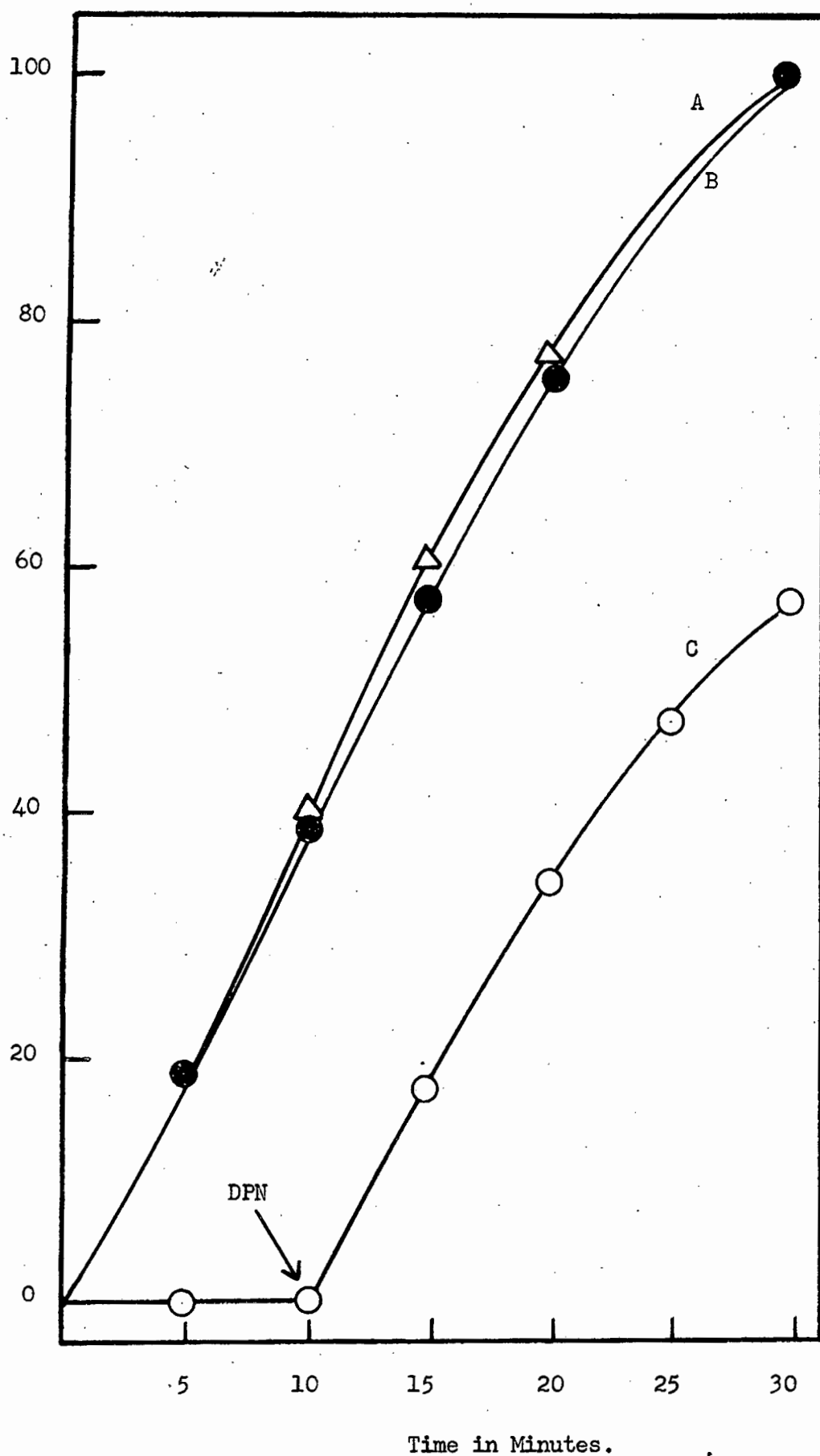


Fig. 13. Oxygen uptake of aged Ehrlich ascites mitochondria.

Reaction mixture as in Fig. 3. Curve A,  $2.4 \times 10^{-4}$  M DPN. Curve B,  $2.4 \times 10^{-3}$  M DPN, and Curve C,  $2.4 \times 10^{-3}$  M DPN added at 10 min. ( $\rightarrow$ ). Mitochondria, 2.63 mg protein, were aged 5 hours at 0°C. prior to use.

oxidation and associated phosphorylations in rat liver mitochondria by Kaplan and his co-workers (9) have shown that phosphorylation takes place only after transfer of electrons from TPNH to DPN, via the transhydrogenase. Vignais, Vignais and Bartley (51) in their studies of isocitrate oxidation in rat liver mitochondria have reported data concerning phosphorylation coupled to isocitrate oxidation. Their mitochondria were not, however, depleted of endogenous pyridine nucleotides. Vignais and Vignais (36) have subsequently studied the phosphorylations associated with DPNH and TPNH oxidation in the mitochondria of rat liver, kidney and brain. Joshi, Newburgh and Cheldelin (52), have studied TPNH oxidation in beef heart mitochondria, and in the high speed supernatant fraction of beef heart homogenates. Phosphorylation was found to be coupled to TPNH oxidation when a combination of these two fractions was used. Pyridine nucleotide transhydrogenase was not implicated in these reactions.

## 11.2 Method

Mitochondria were isolated in 0.25 M sucrose as described in the Experimental Section. They were depleted of endogenous pyridine nucleotides in one of two ways.

- (i) Depletion by ageing the mitochondria in 0.25 M sucrose for 5 - 6 hours at 0°C.
- (ii) Complete depletion of pyridine nucleotides by incubation in 0.25 M sucrose + 0.05 M phosphate buffer, pH 7.4, under aerobic conditions at 30° for 35 - 40 minutes.

### Measurement of oxidative phosphorylation

The following medium in a final volume of 3.0 ml. was used for measurement of oxidative phosphorylation or oxygen uptake: 0.045 M K<sub>2</sub>HPO<sub>4</sub>-phosphate buffer (pH 7.4), 0.034 M D-glucose, 0.015 M KP, 0.008 M MgCl<sub>2</sub>, 0.0025 M ADP, 0.001 M ALP, 0.002 M EDTA, 0.01 M KCl,

0.062 M sucrose,  $9.8 \times 10^{-6}$  M cytochrome c, 0.0033 M DL-isocitrate (pH 7.4), hexokinase (1 - 2 mg.), mitochondrial suspension (0.4 ml.). Temp. 30°. Equilibration time, 9 - 12 minutes. (DHN, TPN concentrations given in legends.)

### 11.3 Oxidation and Associated Phosphorylations with Isocitrate as Substrate

Table 5 shows that isocitrate oxidation is coupled to phosphorylation. In mitochondria completely depleted of endogenous pyridine nucleotides, P:O ratios associated with the DHN pathway lie in the range 1.11 - 1.31, while those associated with the TPN pathway lie in the range 1.07 - 1.31. The highest P:O ratio observed in any experiment was 1.31. It is of interest to note that, when mitochondria were depleted of pyridine nucleotides by ageing for 5 - 6 hours at 0°C., P:O ratios were lower than those found in completely depleted mitochondria. It is difficult to find a reasonable explanation for this lowering in P:O ratio.

## 12 TPNH OXIDATION IN EHRLICH ASCITES TUMOUR CELL MITOCHONDRIA

In view of the findings that externally added TPNH is oxidised by Ehrlich ascites mitochondria using either 2,6-dichlorophenol-indophenol or cytochrome c as terminal electron acceptor (Figs. 1 and 2), experiments were conducted to determine whether TPNH could be oxidised with oxygen as electron acceptor.

### 12.1 Method

In these experiments mitochondria were isolated and depleted of endogenous pyridine nucleotides as described in the section on oxidative phosphorylation (sub-section 11.2). TPNH was generated in situ by the glucose-6-phosphate dehydrogenase reaction. Oxygen uptake was measured in Warburg manometers at 30°C.

TABLE 5

Oxidation and Associated Phosphorylations with  
Isocitrate as Substrate in Ehrlich Ascites Cell Mitochondria

For oxidative phosphorylation studies, mitochondria were isolated as described and twice washed in 0.25 M sucrose. In one series of experiments they were depleted by ageing 5-6 hours in 0.25 M sucrose at 0°, while in further experiments they were completely depleted of pyridine nucleotides by incubation in 0.25 M sucrose + 0.03 M phosphate buffer, pH 7.4, under aerobic conditions at 30° for 35-40 minutes. Reaction mixture in a final volume of 3.0 ml: 0.045 M K<sub>2</sub>Na-phosphate buffer (pH 7.4), 0.034 M D-glucose, 0.015 M KF, 0.008 M MgCl<sub>2</sub>, 0.0025 M ADP, 0.001 M AMP, 0.002 M EDTA, 0.01 M KCl, 0.062 M sucrose,  $9.8 \times 10^{-6}$  M cytochrome C, 0.0033 M DL-isocitrate, hexokinase (1-2 mg.), mitochondria (1.80-2.63 mg. protein). Temp. 30°.

| Pretreatment conditions                                                                                         | Co-factor<br>DFN<br>moles/l. | Co-factor<br>THH<br>moles/l. | O <sub>2</sub><br>μ atoms | PO <sub>4</sub><br>μ moles | P:O               |
|-----------------------------------------------------------------------------------------------------------------|------------------------------|------------------------------|---------------------------|----------------------------|-------------------|
| 0.25 M sucrose<br>aged 5½ hours at 0°                                                                           | $2.4 \times 10^{-3}$         |                              | 8.22                      | 4.65                       | 0.57              |
|                                                                                                                 | $2.8 \times 10^{-3}$         |                              | 8.55                      | 5.60                       | 0.66              |
| 0.25 M sucrose +<br>0.03 M phosphate<br>buffer, pH 7.4,<br>incubated 35-40<br>min. at 30°,<br>then stored at 0° |                              | $1.2 \times 10^{-3}$         | 3.00                      | 5.22                       | 1.07 <sup>2</sup> |
|                                                                                                                 |                              | $1.4 \times 10^{-3}$         | 2.46                      | 3.22                       | 1.31              |
|                                                                                                                 | $1.2 \times 10^{-3}$         |                              | 8.24                      | 9.68                       | 1.18 <sup>2</sup> |
|                                                                                                                 | $1.4 \times 10^{-3}$         |                              | 8.17                      | 9.07                       | 1.11              |
|                                                                                                                 | $2.4 \times 10^{-3}$         |                              | 5.28                      | 6.93                       | 1.31              |

<sup>2</sup>Similar results were obtained with 4 different mitochondrial preparations. Two further mitochondrial preparations showed reasonable oxygen uptakes but were devoid of phosphorylative activity.

## 12.2 TPNH Oxidation

Table 6 shows that oxygen uptake was negligible with externally added TPNH as substrate. In a control flask with isocitrate plus TPN, oxygen uptake was approximately seven times as great. Concentrations of TPN were identical ( $1 \times 10^{-3}$  M) in these two flasks. Additional experiments to test for the presence of possible intramitochondrial inhibitors of TPNH oxidation are reported in Table 6. For this purpose, the protein ovalbumin was used as an adsorbent. Albumins are well known for their ability to remove inhibitory substances by adsorption from certain enzyme systems (61). Ovalbumin at a concentration of 10 mg. per 1.0 ml. of reaction mixture was found to lower DPN-linked oxidation by approximately 13%. An increase in the concentration of DPN from  $2.4 \times 10^{-4}$  M to  $2.4 \times 10^{-5}$  M, had little effect upon oxygen uptake with isocitrate as substrate (cf. Table 6 and Fig. 13).

## 12.3 Discussion

The results clearly indicate that TPNH cannot be oxidised at an appreciable rate in Ehrlich ascites mitochondria with oxygen as final electron acceptor. This is in contrast to the results obtained with isocitrate in the presence of TPN, where oxygen uptake was several times as rapid. However, TPNH is oxidised at an appreciable rate with either 2,6-dichlorophenol-indophenol or cytochrome c as electron acceptor (cf. Figs. 1 and 2). Apparently when TPNH is oxidised, electron transfer takes place only along a portion of the electron transfer chain. A natural metabolite may act in vivo as terminal electron acceptor, intercepting electrons somewhere along the cytochrome chain, but there remains a block in electron transport between cytochrome c and oxygen.

TABLE 6

Oxygen Uptake of Ehrlich Ascites Cell Mitochondria  
with Isocitrate and Reduced Triphosphopyridine  
Nucleotide as Substrates

Reaction mixture in a final volume of 3.0 ml: 0.045 M K<sub>2</sub>Na-phosphate buffer (pH 7.4), 0.034 M D-glucose, 0.015 M KF, 0.008 M MgCl<sub>2</sub>, 0.0025 M ADP, 0.004 M ATP, 0.002 M EDTA, 0.01 M KCl, 0.062 M sucrose,  $9.8 \times 10^{-6}$  M cytochrome c, 0.0033 M DL-isocitrate, hexokinase (1.3 mg.). Extra special additions were as follows: For the generation of TPNH, D-glucose-6-phosphate dehydrogenase (0.8 mg.), D-glucose-6-phosphate 0.0033 M, ovalbumin (10 mg./1.0 ml. incubation medium), DPN and TPN, concentrations given in table. Temp. 30°.

| System oxidised                                                      | mg. protein<br>per flask | O <sub>2</sub><br>µg. atoms |
|----------------------------------------------------------------------|--------------------------|-----------------------------|
| TPNH (TPN, $1.2 \times 10^{-3}$ M) enzymatically<br>produced in situ | 1.91                     | 0.51 +                      |
| TPN ( $1.2 \times 10^{-3}$ M) + isocitrate                           | 1.91                     | 3.41                        |
| DPN ( $1.2 \times 10^{-3}$ M) + isocitrate                           | 2.02                     | 9.68                        |
| DPN ( $1.2 \times 10^{-3}$ M) + isocitrate + ovalbumin               | 2.02                     | 8.44                        |
| DPN ( $2.4 \times 10^{-3}$ M) + isocitrate                           | 2.63                     | 7.92 H                      |
| DPN ( $2.4 \times 10^{-4}$ M) + isocitrate                           | 2.63                     | 8.10                        |

\*Mitochondria completely depleted of pyridine nucleotides prior to assay.

\*\*Mitochondria depleted by ageing 5 hours at 0°.

Wenner and associates (23, 24, 25, 26) have shown that, in a number of different tumour cells, TPNH produced by shunt pathway oxidation is not oxidised via the mitochondrial system by oxygen. The results of the experiments reported in this thesis fully support Wenner's findings.

In mitochondria which had been depleted of pyridine nucleotides by ageing for 5 hours at 0°C., increasing the concentration of DPN from  $2.4 \times 10^{-4}$  M to  $2.4 \times 10^{-3}$  M had no effect on oxygen uptake with isocitrate as substrate.

### 13 THE EFFECTS OF INHIBITORS AND UNCOUPLERS ON ISOCITRATE OXIDATION

#### 13.1 Method

Mitochondria were completely depleted of pyridine nucleotides as described previously. The reaction medium was the same as indicated in Fig. 1, with the exception that cytochrome c (2.0 mg.) was used as electron acceptor. Mitochondria were not pre-incubated with inhibitors or uncouplers prior to assay. Reactions were assayed immediately after addition of the inhibitor or uncoupler.

#### 13.2 The Effects of Inhibitors and Uncouplers

Isocitrate oxidation with either DPN or TPN as co-factor was studied using cytochrome c as electron acceptor (Table 7). Amytal ( $2 \times 10^{-3}$  M), inhibited cytochrome c reduction by approximately 30% in both the DPN and TPN linked reactions. Chlorpromazine ( $2 \times 10^{-4}$  M) had no effect on DPN-linked isocitrate oxidation, but increased the rate of the TPN-linked system approximately 100%. Dinitrophenol ( $2 \times 10^{-4}$  M), a potent uncoupler of oxidative phosphorylation, had no effect on the rate of cytochrome c reduction with DPN as co-factor, but inhibited the TPN-linked reduction by about 50%.

TABLE 7

Effect of Respiratory Chain Inhibitors  
and Uncouplers on Electron Transport  
Resulting from Isocitrate Oxidation

Cuvettes contained the following medium in a final volume of 3.0 ml: 0.10 M K<sub>2</sub>Na-phosphate buffer (pH 7.4), 0.001 M KCN, 0.003 M MgCl<sub>2</sub>, 0.0023 M DL-isocitrate (K<sup>+</sup> salt, pH 7.4), 0.02 M nicotinamide, cytochrome c (2 mg.), DPN and TPN, 1,000 µg. each, depleted mitochondria (0.35 mg. protein).

| Inhibitor<br>or<br>uncoupler | Concentration        | Cytochrome c as electron acceptor            |                    |
|------------------------------|----------------------|----------------------------------------------|--------------------|
|                              |                      | $\Delta OD_{550 \text{ m}\mu}$ per 2 minutes |                    |
|                              |                      | DPN-isocitrate                               | TPN-isocitrate     |
| Control                      | -                    | 0.239                                        | 0.030              |
| Amytal                       | $2 \times 10^{-3}$ M | 0.162                                        | 0.020              |
| Chlorpromazine               | $2 \times 10^{-4}$ M | 0.237                                        | 0.057              |
| 2,4-dinitrophenol            | $2 \times 10^{-4}$ M | 0.227                                        | 0.014              |
| Antimycin A                  | 5 µg.                | -                                            | 0.003 <sup>*</sup> |

<sup>\*</sup>After 2 minutes, the rate of cytochrome c reduction increased, with the  $OD_{550 \text{ m}\mu}$  (15 minutes) being equal to the control.

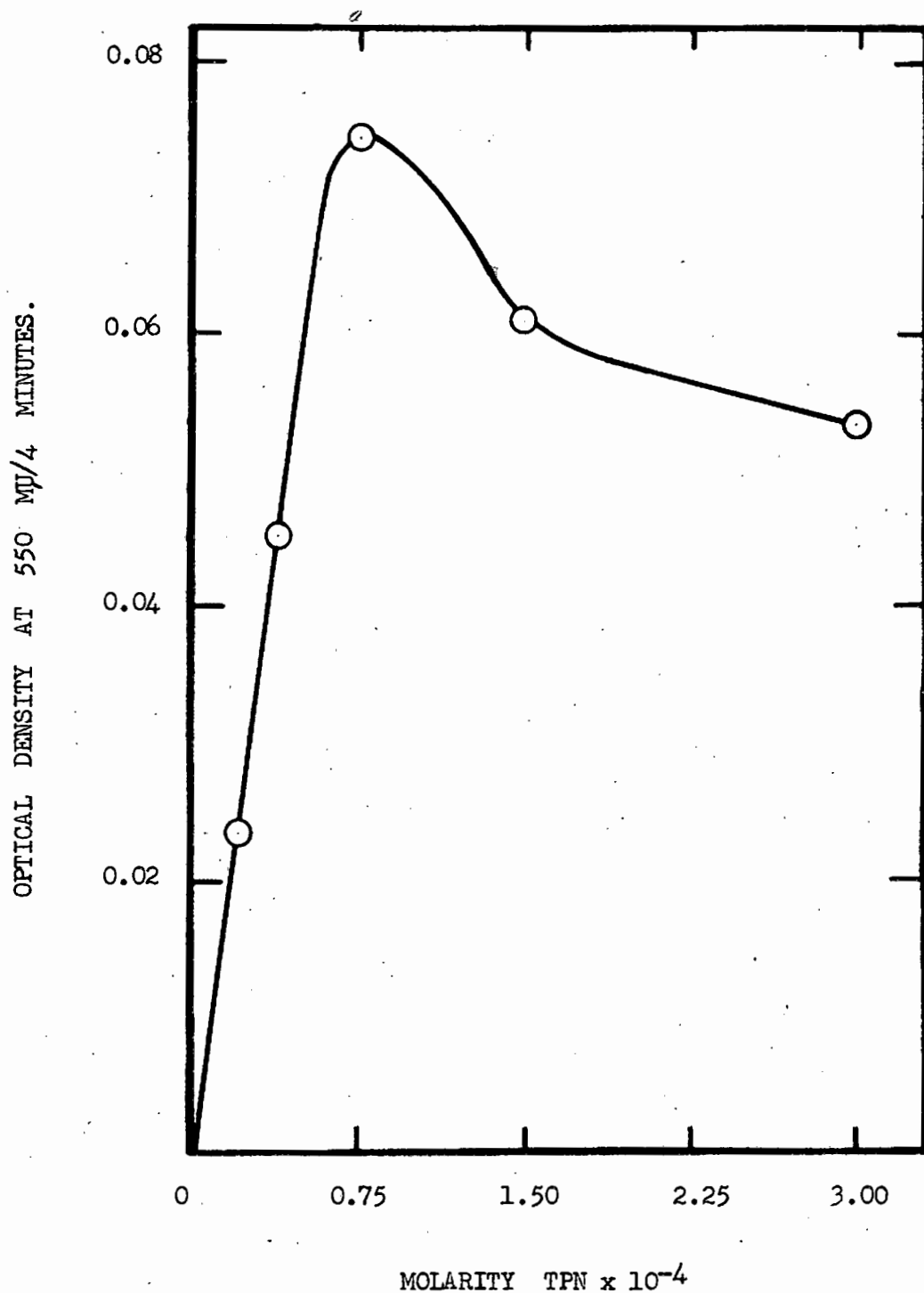


Fig. 10. Effect of TPN concentration on the rate of cytochrome c reduction coupled to the oxidation of DL-isocitrate in pyridine-nucleotide depleted mitochondria.

Reaction medium in a final volume of 3.0 ml: K,Na phosphate buffer (pH 7.4), 0.10 M; KCN, 0.001 M;  $MgCl_2$ , 0.003 M; DL-isocitrate, 0.0023 M; nicotinamide, 0.02 M; cytochrome c (2 mg); depleted mitochondria (0.31 mg protein).

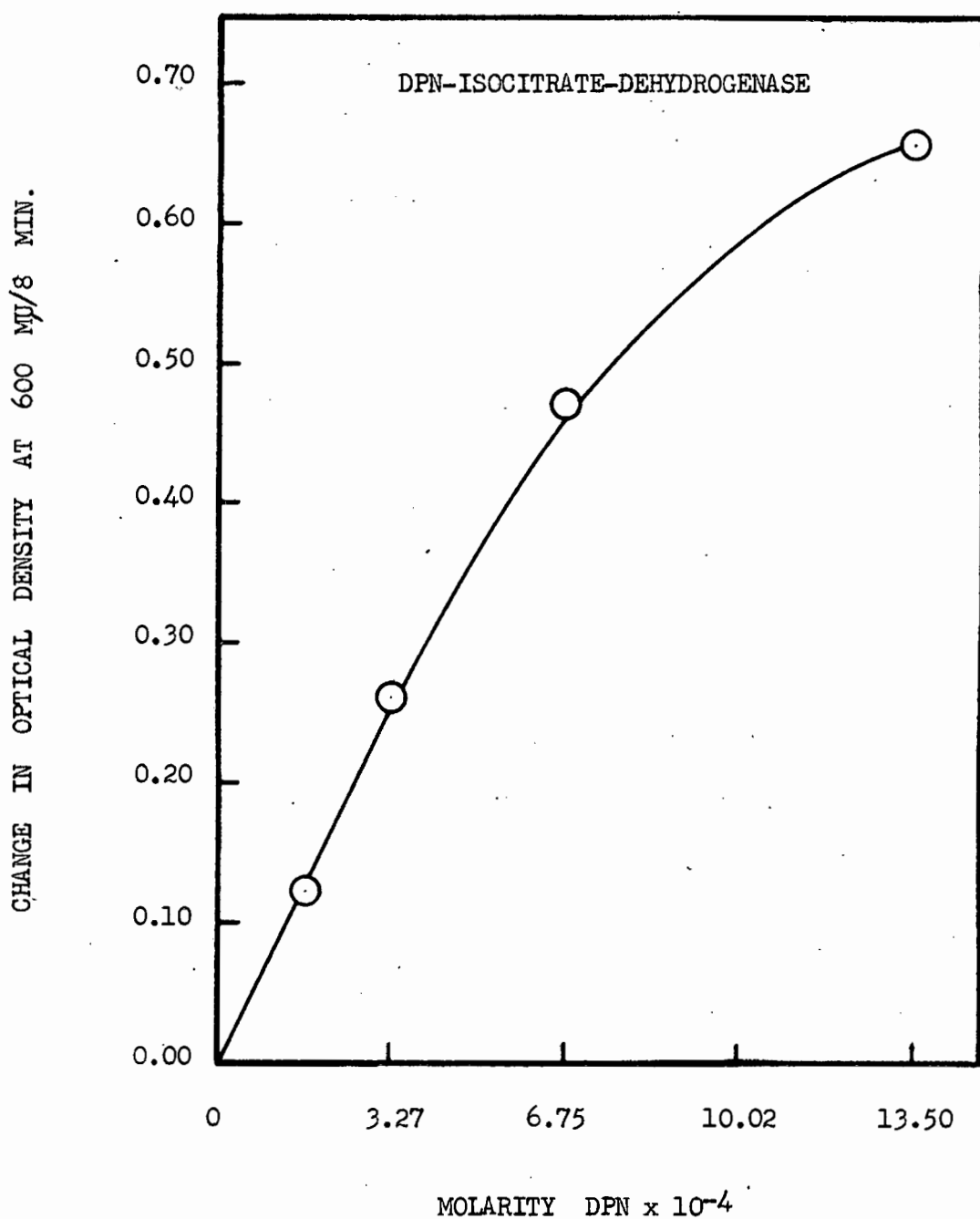


Fig. 11. Effect of DPN concentration on the rate of 2,6-dichlorophenol-indophenol reduction by depleted Ehrlich ascites tumour mitochondria.

Reaction medium in a final volume of 3.0 ml. The components are as given in Fig. 1. Each cuvette contained 0.31 mg mitochondrial protein.

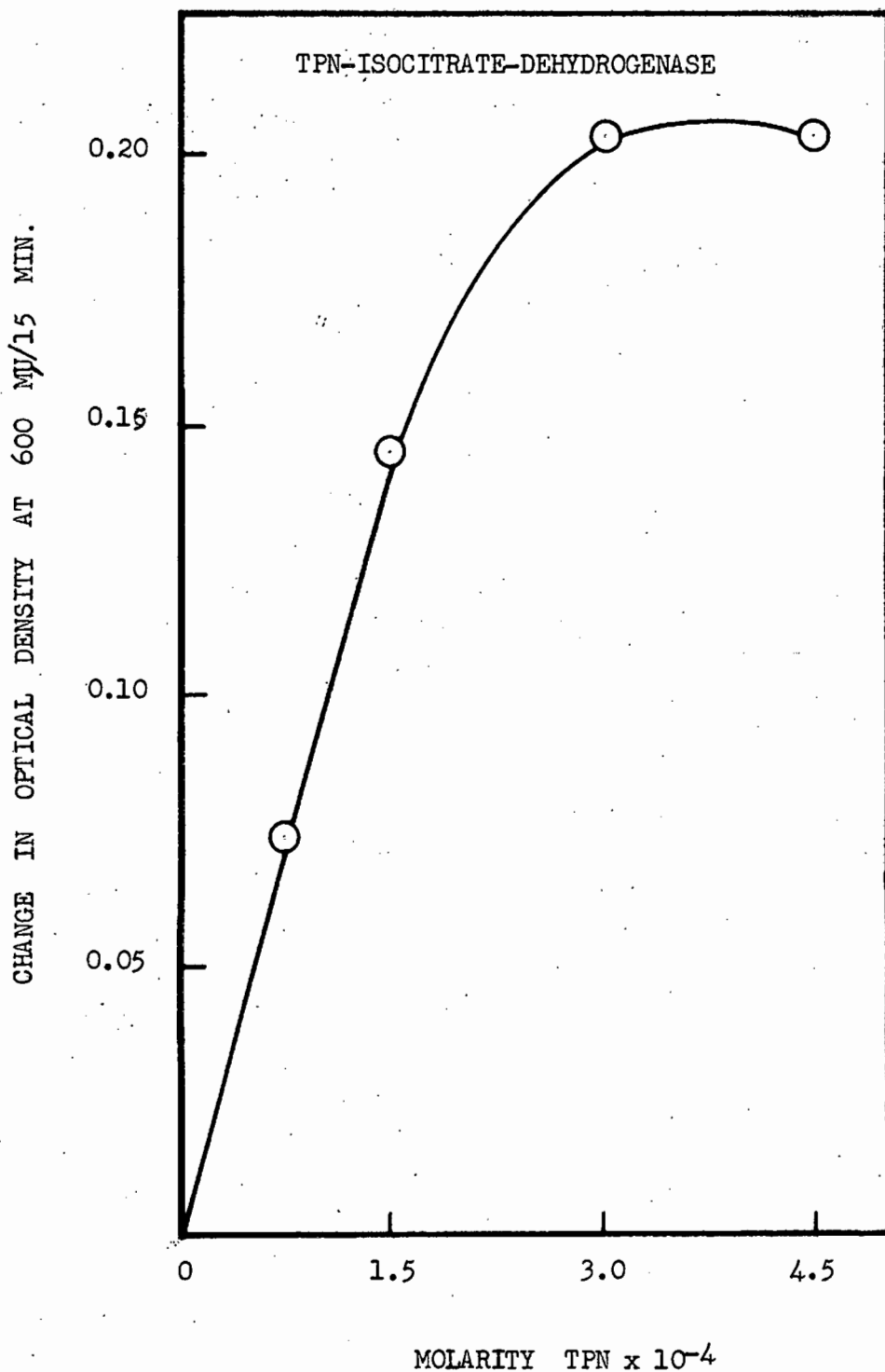
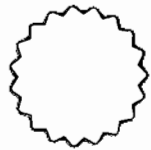


Fig. 12. Effect of TPN concentration on the rate of  
2,6-dichlorophenolindophenol reduction by depleted  
Ehrlich ascites mitochondria.

Reaction mixture in a final volume of 3.0 ml. The components are as given in Fig. 1. Each cuvette contained 0.31 mg mitochondrial protein.

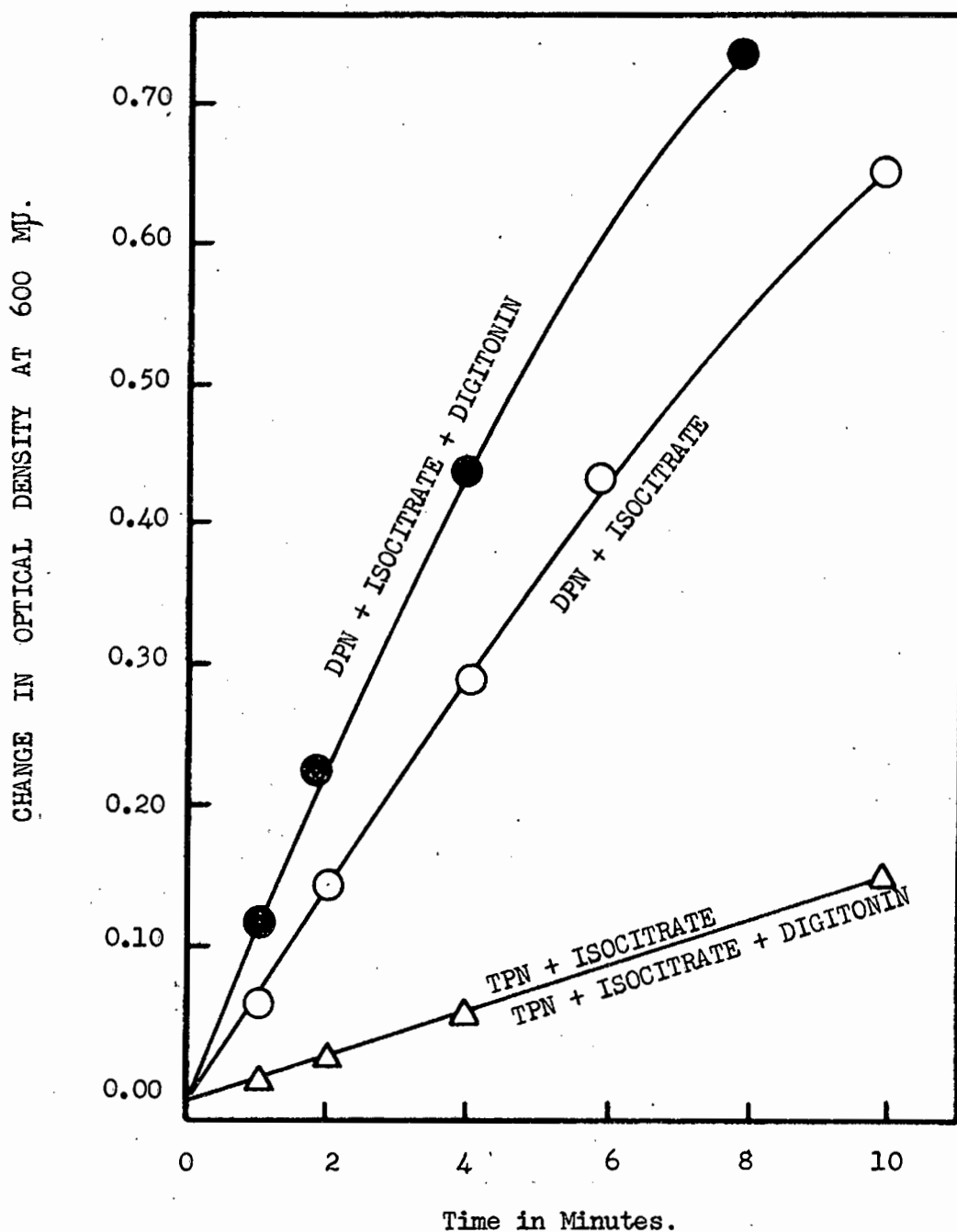


Fig. 7. Effect of Digitonin on the diaphorase activities associated with TPN and DPN-linked isocitrate oxidation.

Pyridine-nucleotide depleted mitochondria were pre-treated with 0.5% digitonin (pH 7.4) in 0.12 M sucrose for 20 minutes aerobically at 0°C. Following this incubation they were immediately assayed in the standard system of Fig. 1 with 2,6-dichlorophenolindophenol as electron acceptor. TPN (400 μg); DPN (1500 μg). Each Beckmann cuvette contained 0.29 mg protein.

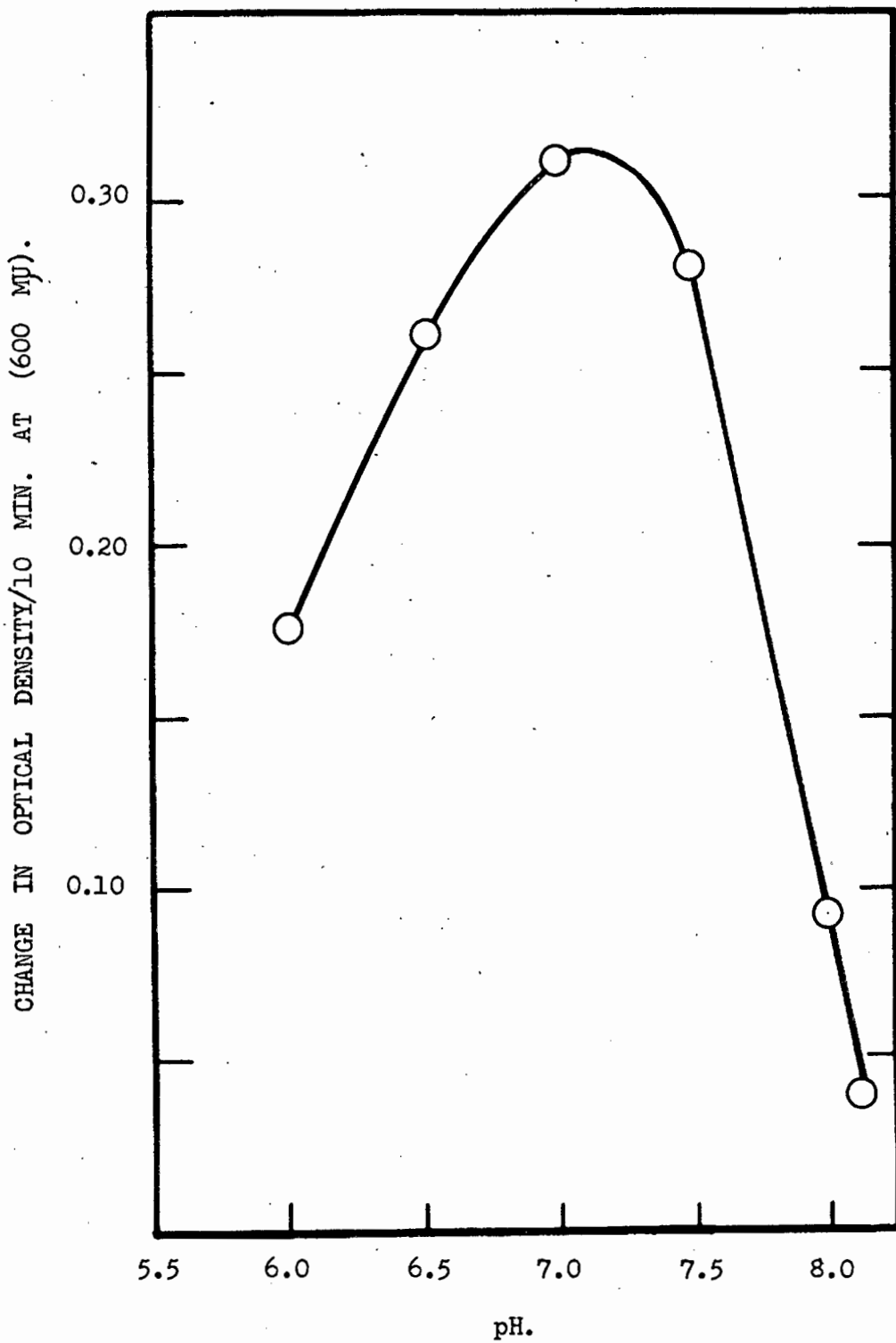


Fig. 8. Variation of Isocitric-Dehydrogenase activity with pH.

The DPN-linked dehydrogenase.

Reaction mixture in a final volume of 3.0 ml:- As in Fig. 1 except that 0.10 M tris(hydroxymethyl) - aminomethane acetate buffer replaced the usual phosphate buffer. DPN, 1500 μg. Depleted mitochondria (0.24 mg protein).

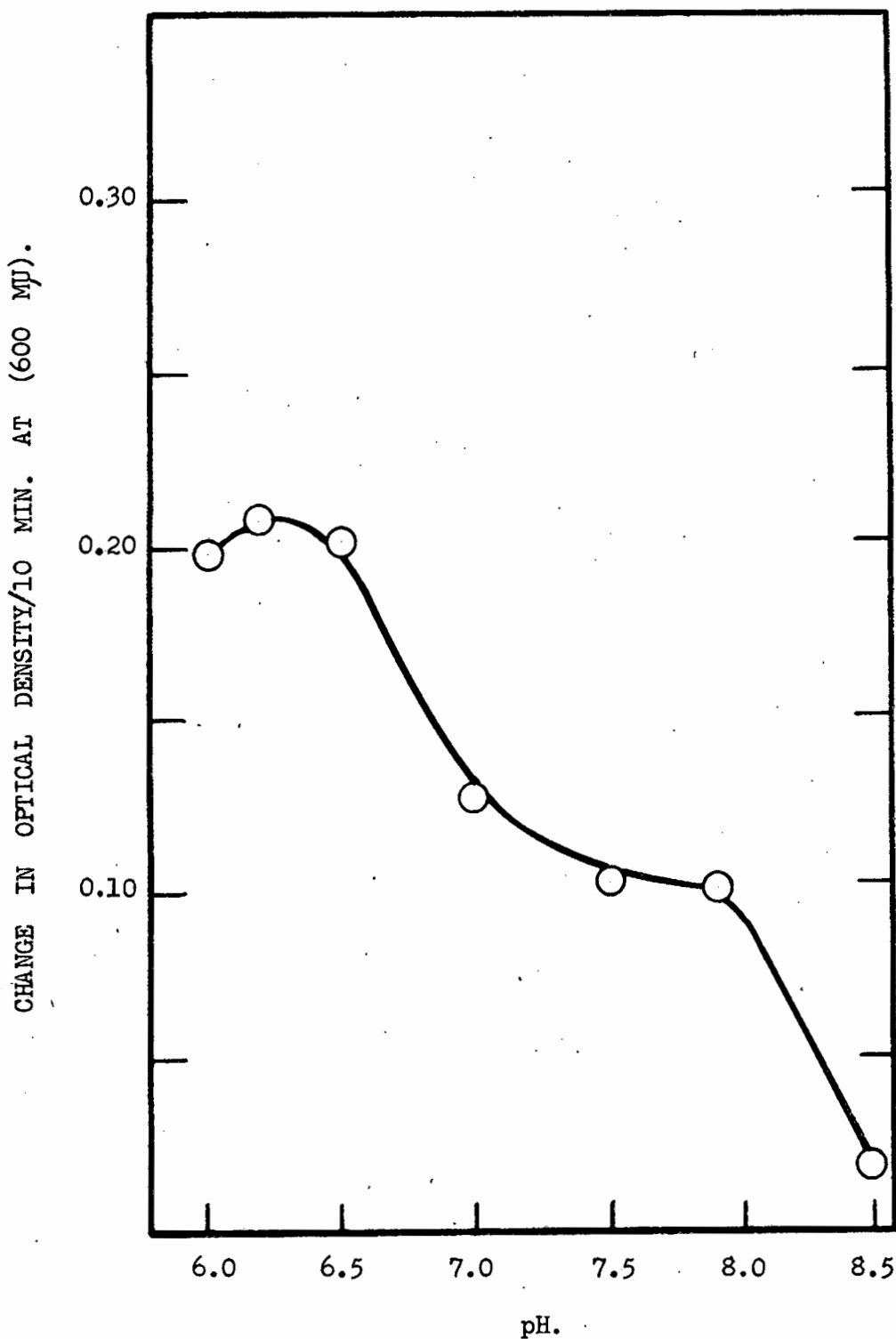


Fig. 9. Variation of Isocitric-Dehydrogenase activity with pH.

The TPN-linked dehydrogenase.

Reaction mixture in a final volume of 3.0 ml:- As in Fig. 1 except that 0.10 tris(hydroxymethyl) - aminomethane acetate buffer replaced the usual phosphate buffer. TPN, 400  $\mu$ g. Depleted mitochondria (0.24 mg protein).

TABLE 3

The Effect of Different Buffers on Isocitrate Oxidation  
in Ehrlich Ascites Cell Mitochondria

The reaction medium contained the following in a final volume of 3.0 ml: 0.10 M  $\text{KH}_2\text{PO}_4$ - $\text{Na}_2\text{HPO}_4$  buffer (pH 7.4), or 0.10 M tris-acetate buffer (pH 7.4), 0.004 M KCN, 0.003 M  $\text{HgCl}_2$ , 0.0023 M DL-isocitrate ( $\text{K}^+$  salt, pH 7.4), 0.02 M nicotinamide, 2,6-dichlorophenol-indophenol (50  $\mu\text{g.}$ ), DPN (1,500  $\mu\text{g.}$ ), TPN (400  $\mu\text{g.}$ ), depleted mitochondria (0.18 mg. protein).

| Buffer used<br>in assay                                                | Electron acceptor 2,6-dichlorophenol-indophenol      |                  |
|------------------------------------------------------------------------|------------------------------------------------------|------------------|
|                                                                        | DPN + isocitrate                                     | TPN + isocitrate |
|                                                                        | $\Delta \text{OD}_{600 \text{ m}\mu}$ per 10 minutes |                  |
| 0.1 M tris-<br>acetate (pH 7.4)                                        | 0.283                                                | 0.100            |
| 0.1 M $\text{KH}_2\text{PO}_4$ -<br>$\text{Na}_2\text{HPO}_4$ (pH 7.4) | 0.478                                                | 0.172            |

TABLE 4

Effect of Ageing on DEN and TFN-isocitric  
Dehydrogenase Activities in Pyridine Nucleotide  
Depleted Ehrlich Ascites Tumour Cell Mitochondria

The experimental reaction system contained the following in a final volume of 3.0 ml: 0.10 M K<sub>2</sub>Na-phosphate buffer (pH 7.4), 0.001 M KCN, 0.003 M MgCl<sub>2</sub>, 0.0025 M DL-isocitrate, 0.02 M nicotinamide, 2,6-dichlorophenol-indophenol (50 µg.), DEN (1,500 µg.), TFN (400 µg.), depleted mitochondria (0.26 mg. protein). Mitochondria were aged by storage in ice at 0° for the times given. Diaphorase activities were measured.

| Hours<br>stored at 0° | Electron acceptor 2,6-dichlorophenol-indophenol |                  |
|-----------------------|-------------------------------------------------|------------------|
|                       | DEN + isocitrate                                | TFN + isocitrate |
|                       | △ OD <sub>600 mμ</sub> per 12 minutes           |                  |
| 1                     | 0.548                                           | 0.200            |
| 24                    | 0.404                                           | 0.190            |
| 50                    | 0.306                                           | 0.188            |
| 72                    | 0.228                                           | 0.196            |
| 84                    | 0.167                                           | 0.191            |

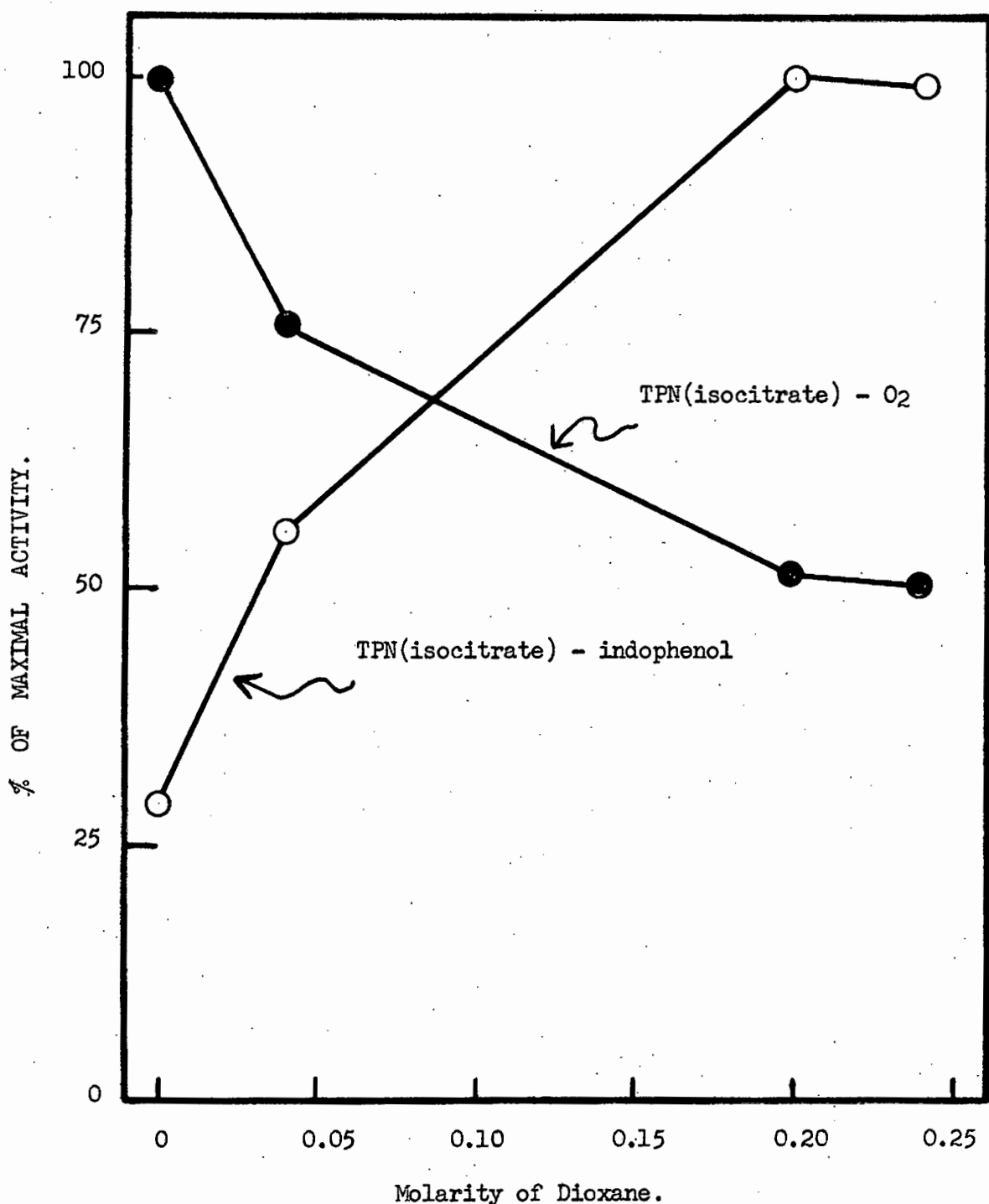


Fig. 6. The effect of concentration of Dioxane on DPN and TPN-linked Isocitrate oxidations in depleted Ehrlich ascites tumour mitochondria.

Conditions of experiment: Each cuvette contained 0.20 mg of mitochondrial protein. The reaction medium when 2,6-dichlorophenolindophenol was used as acceptor is that described in Fig. 1. TPN (400  $\mu$ g). With oxygen as terminal electron acceptor the medium used is that given in Fig. 3. TPN, ( $1.2 \times 10^{-3}$  M). Oxygen uptakes were measured with Warburg manometers at 30°C. over a reaction time of 25 minutes.

## 9 VARIATION OF DEHYDROGENASE ACTIVITY WITH CO-ENZYLE CONCENTRATION

### 9.1 Method

The velocity of isocitrate oxidation with varying concentrations of DEN and TFN as co-enzymes was measured in the standard reaction system of Fig. 1 with 2,6-dichlorophenol-indophenol as electron acceptor.

### 9.2 DEN-linked Dehydrogenase

Fig. 11 illustrates the effect of DEN concentration on isocitrate oxidation. With increasing co-enzyme concentration a linear rate of dye reduction was observed which began to decline at a concentration of  $6.7 \times 10^{-4}$  M DEN.

### 9.3 TFN-linked Dehydrogenase

With TFN as co-enzyme, the maximum rate of dye reduction occurred at a concentration of  $3 \times 10^{-4}$  M TFN (Fig. 12). The system appeared to be saturated by a low concentration of co-enzyme.

## 10 TFN-ISOCITRATE- CYTOCHROME C REDUCTASE

The effect of TFN concentration on TFN-isocitrate-cytochrome c reductase activity is shown in Fig. 10. Maximum activity occurred at a concentration of  $7.5 \times 10^{-5}$  M TFN, with inhibition of cytochrome c reduction above this value.

## 11 PHOSPHORYLATION ASSOCIATED WITH THE OXIDATION OF ISOCITRATE

### 11.1 Introduction

A study was made of the phosphorylations coupled to both the DEN and TFN-linked isocitric dehydrogenase systems found in Ehrlich ascites cell mitochondria. Investigations of isocitrate

TABLE 1

The Effect of Dioxane on DPN and TEN-linked  
Isocitrate Oxidation in Ehrlich Ascites Cell Mitochondria  
with Cytochrome c and Oxygen as Electron Acceptors

With cytochrome c as electron acceptor, the reaction medium contained the following in a volume of 3.0 ml: 0.10 M K<sub>2</sub>H<sub>2</sub>PO<sub>4</sub> buffer (pH 7.4), 0.001 M KCN, 0.003 M MgCl<sub>2</sub>, 0.0023 M DL-isocitrate (K<sup>+</sup> salt, pH 7.4), 0.02 M nicotinamide, cytochrome c (2 mg.), DPN and TEN, 1,000 µg. each, depleted mitochondria (0.20 mg. protein), 0.19 M dioxane (when present). In the reactions with oxygen as electron acceptor, measurements were carried out in Warburg manometers at 30°. Components of the reaction medium are the same as those given in the text. DPN and TEN,  $1.2 \times 10^{-3}$  M; dioxane when present, 0.19 M. Depleted mitochondria (1.90 mg. protein), equilibration time, 9 minutes.

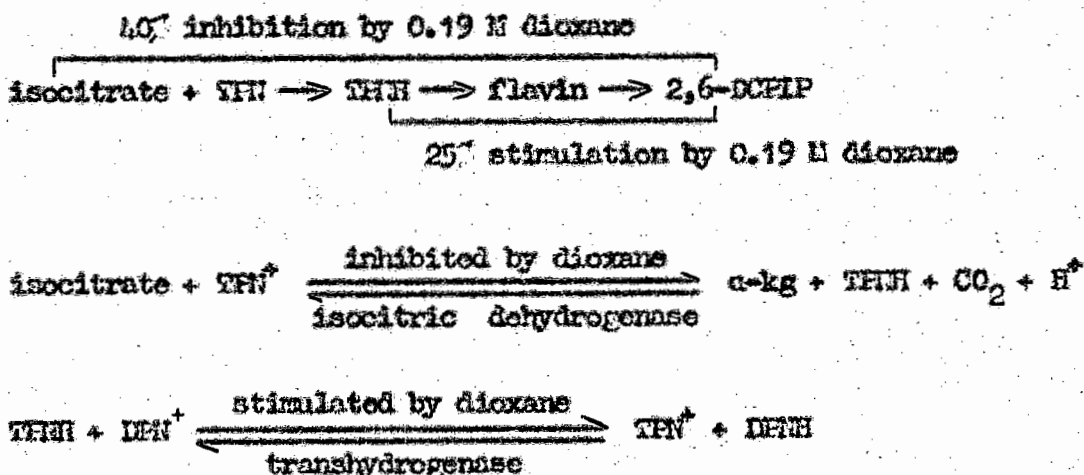
| System studied             | Terminal electron acceptor                      |                                    |
|----------------------------|-------------------------------------------------|------------------------------------|
|                            | Cytochrome c                                    | Oxygen                             |
|                            | $\Delta OD_{550 \text{ m}\mu} / 2 \text{ min.}$ | $\mu\text{g. atox/mg. protein/hr}$ |
| TEN + isocitrate           | 0.024                                           | 3.21                               |
| TEN + isocitrate + dioxane | 0.010                                           | 1.70                               |
| DPN + isocitrate           | 0.165                                           | 10.13                              |
| DPN + isocitrate + dioxane | 0.052                                           | 4.13                               |

#### 4.5 Isocitric Dehydrogenase of Rat Liver Mitochondria

Experiments were carried out to test the effect of dioxane on isocitrate oxidation in rat liver mitochondria. Mitochondria were isolated in 0.4 M sucrose by the method of Dounce (60), and depleted of endogenous pyridine nucleotides by incubation in 0.05 M sucrose + 0.03 M phosphate buffer (pH 7.4) at 30°C. for 60 minutes.

The following results were obtained:

- (1) Isocitric dehydrogenase of rat liver mitochondria was shown to be TPN-specific (Table 2), thus confirming the results obtained by Furvis (10, 11) and Kaplan (9).
- (ii) Results in Table 2 show that TPN-isocitrate oxidation was inhibited by approximately 40% in the presence of dioxane. Assays were carried out in phosphate buffer (pH 7.4) with 2,6-dichlorophenol-indophenol as electron acceptor. With TPN and DPN together as co-factors, isocitrate oxidation was stimulated to the extent of approximately 10%. This latter result indicating that dioxane causes some stimulation of transhydrogenase activity in rat liver mitochondria. Dioxane caused a stimulation of approximately 25% in the oxidation of externally added TPNH with 2,6-dichlorophenol-indophenol as electron acceptor. The results are diagrammatically illustrated below:

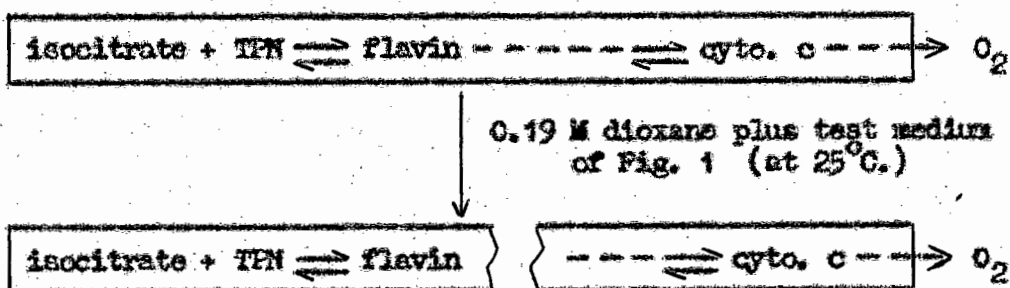


(iii) Pyridine nucleotide transhydrogenase was found to be present in rat liver mitochondria (Table 2).

(iv) Isocitrate oxidation with either TPN, or TPN + DEN together as co-factors, was more rapid in tris-acetate than phosphate buffer of the same pH. This is in contrast to the results obtained with Ehrlich ascites cell mitochondria.

#### 4.6 Discussion

Reasons for the stimulation by dioxane of TPN plus isocitrate-diaphorase activity in Ehrlich ascites tumour mitochondria are not as yet understood. It would seem that dioxane either dissociates the TPN-isocitrate linked diaphorase enzyme complex from the mitochondrial matrix, or else in some way opens up the electron transport chain. It may be that, as the flavin associated with diaphorase activity is unmasked, a simultaneous opening of the electron transfer chain occurs between flavin and cytochrome c (cf. Fig. 6). This possibility is diagrammatically illustrated below:



No experiments were undertaken to test whether dioxane had dissociated the TPN-isocitrate linked diaphorase into a soluble form. A further possibility exists that the increased activity might be associated with smaller particles produced through fragmentation of the parent mitochondria. A stimulation of one activity (diaphorase) with simultaneous inhibition of a second

TABLE 2

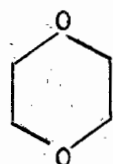
The Effect of Dioxane on Isocitrate Oxidation  
in Rat Liver Mitochondria with  
2,6-dichlorophenol-indophenol as Electron Acceptor

Cuvettes contained the following in a final volume of 3.0 ml: 0.10 M K,H<sub>2</sub>A-phosphate buffer (pH 7.4), 0.001 M KCN, 0.003 M MgCl<sub>2</sub>, 0.0023 M DL-isocitrate (K<sup>+</sup> salt, pH 7.4), 0.02 M nicotinamide,  $3.6 \times 10^{-5}$  M 2,6-dichlorophenol-indophenol, depleted mitochondria (1.05 mg. protein), DEN and TFN, 400 µg. each, TFNH (400 µg.), 0.19 M dioxane. Mitochondria were isolated in 0.44 M sucrose by the method of Dounce (60), twice washed in 0.25 M sucrose and depleted of pyridine nucleotides by incubation in 0.05 M sucrose + 0.03 M phosphate buffer (pH 7.4) at 30°C. for 60 minutes. They were finally suspended in 0.25 M sucrose and stored at 0°C.

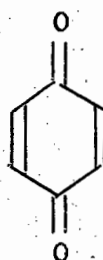
| System studied                   | Electron acceptor<br>2,6-dichlorophenol-indophenol     |
|----------------------------------|--------------------------------------------------------|
|                                  | $\Delta \text{OD}_{600 \text{ m}\mu} / 8 \text{ min.}$ |
| TFN + isocitrate                 | 0.380                                                  |
| TFN + isocitrate + dioxane       | 0.223                                                  |
| DEN + isocitrate                 | 0.016                                                  |
| DEN + isocitrate + dioxane       | 0.018                                                  |
| DEN + TFN + isocitrate           | 0.464                                                  |
| DEN + TFN + isocitrate + dioxane | 0.514                                                  |
| TFNH                             | 0.386                                                  |
| TFNH + dioxane                   | 0.482                                                  |

(cytochrome c reductase or oxidase) is analogous to the effect of desoxycholate on electron transfer in the Electron Transport Particle (ETP) discovered by Mackler and Green (31). This type of effect has also been observed by Slater (32, 33) working with a Keilen and Hartree heart muscle preparation. In these preparations a decrease in DHE-oxidase activity with a simultaneous increase in DHE-cytochrome c reductase activity was observed with increasing desoxycholate concentration.

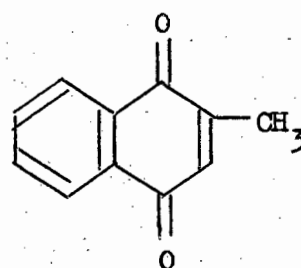
In structure, the dioxane molecule bears some resemblance to a quinone. However, unlike a quinone it possesses a saturated ring structure which is capable of assuming both boat and chair conformational structures. The two oxygen atoms in the 1 and 4 positions of the dioxane ring can function as nucleophilic centres because of their lone electron pairs.



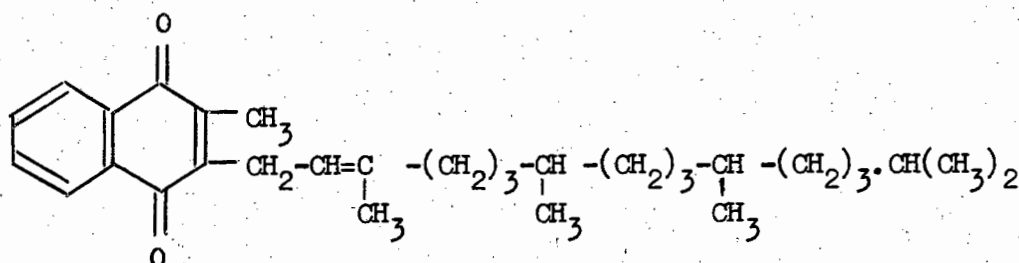
Dioxane



p-Benzoquinone



2-methyl-1,4-naphthoquinone  
(menadione)

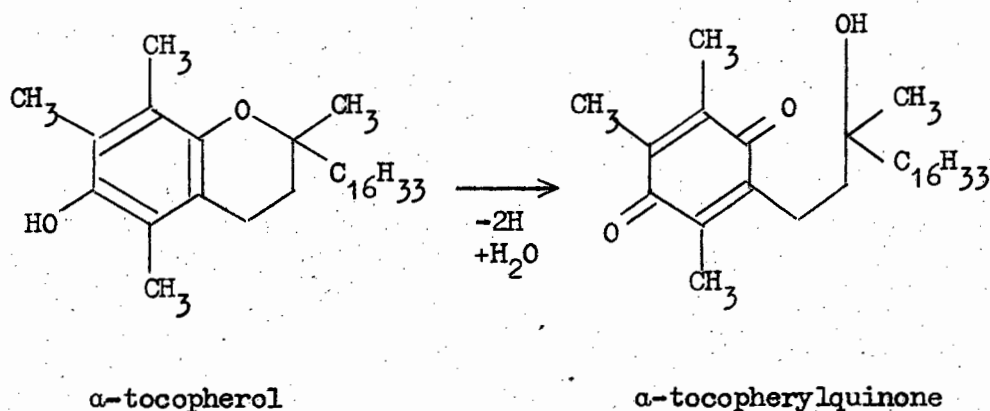


Vitamin K<sub>1</sub> (2-methyl-3 phytyl-1,4-naphthoquinone)

Certain of the vitamin K compounds have been shown to be involved in electron transport and photosynthetic phosphorylation occurring in plant chloroplasts (41, 42, 43). Ubiquinone, known as co-enzyme Q or  $Q_{275}$  and discovered independently by Morton and co-workers in England (44), and by Crene and associates at Wisconsin in the United States (45, 46, 47, 48), has been shown to act as a co-enzyme of electron transport in mitochondria. It is reversibly oxidised and reduced during electron transport in mitochondria, and also appears to be connected with oxidative phosphorylation.

Slater and Bauman (49) have isolated  $\alpha$ -tocopherol (vitamin E) in significant amounts from pig heart mitochondria. They state that:

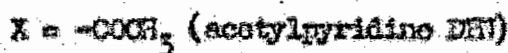
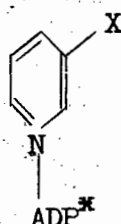
"The oxidation of  $\alpha$ -tocopherol to its quinone is not simply a removal of two hydrogen atoms (as for example with vitamin  $K_1$ ) but involves also the addition of a molecule of water and the opening of the chromen ring of  $\alpha$ -tocopherol." These reactions are shown below:



Dioxane may function as a type of quinone in electron transport. Alternatively, it may unmask activity through solubilisation of lipo-protein material. It would be of interest to test other compounds with a cyclic ether structure such as tetrahydrofuran, pyran and morpholine to simulate the dioxane effect.

## 5 THE EFFECT OF DIGITONIN ON ISOCITRATE OXIDATION

Digitonin has been used by Kaplan and co-workers (34, 37) to extract transhydrogenase activity in a non-sedimentable form from beef heart particles. These workers (35) have also observed that digitonin treatment of pigeon liver mitochondria causes a remarkable increase in the transhydrogenase activities (1) and (2).



<sup>\*</sup>The ADP ribosyl moiety of DEH

Vignais and Vignais (36) have reported similar effects of digitonin on certain enzymatic reactions in mitochondria.

### 5.1 Method

Pyridine nucleotide depleted mitochondria were prepared as described in the Experimental Section, and treated with 0.5% digitonin in 0.12 M sucrose (pH 7.4) at 0°C. for 20 minutes. They were then stored at 0°C. and used as quickly as possible. This treatment causes considerable solubilisation of the mitochondria as judged by clearing of a particulate suspension. The activity of digitonin treated mitochondria was assayed in the standard system given in Fig. 1, with 2,6-dichlorophenol-indophenol as electron acceptor.

## 5.2 Diaphorase Activities of Digitonin Treated Mitochondria

The curves in Fig. 7 show a stimulation of diaphorase activity associated with DPN-isocitrate oxidation, comparable to that obtained by dioxane treatment. TPN-isocitrate oxidation is, however, unaffected by digitonin treatment in contrast to the result found with dioxane. From these results it may be concluded that digitonin and dioxane produce different effects on Ehrlich ascites tumour mitochondria.

It is of interest to note that Lehninger and his co-workers (38, 39, 40) have had some success in preparing sub-mitochondrial fragments of rat liver mitochondria by digitonin treatment. These fragments are capable of oxidising D- $\beta$ -hydroxybutyrate with coupled phosphorylation.

## 6 VARIATION OF ISOCITRIC DEHYDROGENASE ACTIVITY WITH pH

### 6.1 Method

The isocitric dehydrogenase activity of pyridine nucleotide depleted mitochondria was assayed in tris-acetate buffer of different pH values, with 2,6-dichlorophenol-indophenol as electron acceptor.

### 6.2 DPN-Isocitric Dehydrogenase

Fig. 8 illustrates the effect of pH on diaphorase activity associated with DPN + isocitrate oxidation. Optimum activity occurred at pH 7.2, with a fall in rate on either side of this value.

### 6.3 TPN-Isocitric Dehydrogenase

The effect of pH on diaphorase activity associated with TPN + isocitrate oxidation is shown in Fig. 9. The reaction has an optimum pH of 6.2 with a point of inflection at pH 7.8. The optimum activity at pH 6.2 is approximately two-thirds that of the DPN-linked system at pH 7.2.

## 7 DIAPHORASE ACTIVITIES IN PHOSPHATE AND TRIS-ACETATE BUFFERS

Diaphorase activities assayed in phosphate and tris-acetate buffers are shown in Table 3. Reactions were carried out in 0.1 M buffers (pH 7.4) with 2,6-dichlorophenol-indophenol as terminal electron acceptor. When tris-acetate was employed as the buffer system, diaphorase activities were inhibited by approximately 50% in both the DPN and TPN-linked isocitrate oxidations. The reason for this lowering of activity in tris-acetate buffer is not known.

## 8 EFFECT OF AGEING ON DPN AND TPN-LINKED ISOCITRATE OXIDATION

### 8.1 Method

Pyridine nucleotide depleted mitochondria were aged for 84 hours at 0° in 0.25 M sucrose + 0.03 M phosphate buffer, pH 7.4. Aliquot portions of the mitochondrial suspension were removed for assay after 1, 24, 50, 72 and 84 hours storage. Activity was measured in the standard system of Fig. 1 with 2,6-dichlorophenol-indophenol as electron acceptor.

### 8.2 Effect of ageing on isocitrate oxidation

Results in Table 4 show that the DPN-linked isocitrate dehydrogenase activity, measured as diaphorase, dropped approximately 70% on 84 hours storage at 0°, while that of the TPN-linked enzyme system remained practically unchanged. Ageing experiments thus established a distinction between the two dehydrogenase systems.

in rate after two minutes. Isocitrate + TFH gave a much slower rate of reduction. However, when both DPH and TFH were present together with isocitrate, cytochrome c reduction was even more rapid than that observed with isocitrate + DPH. The latter result is in contrast to that observed with 2,6-dichlorophenol-indophenol as electron acceptor, and would suggest that there are separate DPH and TFH-linked isocitric dehydrogenases functioning in Ehrlich ascites mitochondria. Externally added TFH was oxidised at a greater rate than TFH generated by isocitrate oxidation, in conformity with the results obtained using 2,6-dichlorophenol-indophenol as electron acceptor.

#### 3.4 Results with Oxygen as Terminal Electron Acceptor

Curves obtained manometrically with oxygen as electron acceptor are shown in Fig. 3. A high rate of oxygen uptake was observed with DPH as co-factor, but in the presence of DPH and TFH together the rate was decreased to some extent. The lowest rate of oxygen uptake was observed with TFH as co-factor. Although respiration was measured in a medium capable of supporting oxidative phosphorylation, the rate of oxygen uptake decreased considerably after twenty-five minutes in all experiments.

#### 3.5 Discussion

##### Co-factor requirement of isocitric dehydrogenase

The experimental results with all electron acceptors are clearly different from those of Purvis (11) and Kaplan(9), and indicate the existence of both DPH and TFH-linked isocitric dehydrogenase systems in the mitochondria of the Ehrlich ascites tumour. However, Ernster and Navazio (29) state that in rat liver mitochondria, isocitrate is oxidised by two enzymes, a DPH and TFH-specific isocitric dehydrogenase. They also conclude

that the former is exclusively in the mitochondria, while the latter is distributed between mitochondria and the soluble fraction of the cytoplasm in a rough proportion of 1:9.

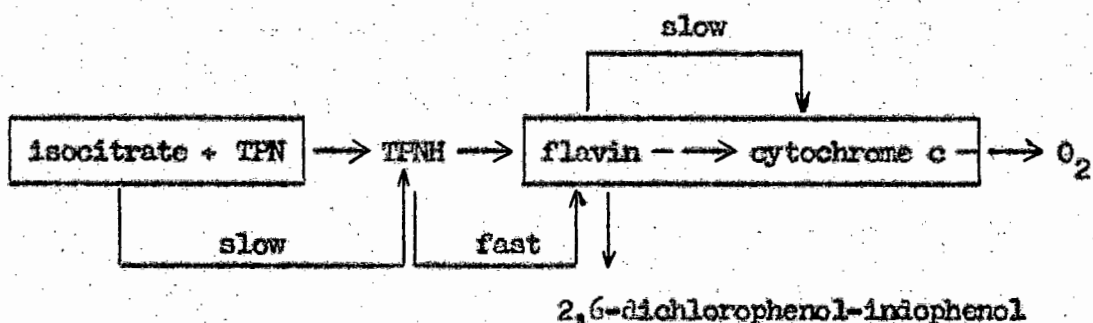
Grant and Longkolkul (27) have reported the presence of both DPN and TPN-dependent isocitric dehydrogenases in ox-adrenal mitochondria. Other naturally occurring enzymes are known which can function with either DPN or TPN as co-enzyme. For example, Hochster and Katznelson (28) have shown that extracts of the plant pathogenic organism Xanthomonas phaseoli contain both DPN and TPN-linked glucose-6-phosphate dehydrogenases.

#### Pyridine nucleotide transhydrogenase

The absence of this enzyme from Ehrlich ascites tumour mitochondria is clearly illustrated by the results presented in Fig. 1. This is in contrast to the findings with liver mitochondria and heart muscle sarcosomes which have been shown to contain a transhydrogenase (9, 11).

#### Rates of electron transfer

Examination of the experimental curves in Figs. 1, 2 and 3, shows that the rate of oxidation of TPNH is almost as rapid as that observed for isocitrate in the presence of DPN using 2,6-dichlorophenol-indophenol as electron acceptor. However, the rates of oxidation of these substrates with cytochrome c as terminal electron acceptor are vastly different, suggesting the existence of a second rate-limiting step between flavin and cytochrome c in the electron transfer pathway operating between TPN-isocitric dehydrogenase and oxygen. A diagram illustrating these observations is shown below:



As previously mentioned, a rate-limiting step also occurs at the TPNH-isocitric dehydrogenase level (sub-section 3.2).

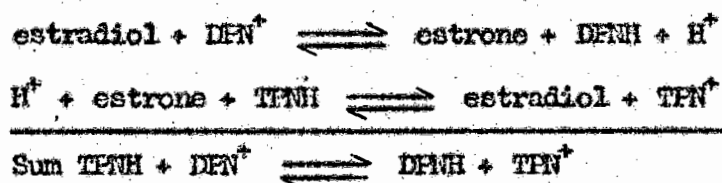
#### TPNH-cytochrome c reductase

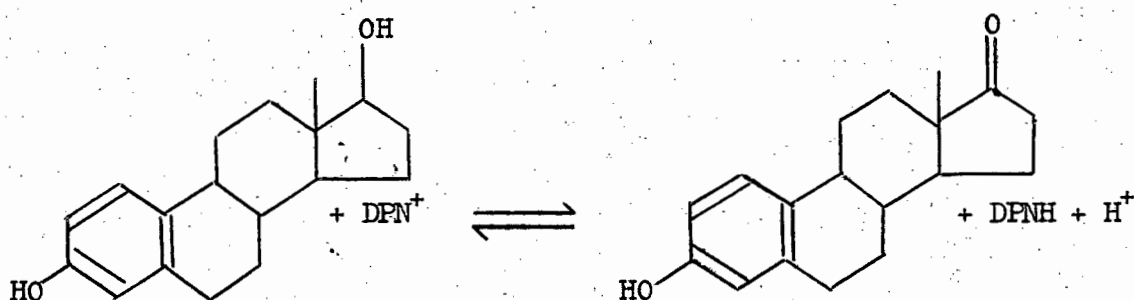
Studies of the oxidation of externally added TPNH have shown that Ehrlich ascites tumour mitochondria possess TPNH-cytochrome c reductase activity (Fig. 2). Novikoff and his co-workers (15) have reported the presence of a TPNH-cytochrome c reductase in an ascites form of the Novikoff hepatoma. They find this activity to be present in the solid tumour as well.

#### 4 STUDIES OF THE EFFECT OF DIOXANE ON ISOCITRATE OXIDATION IN EHRlich ASCITES TUMOUR CELL MITOCHONDRIA

Talalay and co-workers (30) have shown that the high speed supernatant fraction of rat liver homogenate contains a series of enzymes which catalyse the transfer of hydrogen between DPN and TPN through a series of reactions involving the steroids estrone and estradiol.

This transhydrogenase reaction is shown below:





In this series of coupled reactions the steroid functions as a co-enzyme for the transfer of hydrogen between DPN and TPN. Although the steroid-dependent transhydrogenase enzymes have been found in the soluble supernatant fraction of homogenates, the possibility exists that a steroid such as estradiol, might also function as a component of the isocitric dehydrogenase - pyridine nucleotide transhydrogenase enzyme complex which is found in mitochondria. Experiments were accordingly undertaken to test this hypothesis.

It was found that the steroids estrone and estradiol were without effect on isocitrate oxidation in Ehrlich ascites tumour mitochondria. However, the organic compound dioxane used as solvent for these substances, stimulated TPN-dependent isocitrate diaphorase activity to a remarkable extent. This effect of dioxane was further investigated as follows:

#### 4.1 Method

Pyridine nucleotide depleted Ehrlich ascites tumour mitochondria were prepared as described in the Experimental Section. Assays with different electron acceptors were carried out as described for the co-factor requirement experiments (sub-section 3.1).

#### 4.2 The Effect of Dioxane on Isocitrate Oxidation with 2,6-dichlorophenol-indophenol as Electron Acceptor

Curves in Figs. 4 and 5 show the effect of dioxane on diaphorase activities associated with isocitrate oxidation using 2,6-dichlorophenol-indophenol as electron acceptor. Dioxane was added to a final concentration of 0.19 M. The rate of dye reduction was increased approximately 250% when dioxane was present in the TPN-isocitrate reaction. The DPN-isocitrate reaction was also stimulated, but only by about 35 - 40% under these conditions.

#### 4.3 The Effect of Dioxane on Isocitrate Oxidation with Cytochrome c and Oxygen as Electron Acceptors

In experiments with either cytochrome c or oxygen as electron acceptor, dioxane (0.19 M) inhibited electron transfer resulting from isocitrate oxidation. Table 1 shows that this inhibition was observed with either DPN or TPN as co-factor.

#### 4.4 The Effect of Dioxane Concentration on Electron Transport

Figure 6 shows the effect of increasing dioxane concentration on TPN-isocitrate oxidation with both 2,6-dichlorophenol-indophenol and molecular oxygen as electron acceptors. A concentration of 0.19 M dioxane caused both maximum stimulation of 2,6-dichlorophenol-indophenol reduction, and maximum inhibition of oxygen uptake. These results suggest that dioxane probably reacts with the electron transfer chain at a single locus.

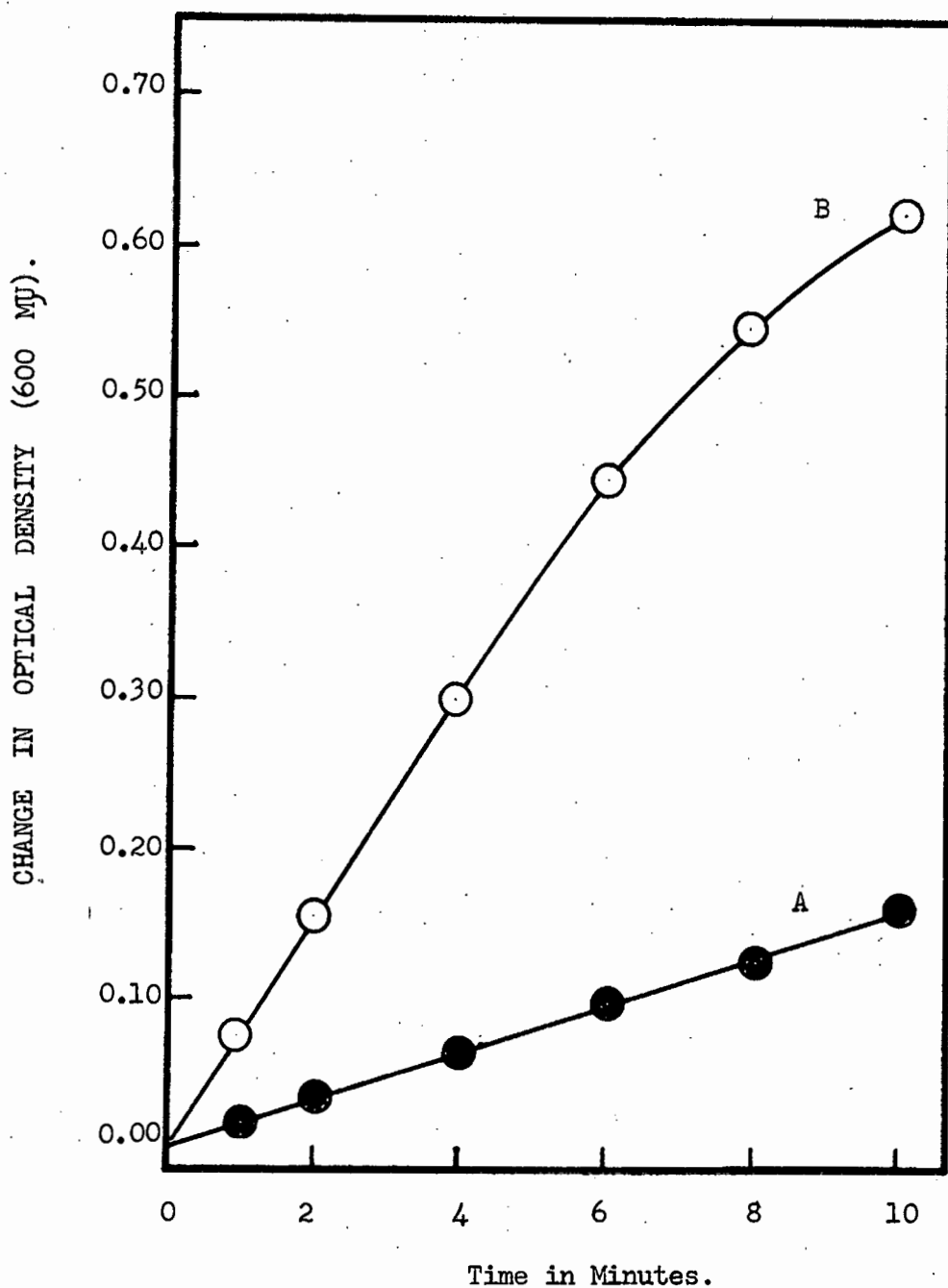


Fig. 4. TPN-Isocitric Diaphorase. Stimulation of the rate of 2,6-dichlorophenolindophenol reduction by Dioxane.

Reaction mixture the same as in Fig. 1. Curve A, control with TPN (400 μg) and DL-isocitrate, 0.0023 M. Curve B, as for Curve A plus 0.19 M dioxane. The dioxane was added initially to both Beckman cuvettes. Each cuvette contained 0.20 mg protein.

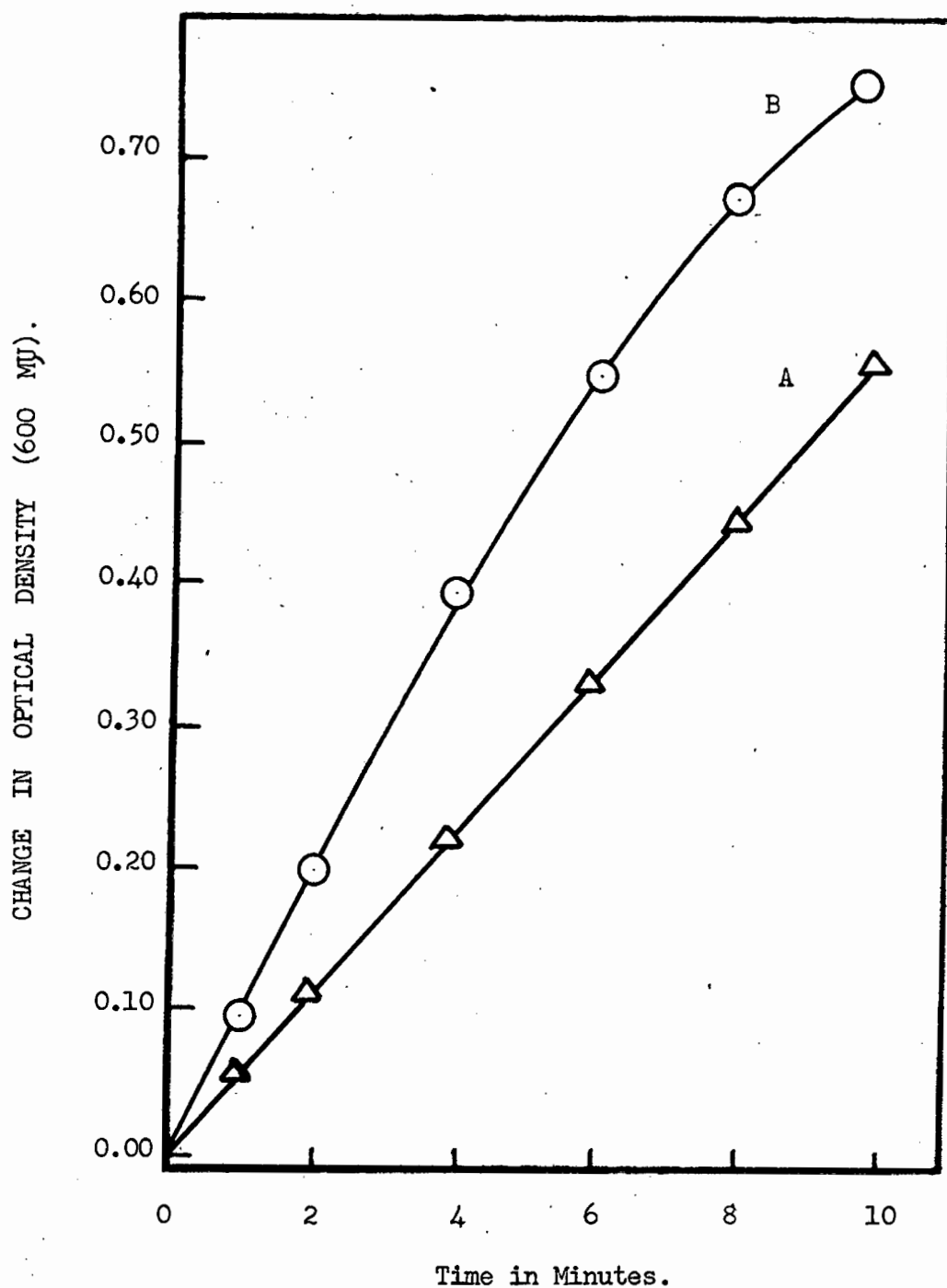


Fig. 5. DPN-Isocitric Diaphorase. Stimulation of the rate of 2,6-dichlorophenolindophenol reduction by Dioxane.

Reaction mixture as in Fig. 1. Curve A, control with DPN (1000  $\mu$ g) and DL-isocitrate, 0.0023 M, pH 7.4. Curve B, same as Curve A plus 0.19 M dioxane. Dioxane was added to both Beckman cuvettes each of which contained 0.20 mg protein.

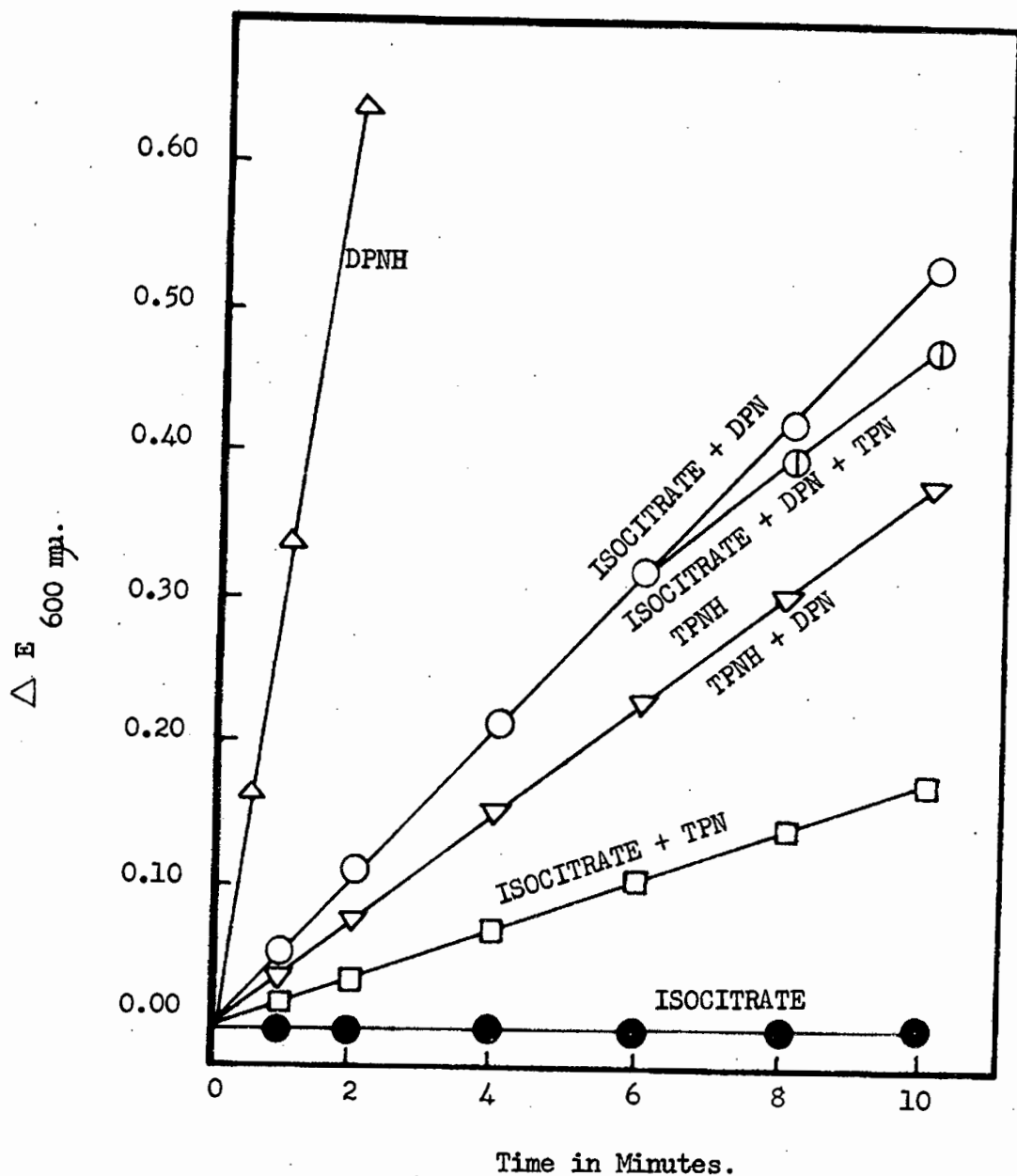


Fig. 1. Reduction of 2,6-dichlorophenolindophenol by pyridine-nucleotide depleted Ehrlich ascites tumour cell mitochondria.

Reaction mixture in a final vol of 3.0 ml: K,Na phosphate buffer (pH 7.4), 0.10 M; KCN, 0.001 M;  $MgCl_2$ , 0.003 M; DL-isocitrate ( $K^+$  salt), 0.0023 M; nicotinamide, 0.02 M; 2,6-dichlorophenolindophenol, 50  $\mu$ g; depleted Mitochondria (0.26 mg protein); DPN and TPN, 1000  $\mu$ g each; DPNH, generated from DPN (1000  $\mu$ g) by addition of ethanol (0.04 ml) and alcohol dehydrogenase (0.5 mg) to the reaction mixture; TPNH, generated from TPN (1000  $\mu$ g) by addition of glucose-6-phosphate (0.0017 M) and glucose-6-phosphate dehydrogenase (1.0 mg) to the reaction mixture.

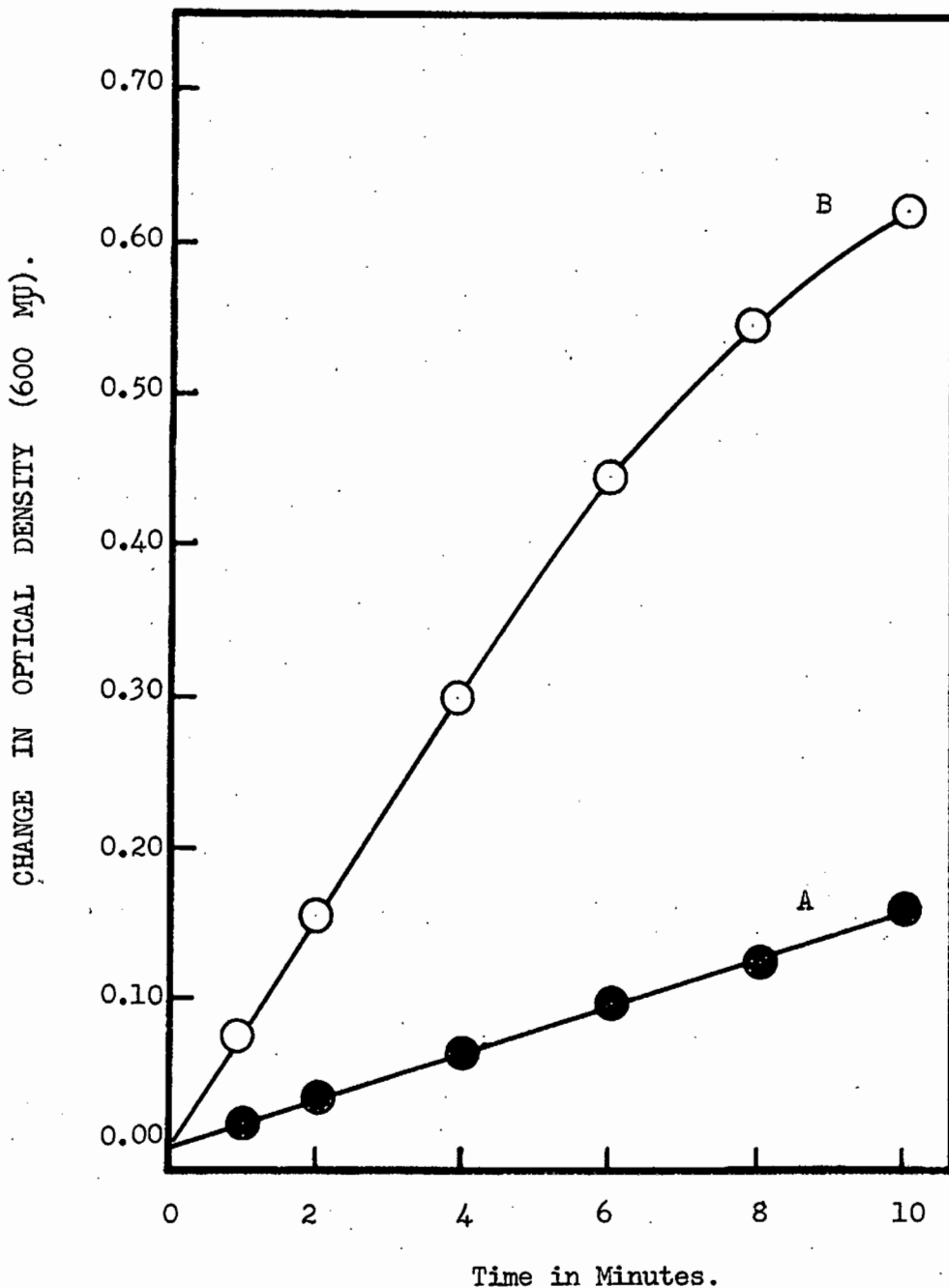


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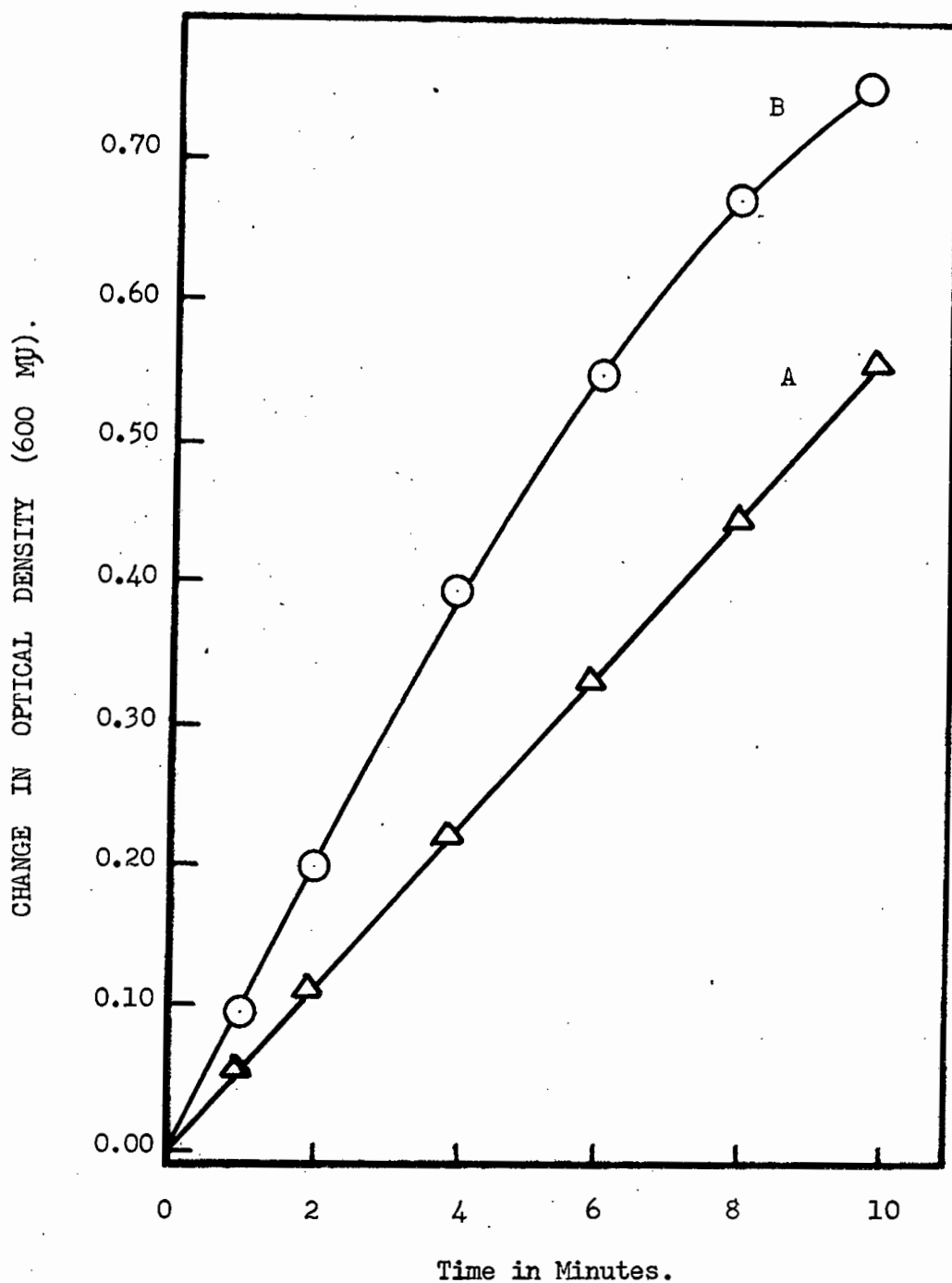


Fig. 5. DPN-Isocitric Diaphorase. Stimulation of the rate of 2,6-dichlorophenolindophenol reduction by Dioxane.

Reaction mixture as in Fig. 1. Curve A, control with DPN (1000  $\mu$ g) and DL-isocitrate, 0.0023 M, pH 7.4. Curve B, same as Curve A plus 0.19 M dioxane. Dioxane was added to both Beckman cuvettes each of which contained 0.20 mg protein.

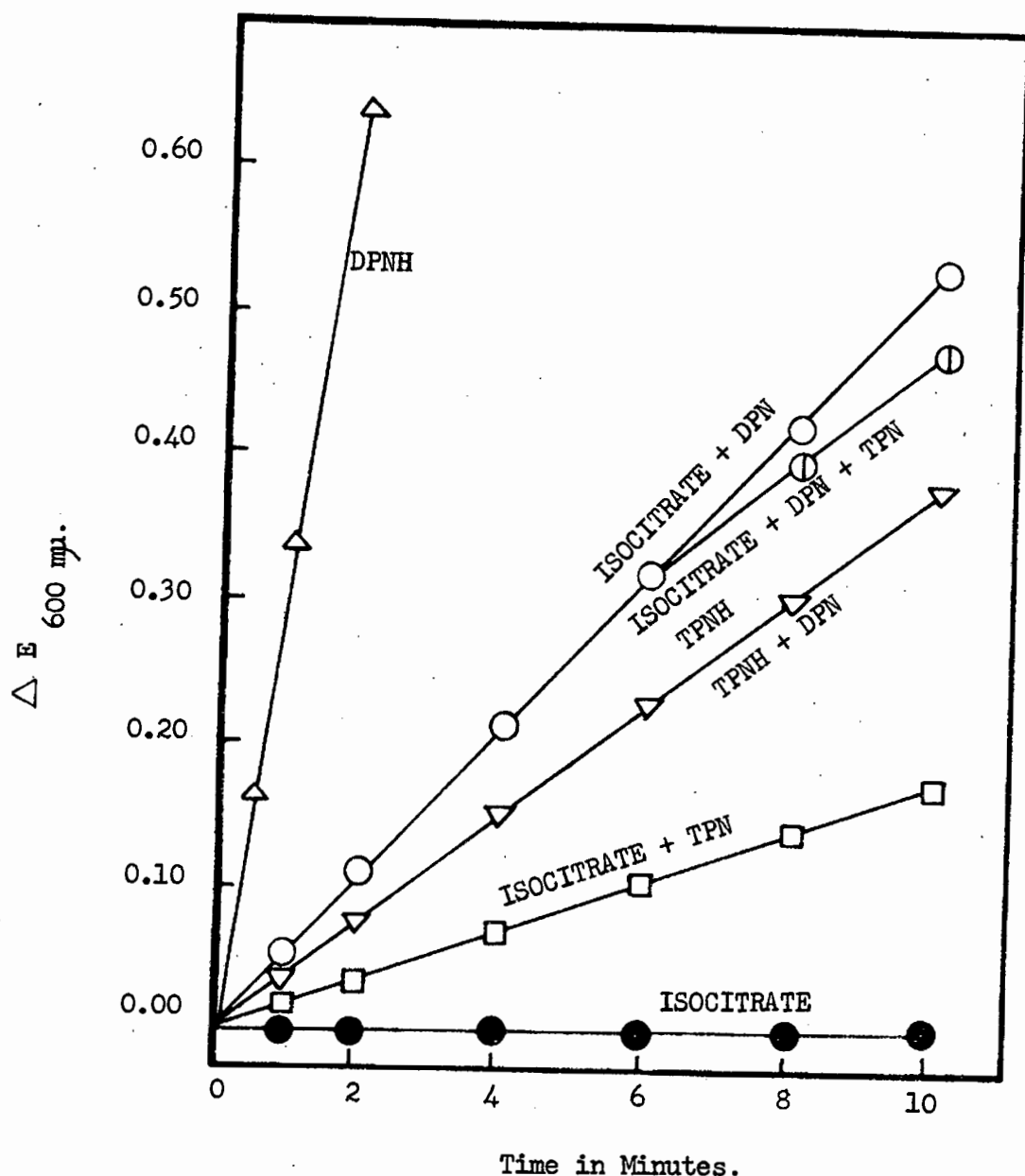
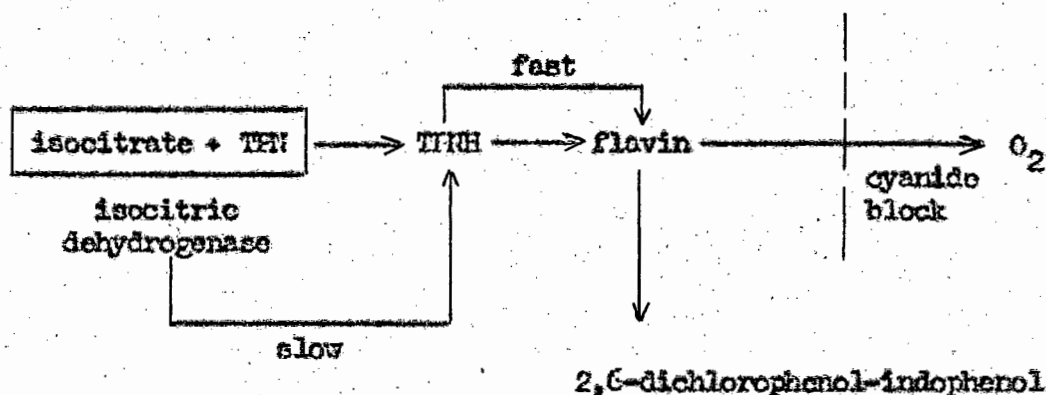


Fig. 1. Reduction of 2,6-dichlorophenolindophenol by pyridine-nucleotide depleted Ehrlich ascites tumour cell mitochondria.

Reaction mixture in a final vol of 3.0 ml: K,Na phosphate buffer (pH 7.4), 0.10 M; KCN, 0.001 M;  $MgCl_2$ , 0.003 M; DL-isocitrate ( $K^+$  salt), 0.0023 M; nicotinamide, 0.02 M; 2,6-dichlorophenolindophenol, 50  $\mu g$ ; depleted Mitochondria (0.26 mg protein); DPN and TPN, 1000  $\mu g$  each; DPNH, generated from DPN (1000  $\mu g$ ) by addition of ethanol (0.04 ml) and alcohol dehydrogenase (0.5 mg) to the reaction mixture; TPNH, generated from TPN (1000  $\mu g$ ) by addition of glucose-6-phosphate (0.0017 M) and glucose-6-phosphate dehydrogenase (1.0 mg) to the reaction mixture.

was fairly rapidly oxidised, but at a much slower rate than DFH. It is noteworthy that oxidation of external THH proceeded at approximately twice the rate of the reaction with isocitrate plus THH. This would indicate that the rate limiting step in the sequence of reactions,



is the primary isocitric dehydrogenase reaction.

In these experiments the assumption was made that electron transfer resulting from both externally added and isocitric dehydrogenase generated THH occurred via the same pathway. The results obtained with THH and THH + DFH confirm the absence of pyridine nucleotide transhydrogenase in Ehrlich ascites tumour cell mitochondria. The DFH-dependent isocitric dehydrogenase activity was approximately three to four times that of its THH-counterpart when assayed in phosphate buffer (pH 7.4), with 2,6-dichlorophenol-indophenol as electron acceptor.

### 3.3 Results with Cytochrome c as Terminal Electron Acceptor

With cytochrome c as electron acceptor, results similar to that found with 2,6-dichlorophenol-indophenol as acceptor were obtained, with the exception of the result with isocitrate + DFH + THH, where the rate was found to be faster than that with isocitrate + DFH. Fig. 2 shows the curves obtained with cytochrome c as electron acceptor. With isocitrate + DFH, reduction of cytochrome c was initially rapid, followed by a considerable decrease

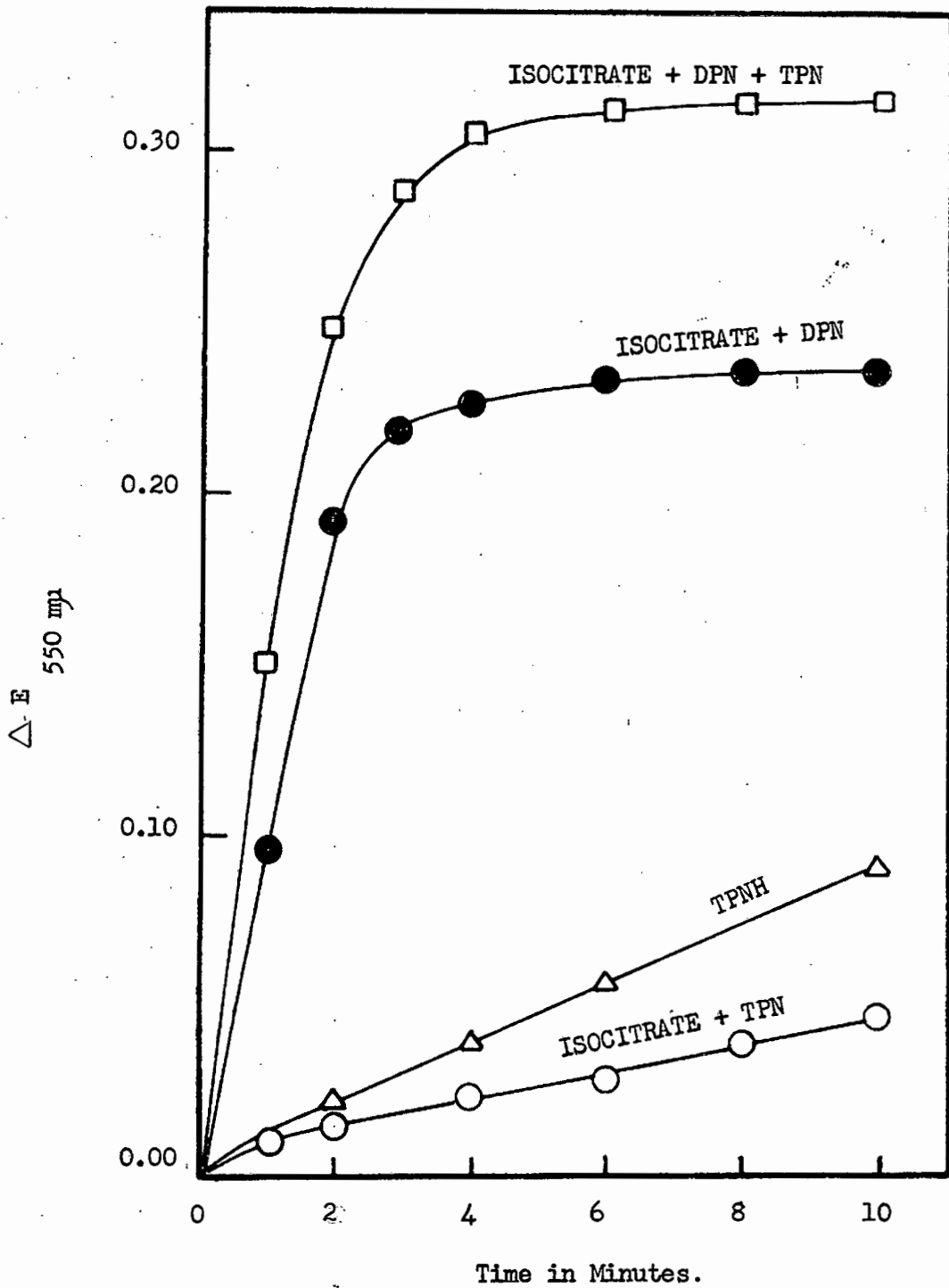


Fig. 2. Reduction of cytochrome c by pyridine-nucleotide depleted Ehrlich ascites tumour cell mitochondria.

Reaction mixture in a final vol of 3.0 ml:- As in Fig. 1 except that cytochrome c (2 mg) was used as electron acceptor in place of 2,6-dichlorophenolindophenol. Depleted mitochondria (0.26 mg protein).

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## CHAPTER IV

### STUDIES ON ISOCITRATE OXIDATION

#### III EHRLICH ASCITES CELL MITOCHONDRIA

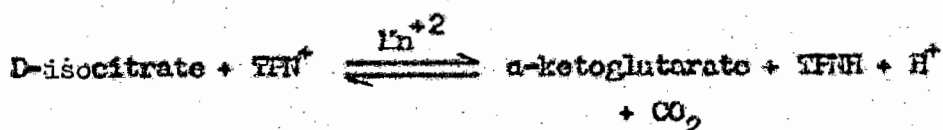
#### INTRODUCTION

##### 1 ISOCITRATE OXIDATION IN MITOCHONDRIA

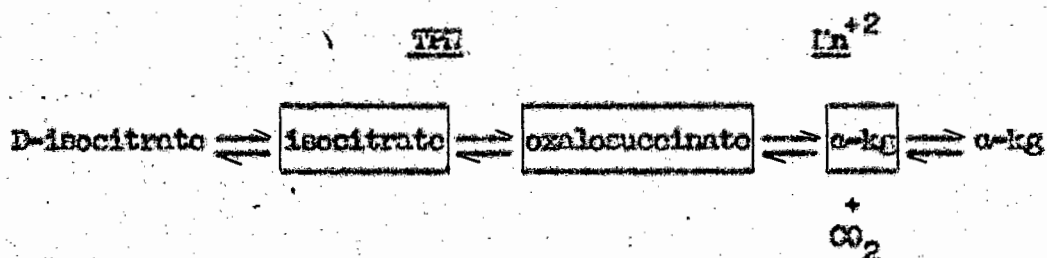
Very few detailed investigations have been reported concerning isocitrate oxidation in tumour mitochondria. Most research on tumours has concerned the formation of reduced triphosphopyridine nucleotide from the oxidations of the hexose-monophosphate shunt pathway.

It is generally assumed that the co-enzyme of isocitric dehydrogenase is TPN (1, 2). This assumption appears to be valid for most normal mammalian tissues such as heart, liver, muscle and spleen. However, Plant and Sung (3, 4) state that in a number of animal tissues a diphosphopyridine nucleotide-dependent isocitric dehydrogenase is also present. The tissues examined included guinea-pig, beef, pigeon heart, pigeon breast muscle, rat kidney and rat liver. In extracts of beef and guinea-pig heart there was found to be ten to thirty times as much TPN-dependent as DPN-dependent isocitric dehydrogenase. It may therefore be concluded that in certain animal tissues both dehydrogenases are present. Various yeasts also possess both DPN and TPN-dependent isocitric dehydrogenases which can be separated from each other (5, 6).

The isocitric dehydrogenase reaction is represented as:

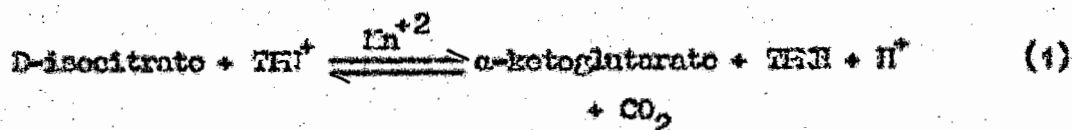


Jennifer L'oye and H. Dixon (7, 8) in a recent investigation of the isocitric dehydrogenase enzyme obtained from acetone powders of pig heart, have shown that both isocitric dehydrogenase and oxalosuccinic decarboxylase activities are the property of a single enzyme, termed by them the "Isocitric Enzyme". Their particular enzyme was shown to be  $\text{Th}^{+}$ -specific. The mechanism of isocitric dehydrogenase action is postulated as follows:



where  $\boxed{\phantom{\text{isocitrate}}}$  = enzyme-substrate complex.

Interest in the problem of  $\text{DH}^{+}$  and  $\text{Th}^{+}$  pathways was stimulated by Kaplan and co-workers (9) who discovered a pyridine nucleotide transhydrogenase enzyme system in the mitochondria of certain animal tissues such as liver and kidney. They were able to demonstrate that isocitrate oxidation in rat liver mitochondria is  $\text{Th}^{+}$ -specific, and that there was an increase in the rate of substrate oxidation when both  $\text{Th}^{+}$  and  $\text{DH}^{+}$  were present together as co-factors. The increased rate of oxidation was shown to be due to the presence of a transhydrogenase which converted  $\text{Th}^{+}$  to  $\text{Th}^{+}$  with a simultaneous reduction of  $\text{DH}^{+}$  to  $\text{DTh}^{+}$ , followed by oxidation of the latter via a cytochrome system with oxygen as the terminal electron acceptor. These reactions are as follows:



It should be noted that the TPNH produced by the isocitric dehydrogenase reaction is not directly oxidised via a cytochrome pathway of enzymes linked to oxygen, but functions instead as substrate for the transhydrogenase enzyme as shown in equation (2). Phosphorylation of ADP to ATP occurs during the oxidation of DPNH via the cytochrome series of enzymes with oxygen as terminal electron acceptor, but it is doubtful whether any phosphorylation occurs at the primary dehydrogenase or transhydrogenase level. Kaplan's experiments (9) indicated the complete absence of DPNH-dependent isocitric dehydrogenase activity in rat liver mitochondria.

Investigations by Purvis (10, 11) in Slater's laboratory have supported and verified the results obtained by Kaplan and his co-workers. Purvis studied the isocitric dehydrogenase co-factor requirement of rat liver mitochondria and heart muscle sarcosomes by completely depleting the isolated mitochondria of their endogenous pyridine nucleotides. TPN-specificity was clearly demonstrated for the isocitric dehydrogenase reaction in both types of mitochondria. Purvis's experiments also established the presence of the transhydrogenase enzyme in these mitochondria.

The transhydrogenase enzyme system in mitochondria is of importance since it probably serves to maintain an equilibrium between energy-yielding phosphorylations and the generation of active hydrogen for reductive synthesis in the cytoplasm of the cell. The enzyme has been found in mitochondria of liver, heart, muscle and kidney, and at lower concentrations in brain, spleen and testes (12). It has not been detected in cells of the Ehrlich carcinoma (13) or Novikoff hepatoma (14). The lack of a transhydrogenase in these two tumours is significant, and would suggest that the TPN-dependent isocitric dehydrogenase in tumour mitochondria may be linked directly to oxygen via a cytochrome pathway.

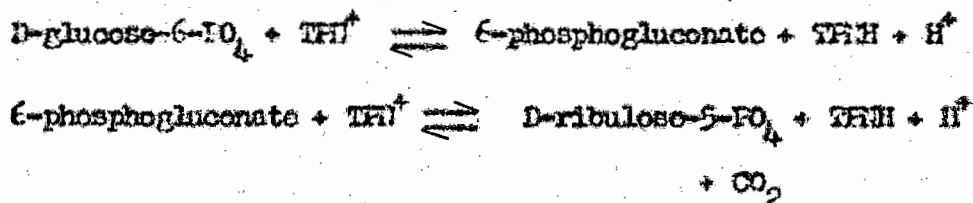
## CHAPTER IV

### STUDIES ON ISOCITRATE OXIDATION

#### IN EHRLICH ASCITES CELL MITOCHONDRIA

2 THE HEXOSE-MONOPHOSPHATE  
SHUNT PATHWAY IN TUMOURS

Reduced triphosphopyridine nucleotide is known to be produced in the oxidation of isocitrate at the mitochondrial level. Synthesis of this important compound also occurs in the soluble fraction of the cytoplasm as a result of oxidative degradation of glucose by the enzymes of the hexose-monophosphate shunt pathway. The important reactions producing TPNH in this pathway, are the oxidation of D-glucose-6-phosphate and 6-phosphogluconate which are shown below.



The reduced triphosphopyridine nucleotide produced in these reactions may either be used for reductive synthesis in the cell, or transferred to the mitochondria where it is oxidised with the production of ATP.

Dosch, van Vals and Emmelot (16, 17), Glock and McLean (18, 19) and many other workers, have demonstrated that tumours possess an active hexose-monophosphate shunt pathway. Glock and McLean have also measured the amounts of pyridine nucleotides present in a variety of tumours and found TPN to be present at an extremely low level. They state that:

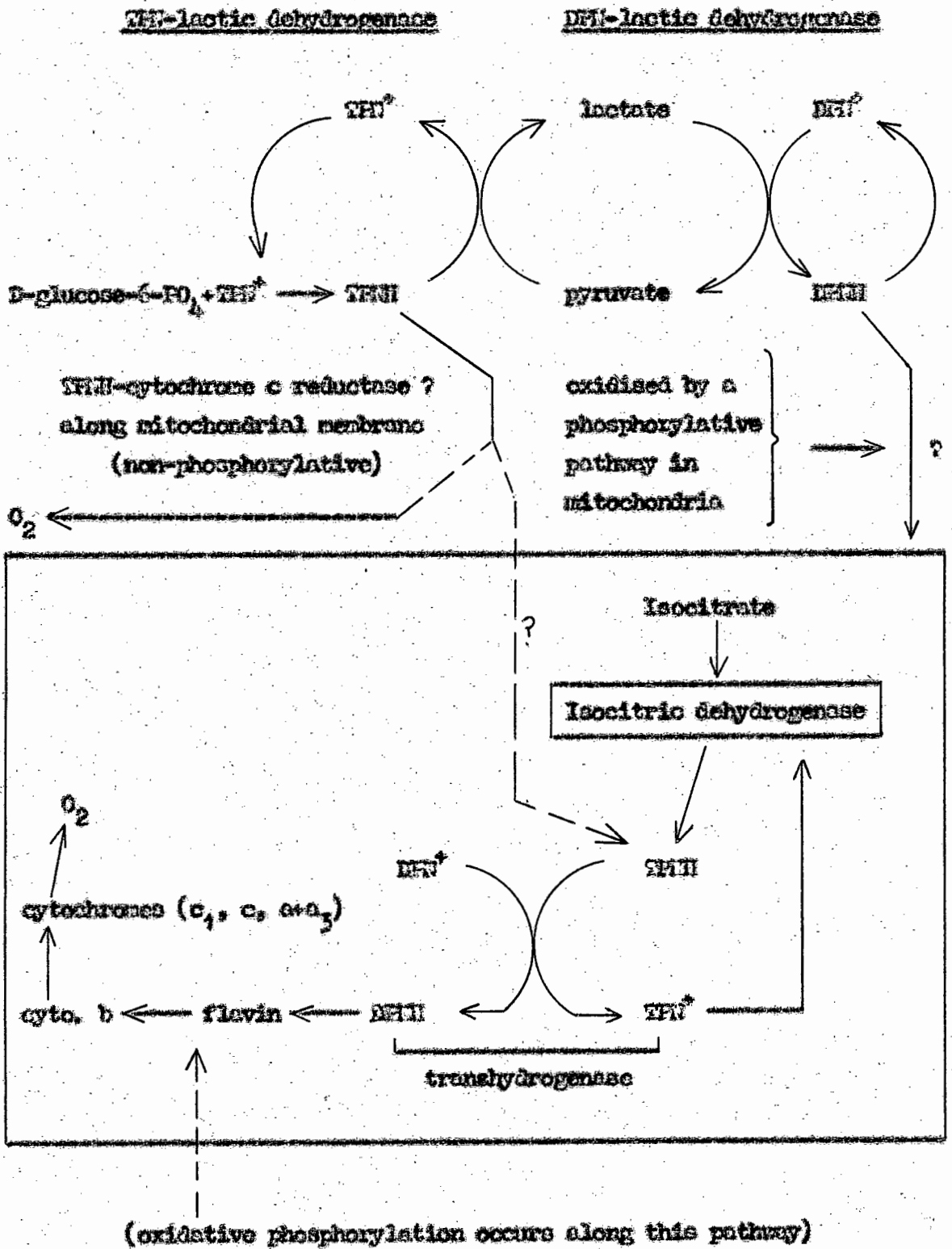
"Previous work on the pyridine nucleotide content of normal tissues indicated that the total TPN contents showed a rough positive correlation with levels of activity of the TPN-dependent 6-phosphogluconate oxidative pathway. This correlation does not seem to apply to tumours, however, since glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities of a variety of tumours were found to fall within the range of normal tissues, although all the tumours used in the present investigation had very low TPN contents.

Additional evidence indicating an active 6-phosphogluconate pathway in neoplastic tissues is provided by experiments using labelled glucose" (20, 21, 22).

The operation of an active hexose-monophosphate shunt pathway in tumours is remarkable in that these reactions occur when the necessary co-factor, TPN, is present at a critically low level. It would thus appear that the rate-limiting factor in the operation of the shunt pathway may be the re-oxidation of TPNH. The nature of the natural enzymatic hydrogen acceptor for TPNH produced in shunt pathway oxidations is, however, unknown. Kit and Clark Griffin (12) have postulated that tumours possess a TPN-dependent lactic dehydrogenase which could couple with the enzymes of the shunt pathway and regenerate TPN. Wenner and associates (23, 24, 25, 26) have shown that a natural metabolite such as pyruvate can act as hydrogen acceptor for TPNH "in vitro". However, TPNH produced in shunt pathway oxidations may be oxidised via several different enzyme systems in normal cell mitochondria. In certain tumour cells, Wenner and his co-workers have failed to demonstrate TPNH oxidation with molecular oxygen as acceptor.

The much higher level of DPN by comparison with TPN in tumours would suggest that DPN may also serve as co-factor for the enzymes of the shunt pathway.

PATHWAY OF TPNH OXIDATION IN RAT LIVER CELLS



## 2 CO-FACTOR REQUIREMENT OF ISOCITRIC DEHYDROGENASE IN EHRLICH ASCITES TUMOUR CELL MITOCHONDRIA

### 3.1 Method

Ehrlich ascites tumour cell mitochondria were isolated and depleted of endogenous pyridine nucleotides as described in the Experimental Section. Co-factor requirement and specificity were studied by the addition of the appropriate co-enzyme, or mixture of co-enzymes to a reaction medium containing isocitrate, electron acceptor and other necessary reagents.

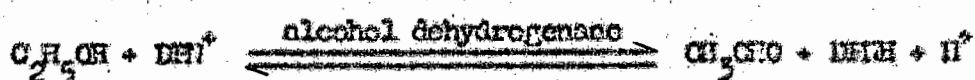
#### Media for determination of co-factor requirements

- (a) With 2,6-dichlorophenol-indophenol and cytochrome c as terminal electron acceptors; the following medium in a final volume of 3.0 ml. was used: K<sub>2</sub>Na phosphate buffer (pH 7.4), 0.10 M; KCl, 0.001 M; MgCl<sub>2</sub>, 0.003 M; DL-isocitrate (K<sup>+</sup> salt), 0.0025 M; nicotinamide, 0.02 M; 2,6-dichlorophenol-indophenol, 50 µg.; or cytochrome c (2 mg.); depleted mitochondria (0.26 mg. protein); DPN and TPN, 400 µg. each; DPNH, generated from DPN (400 µg.) by addition of ethanol (0.04 ml.) and alcohol dehydrogenase (0.5 mg.) to the reaction mixture; TPNH, generated from TPN (400 µg.) by addition of glucose-6-phosphate (0.0017 M) and glucose-6-phosphate dehydrogenase (1.0 mg.) to the reaction mixture.
- (b) With oxygen as terminal electron acceptor; the following medium in a final volume of 3.0 ml. was used: K<sub>2</sub>Na phosphate buffer (pH 7.4), 0.045 M; D-glucose, 0.034 M; KP, 0.015 M; MgCl<sub>2</sub>, 0.008 M; ADP, 0.0025 M; AMP, 0.001 M; ethylenediamine tetraacetic acid (pH 7.4), 0.002 M; KCl, 0.01 M; sucrose 0.067 M; DL-isocitrate, 0.0033 M; cytochrome c,  $9.8 \times 10^{-6}$  M; hexokinase (crystalline), 1.5 mg.; depleted mitochondria (2.02 mg. protein); DPN and TPN,  $1.2 \times 10^{-3}$  M. Temp. 30°.

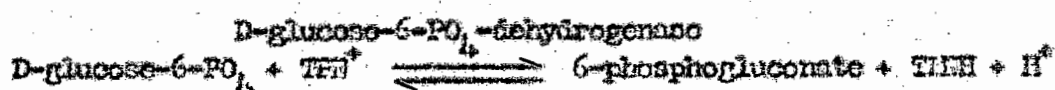
When either 2,6-dichlorophenol-indophenol or cytochrome c was employed as terminal electron acceptor, measurements were made with a Beckman spectrophotometer model DU at room temperature. With oxygen as electron acceptor, measurement of respiration was carried out by Warburg manometry.

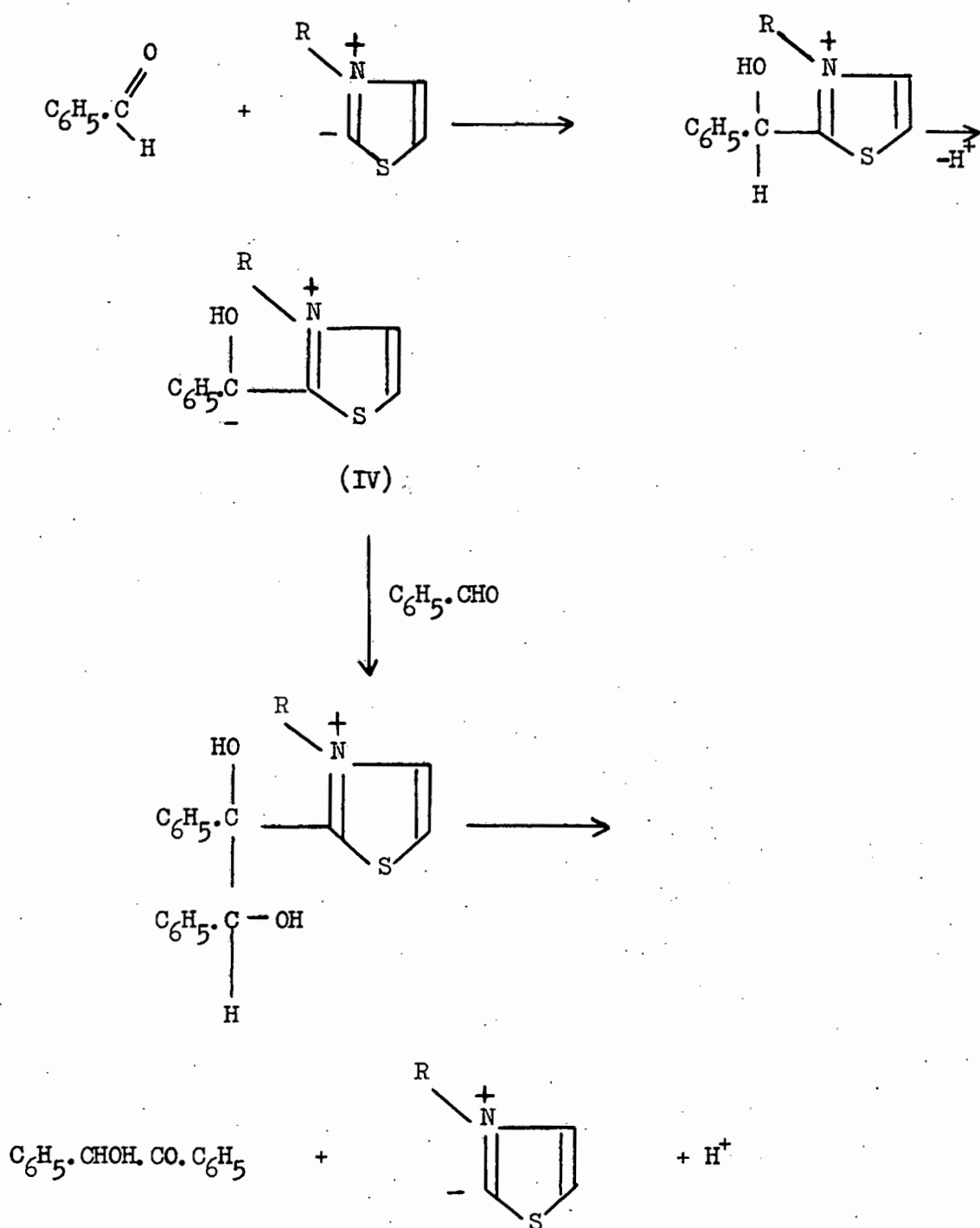
### 3.2 Results with 2,6-dichlorophenol-indophenol as Terminal Electron Acceptor

Graphs shown in Fig. 1 illustrate the rate of reduction of 2,6-dichlorophenol-indophenol as terminal electron acceptor. In the absence of added pyridine nucleotide (DPN or TPN), but in the presence of isocitrate no reduction of the dye occurred, as would be expected with pyridine nucleotide depleted mitochondria. This result confirms the absence of DPN and TPN associated with isocitrate oxidation in depleted mitochondria. Rapid dye reduction occurred with isocitrate + DPN, while a much slower rate of reduction was observed with TPN as co-factor. When isocitrate was fortified with both DPN and TPN together the rate of dye reduction was unchanged, from that of DPN alone, as co-factor. However, after some two-thirds the reaction time, a decline in rate was consistently observed. This may possibly be due to some type of competitive inhibition of the DPN-isocitric dehydrogenase by TPN. Reduced diphosphopyridine nucleotide (DPNH) produced through the alcohol dehydrogenase reaction as follows,



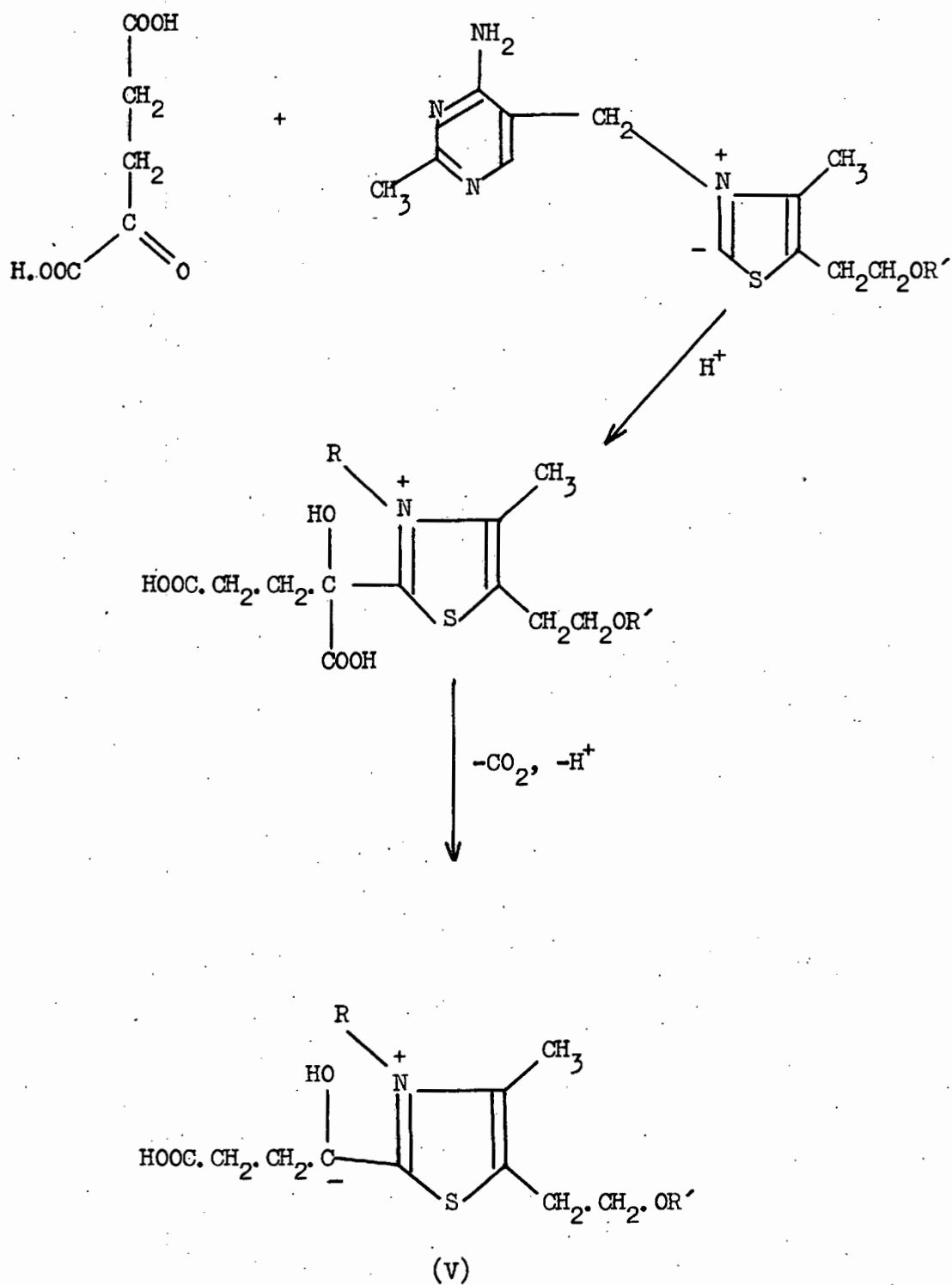
was rapidly oxidised with 2,6-dichlorophenol-indophenol as electron acceptor. Reduced triphosphopyridine nucleotide (TPNH) generated through the glucose-6-phosphate dehydrogenase reaction as follows,





When tested experimentally, thiazolium compounds did in fact catalyse the benzoin condensation. However, only those thiazolium salts with hydrogen at C-2 (unsubstituted) were found to be active.

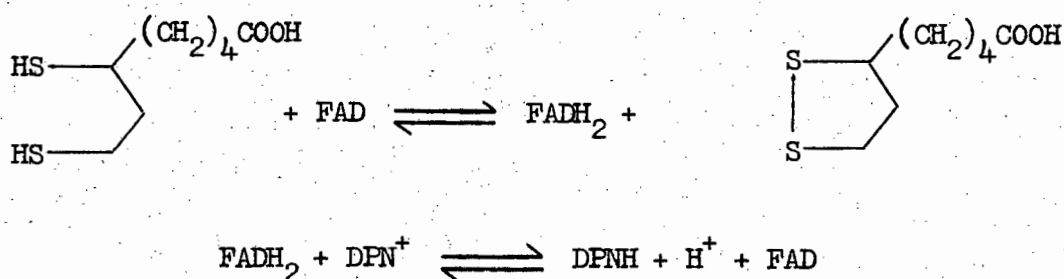
If this type of reasoning is applied to the decarboxylation of  $\alpha$ -ketoglutarate in its reaction with thiamine-pyrophosphate, the following scheme may be postulated:



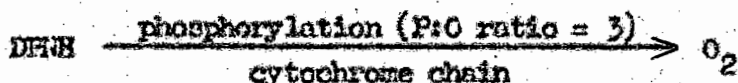
Compound (V) may be regarded as an active acyl carbanion intermediate which reacts with the oxidised form of lipoic acid.



Dihydro-lipoic acid (IX) reduces DPN, undergoing ring closure during the process. However, Massey (31) has recently shown that flavin adenine dinucleotide is a co-factor in this reaction, which must therefore occur as follows:

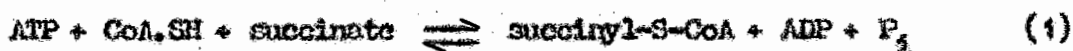


DPNH is oxidised by way of the cytochrome system with oxygen as terminal electron acceptor.



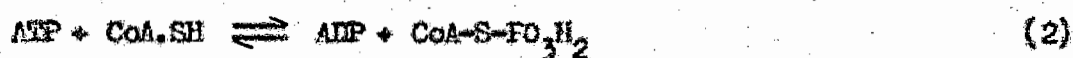
#### Phosphorylation at the substrate level

During the decarboxylation of  $\alpha$ -ketoglutarate, a number of complex reactions occur. The participation of thiamine-pyrophosphate and lipoic acid in these reactions has been discussed in detail. The further reaction of succinyl-S-CoA with GDP and inorganic phosphate to form GTP has, however, been only briefly commented upon. This latter reaction has been studied by Gunsalus and his co-workers (35), who have shown that in extracts of Escherichia coli the following series of reactions occur.

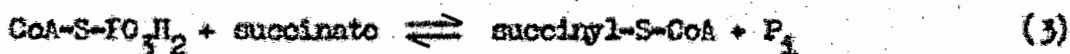


In E. coli, ATP replaces GTP of the mammalian system. Fractionation of the E. coli extracts has demonstrated the above reaction to be the sum of two enzymatic reactions shown below:

Co-enzyme A Thiolkinase



Phosphoryl CoA Transferase



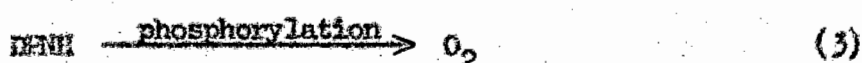
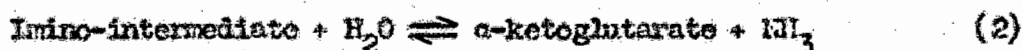
Presumably, a similar reaction mechanism is applicable to the mammalian enzyme system.

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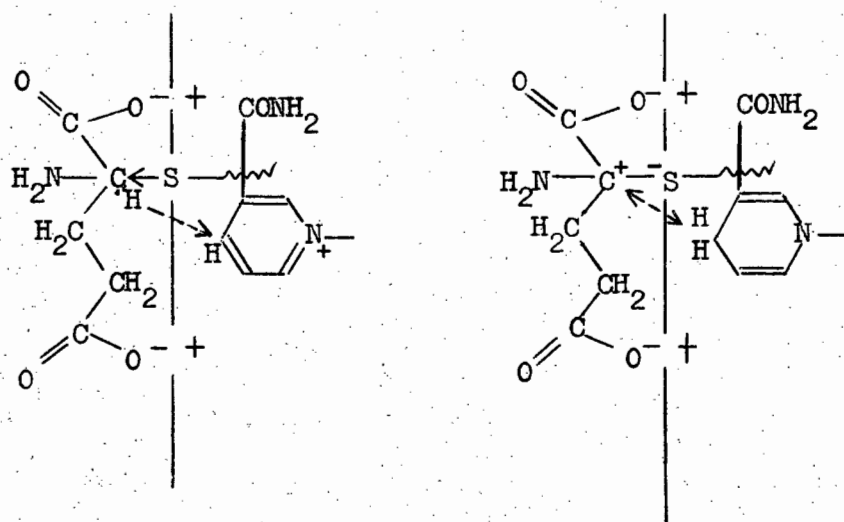
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Oxygen uptakes per mg. protein of various mitochondrial preparations with four different substrates are shown in Fig. 1. Curves are included to show the effect of IEN and nicotinamide additions in the oxidation of  $\alpha$ -ketoglutarate and L-glutamate.

The L-glutamate dehydrogenase reaction is shown below:



Recently a detailed investigation of the L-glutamic dehydrogenase reaction has been carried out by Dellerman and associates (18) using a highly purified enzyme preparation. Their postulated mechanism for the dehydrogenase reaction is shown below:



#### 4. THE EFFECT OF WASHING, ON YIELD AND OXIDATIVE PHOSPHORYLATION OF EHRICH ASCITES CELL MITOCHONDRIA

##### 4.1 Method

Mitochondria were isolated as previously described. They were washed either once or twice in 0.25 M sucrose and

finally resuspended in the same medium. Measurement of oxidative phosphorylation was carried out in Warburg manometers using the reaction medium shown in Table 3.

#### 4.2 Effect of Washing

An examination was made of the effect of washing upon yield and oxidative phosphorylation of Ehrlich ascites cell mitochondria. The results in Table 3 indicate that once washed mitochondria have a low protein content and give unsatisfactory P:O ratios with  $\alpha$ -ketoglutarate and succinate as substrates. The oxygen uptake per mg. protein of once washed mitochondria is, however, comparable with that of twice washed mitochondria, and is even higher in the case where succinate is used as substrate. The yield of twice washed mitochondria is approximately 50% that of once washed material, and represents 4 - 6% of the original wet weight of cell material.

A second washing of the mitochondria with 0.25 M sucrose ensures almost complete removal of material which appears as lipid droplets under the phase contrast microscope. Double washing also ensures removal of a high percentage of contaminating microsomes and soluble enzymes. The low P:O ratios obtained with once washed mitochondria in the presence of succinate or  $\alpha$ -ketoglutarate, may be due to ATP-ase action by contaminating microsomes or other uncoupling agents. The substances causing ATP-breakdown are apparently removed by a second washing which has the additional advantage of yielding a mitochondrial pellet free of fluffy layer. A force of 12,000 x g was found optimal for obtaining a firmly packed mitochondrial pellet with a high protein content. The final mitochondrial suspension after twice washing in 0.25 M sucrose was apparently homogeneous when examined by phase contrast microscopy.

Novikoff and co-workers (19) in a study of the various particulate fractions of the Novikoff hepatoma found that ATP-ase activity was associated mainly with the microsome fraction. Mitochondria showed a relatively low activity.

It is also of interest to note that Devlin (20) working with mitochondria of the Novikoff hepatoma found these particulates incapable of oxidizing certain substrates. Oxidative phosphorylation was demonstrated, on fortification of the medium with bovine serum albumin. An inhibitor of oxidative phosphorylation was found to be present in the high speed supernatant fraction of Novikoff homogenates, although this inhibitor was not present in the high speed supernatant fraction from normal liver. The presence of such an inhibitor may explain the considerable degree of uncoupling of phosphorylation observed with once washed Ehrlich ascites cell mitochondria (Table 3).

L. Wojtczak and A. Wojtczak (21) observed a pronounced stimulation of oxidative phosphorylation with insect mitochondria in the presence of bovine serum albumin. The ATP- $P_i$  exchange reaction was demonstrated only in the presence of the albumin. The inhibitor of the exchange reaction and perhaps oxidative phosphorylation, is removed from insect mitochondria by washing with serum albumin solution. The adsorbed inhibitor is extractable from the denatured albumin with ethanol or benzene.

## 2 FURTHER STUDIES OF PHOSPHORYLATION COUPLED TO $\alpha$ -KETOGLUTARATE OXIDATION IN EHRlich ASCITES CELL MITOCHONDRIA

### 5.1 Method

Mitochondria were isolated in 0.25 M sucrose as described in the Experimental Section. They were allowed to stand at 0°C. under aerobic conditions for periods of 4 - 5.5 hours, in order to deplete them of endogenous pyridine nucleotides under mild conditions.

Measurement of oxidative phosphorylation was carried out in Warburg manometers at 30°C. employing the reaction medium shown in Table 4.

#### 5.2 Effect of Diphosphopyridine Nucleotide and Nicotinamide Additions on Phosphorylation Associated with $\alpha$ -Ketoglutarate Oxidation in Aged Mitochondria

Mitochondria isolated in 0.25 M sucrose were allowed to stand at 0°C. for periods of 4 - 5.5 hours. Such ageing of tumour mitochondria is reported by Kielley (27) to result in a lowered capacity for phosphorylation which can be largely restored by subsequent addition of DPN. Mitochondria of normal cells do not, however, appear to undergo loss of phosphorylation activity on corresponding ageing. In accord with previous results (26) and the findings of other workers concerning tumour mitochondria (27, 28), it has been shown that addition of DPN causes a significant increase in the oxygen uptake of Ehrlich ascites cell mitochondria respiring with  $\alpha$ -ketoglutarate as substrate (Table 4). Oxygen uptake is increased approximately 46% by addition of  $2.4 \times 10^{-3}$  M DPN to the medium. Nicotinamide (0.02 M) on the other hand, causes 1 - 2% lowering of oxygen uptake in both DPN fortified and unfortified media.

The phosphorylation activity of 4 - 5.5 hours aged mitochondria respiring with  $\alpha$ -ketoglutarate is markedly influenced by addition of nicotinamide which raises the P:O ratio by approximately 46% in media fortified with DPN and by approximately 33% in media without DPN. The effect is greatest with low quantities of mitochondrial protein. In a medium without nicotinamide, DPN fortification seems to cause a lowering of approximately 9% in P:O ratio. Although DPN addition raises the oxygen uptake, the esterification of inorganic phosphate is not correspondingly increased, with the result that lower P:O ratios are observed in the presence of DPN (Table 4). Similar results were obtained by Kielley (27) confirming that externally added DPN does not completely restore the phosphorylation activity lost on ageing.

TABLE 4

Effect of DEN and Nicotinamide Additions  
on Phosphorylation Associated with  
 $\alpha$ -Ketoglutarate Oxidation in Aged  
Ehrlich Ascites Cell Mitochondria

Flasks contained the following basic medium in a final volume of 3.0 ml.: K, Na phosphate buffer (pH 7.4), 0.045 M; D-glucose, 0.034 M; KP, 0.015 M;  $\text{MgCl}_2$ , 0.008 M; ADP, 0.0025 M; AMP, 0.001 M; ethylenediaminetetra-acetic acid (pH 7.4), 0.002 M; KCl, 0.01 M; sucrose, 0.062 M;  $\alpha$ -ketoglutarate, 0.0066 M; malonate, 0.02 M; cytochrome c,  $9.8 \times 10^{-6}$  M; hexokinase (crystalline), 1 - 2 mg.; mitochondrial suspension (aged 4 - 5.5 hr in sucrose at 0°) in 0.25 M sucrose (0.4 ml.) equivalent to  $2.11 \pm 0.29$  mg. protein determined according to Cleland and Slater (34). Temp. 30°. The reaction was stopped after 30 - 40 minutes by addition of 1.0 ml. 30% trichloroacetic acid. Phosphate uptake was determined according to Fiske and Subbarow (32). The values given are the means  $\pm$  the average deviations of results obtained in a total of 11 determinations with different batches of mitochondria.

| Special additions                                       | $\text{O}_2$ consumed<br>$\mu\text{atoms/mg.}$<br>protein/hr | P esterified<br>$\mu\text{atoms/mg.}$<br>protein/hr | P:O             |
|---------------------------------------------------------|--------------------------------------------------------------|-----------------------------------------------------|-----------------|
| DEN ( $2.4 \times 10^{-3}$ M)+<br>nicotinamide (0.02 M) | $5.40 \pm 1.02$                                              | $11.60 \pm 2.78$                                    | $2.17 \pm 0.35$ |
| DEN ( $2.4 \times 10^{-3}$ M)                           | $5.51 \pm 1.10$                                              | $8.35 \pm 3.14$                                     | $1.49 \pm 0.40$ |
| Nicotinamide (0.02 M)                                   | $3.43 \pm 1.00$                                              | $7.28 \pm 2.07$                                     | $2.17 \pm 0.49$ |
| None                                                    | $3.77 \pm 1.10$                                              | $6.45 \pm 4.10$                                     | $1.63 \pm 0.92$ |

Mitochondria aged only 1 hour at 0°C. show a P:O ratio of approximately 1.56 (average of 1.49 and 1.64) in the absence of DPN and nicotinamide, while 4 - 5.5 hours aged mitochondria show a P:O ratio of approximately 1.63 in the same medium (Table 4).

# 6 OXIDATION OF DPNH BY AGED EHRLICH ASCITES CELL MITOCHONDRIA

During the studies of  $\alpha$ -ketoglutarate oxidation, it was considered of interest to investigate the oxidation of reduced diphosphopyridine nucleotide by Ehrlich ascites cell mitochondria. Mitochondria were isolated and aged 4 - 5.5 hours at 0°C. as previously described. DPNH was generated in situ by the alcohol dehydrogenase reaction, and its oxidation studied in the medium of Table 4. Results in Table 5 show that externally added DPNH is readily oxidised by aged Ehrlich mitochondria but the oxidation does not appear to be coupled to phosphorylation. In fact a small output of inorganic phosphate was observed in experiments in which the DPNH was generated in situ by addition of DPN, ethanol and alcohol dehydrogenase to the medium.

The results suggest that externally added DPNH is oxidised via a non-phosphorylating pathway in Ehrlich ascites cell mitochondria.

TABLE 5

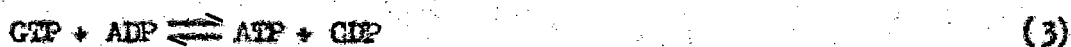
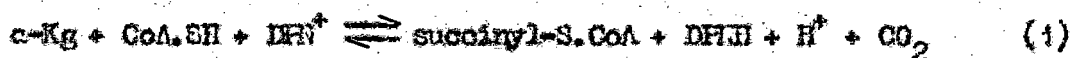
## OXIDATION OF DPNH BY AGED EHRLICH ASCITES CELL MITOCHONDRIA

Flasks contained the basic medium of Table 4 without malonate. Special additions as indicated. Temp. 30°. Inorganic phosphate was liberated instead of esterified.

| Special additions                                                                                          | Mitochondrial protein mg. | O <sub>2</sub> consumed $\mu$ atoms/mg. protein/hr | P esterified $\mu$ atoms/mg. protein/hr |
|------------------------------------------------------------------------------------------------------------|---------------------------|----------------------------------------------------|-----------------------------------------|
| DPN ( $2.4 \times 10^{-3}$ M) + alcohol dehydrogenase (0.4 mg.) + ethanol (0.04 M)                         | 1.44                      | 4.49                                               | + 2.40                                  |
| DPN ( $2.4 \times 10^{-3}$ M) + alcohol dehydrogenase (0.4 mg.) + ethanol (0.04 M) + nicotinamide (0.02 M) | 1.44                      | 4.71                                               | + 1.42                                  |

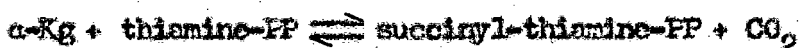
## 7 MECHANISM OF $\alpha$ -KETOGlutARATE OXIDATION

In the light of present knowledge,  $\alpha$ -ketoglutarate oxidation is assumed to proceed as follows (22, 23, 24).



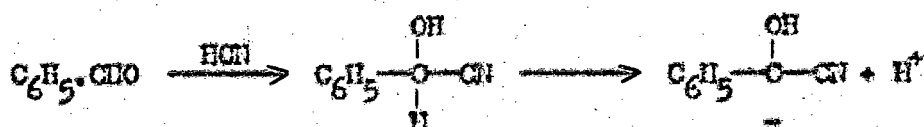
Diphosphopyridine nucleotide, co-enzyme A and guanosine diphosphate are essential co-factors in this sequence of reactions. The requirement for ADP is absolute to ensure regeneration of GDP. Inosine diphosphate can substitute for GDP in reaction (3).

Reaction (1) is complex and consists of a number of steps involving thiamine-pyrophosphate and lipoic acid. The primary reaction is one between  $\alpha$ -ketoglutarate, enzyme and thiamine-pyrophosphate as shown below.



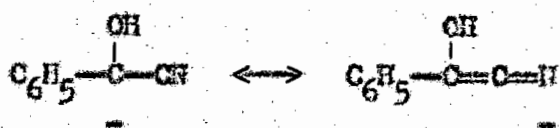
The mechanism of decarboxylation and the function of thiamine-pyrophosphate in this reaction is at present not fully understood. However, in considering the mechanism of thiamine action Breslow (25) has presented evidence which suggests a plausible sequence of reactions. He has shown that a number of model thiazole compounds as well as thiamine and thiamine-pyrophosphate are capable of catalysing the benzoin condensation. The mechanism of condensation is similar to the one presented by Lapworth, in which cyanide ( $\text{CN}^-$ ) is the active catalyst.

Benzoin condensation with cyanide ion as catalyst:

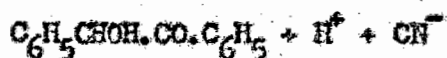
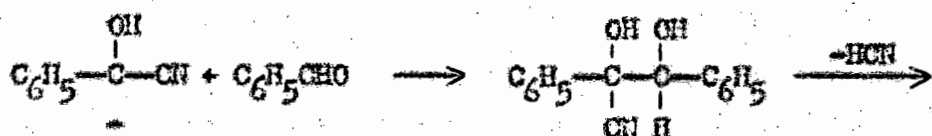


(I)

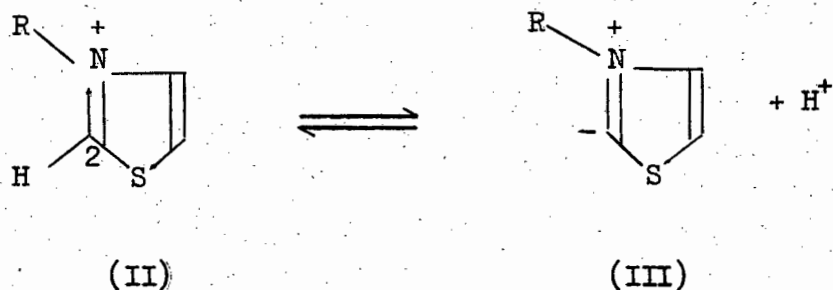
The reactive carbanion (I) is stabilised by resonance.



Further reaction of the carbanion (I) with another molecule of benzaldehyde takes place as follows:



Breslow has shown that under relatively mild conditions, thiazolium salts undergo the following reaction,



resulting in the formation of a reactive carbanion at position 2 of the thiazole ring. The thiazolium zwitterion (III) should be able to catalyse the benzoin condensation in analogous fashion as does the cyanide ion. The probable reaction mechanism is shown below.

however, emphasised that the method is only applicable to small amounts of cellular material.

Since the fractionation achieved depends upon a number of parameters such as density of the various cells present, viscosity and temperature of the sucrose gradient, minor variations in the degree of separation are to be expected. Variation in the composition of ascites fluid at different times after inoculation would furthermore influence the separation.

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## CHAPTER III

ISOLATION OF EHRLICH ASCITES TUMOUR CELL MITOCHONDRIA

AND STUDIES OF OXIDATIVE PHOSPHORYLATION

WITH SUCCINATE, L-ASCORBATE,  $\alpha$ -KETOGLOUTARATE,

L-GLUTAMATE AND DPNH AS SUBSTRATES

### CHAPTER III

#### ISOLATION OF EHRLICH ASCITES TUMOUR CELL MITOCHONDRIA AND STUDIES OF OXIDATIVE PHOSPHORYLATION WITH SUCCHINATE, L-ASCORBATE, $\alpha$ -KETOGLOUTARATE, L-GLUTAMATE AND L-AMINO ACIDS AS SUBSTRATES

##### 1 PREPARATION OF EHRLICH ASCITES TUMOUR CELL MITOCHONDRIA

##### 1.1 Introduction

Isolation of mitochondria from the Ehrlich ascites tumour is complicated by the fact that these cells have an extremely tough membrane which is not easily ruptured. Homogenates have been prepared in 0.25 M sucrose by Kun, Talalay and Williams-Ashman (1) using an all glass Potter homogeniser. Lutolo and Abrignani (2) have similarly obtained homogenates in 0.44 M sucrose with 0.1 M citrate (pH 6.3) and  $10^{-3}$  M versene, using a Potter homogeniser and Teflon plunger. The tumour cells can also be disrupted by the application and sudden release of nitrogen gas pressure in a steel bomb (3). The technique of shaking the cells vigorously with quartz sand in a fortified sucrose medium has been employed by Lindberg, Ljunggren, Ernster and Revess (4), while Wenner and co-workers (5) have obtained broken-cell preparations by homogenisation in a Servall Omni-Mixer at full speed for three minutes at 3°C. This latter method unfortunately causes considerable damage to the nuclei. A more gentle procedure (5) involves disintegration of the ascites cells by mechanical agitation in the presence of grade 10 "glow" beads (60 - 80 mesh, 200  $\mu$  average diameter) in a Servall Omni-Mixer for a period of two minutes at 5,000 r.p.m. No disruption of cells occurs in the absence of glow beads.

Sucrose has been the medium chiefly used by previous workers for homogenisation, and isolation of cell particulates. Recently, however, Carruthers and co-workers (6) have employed 90% glycerol. With this medium approximately 100 passes in an all glass Potter homogeniser are required to effect cell breakage.

Sonic disintegration has been employed to disrupt ascites cells (7), but the method is not suitable for work with mitochondria since these particulates lose their capacity to carry out coupled oxidative phosphorylation after sonic treatment.

Unpublished work by Dounce, Stotz and their co-workers (8) at the University of Rochester, N.Y., U.S.A., has shown that the membrane of ascites cells can be easily ruptured with a Dounce homogeniser if these cells are previously swollen in quarter-strength Ringer phosphate solution for 30 - 60 minutes at 0°C.

In their studies of protein synthesis with isolated microsomes, Littlefield and Keller (9) used the technique of lysing the ascites cells into distilled water. A water cell-mass ratio of 10:1 was found optimum for maximal cell rupture. Gentle homogenisation was carried out for five minutes in a large homogeniser with loose plunger.

For the purpose of the present investigation it was required to devise a method for isolation of mitochondria which would be reasonably quick and simple, and which would afford good yields of twice washed mitochondria capable of oxidative phosphorylation.

## 1.2 Method

Numerous variations of the procedures described by Kun (1) and Mutolo (2) failed to provide satisfactory homogenates of Ehrlich ascites cells. These modifications involved the use of 0.25 M and 0.44 M sucrose with or without 0.001 M versene

(pH 7.4), as the isolation medium; the use of both all glass or teflon Potter homogenisers, variation in the number of passes (10 - 100) and alteration of the cell-mass, sucrose-volume ratio. Attempts to homogenise untreated cells with the Dounce homogeniser also led to negative results. Vigorous shaking of the cells with glass beads, for various lengths of time, and grinding with carborundum powder were likewise tried and proved unsuccessful.

Preliminary experiments designed to pre-stress the cell membranes, showed that treatment for 15 minutes in quarter-strength Ringer phosphate (pH 7.4) at 0°C. followed by the disruption of the cells in pure water by osmotic shock, produced a 90 - 95% homogenate in which the nuclei were completely stripped of adhering cytoplasmic material. Following the osmotic shock treatment for a period of exactly 3 minutes, concentrated sucrose solution was added to the homogenate to a final concentration of 0.25 M. After further homogenisation with both loose and tight plungers, mitochondria were isolated in the customary manner by differential centrifugation. For full details of the isolation procedure see the Experimental Section.

The method, if carefully followed, offers several advantages over previous techniques [Nirenberg and Hogg (3); Kun, Talalay and Williams-Ashman (1); Mitolo and Abrighani (2); and Lindberg, Ljunggren, Ernster and Revel (4)]. The use of Dounce homogenisers, which need not be removed from the ice bath during the isolation procedure, is convenient for proper control of temperature. The homogenisation of pre-stressed cells is a relatively simple matter by comparison with the prolonged grinding of unswollen cells required in previous isolation techniques.

In studies concerning earlier methods, it was observed that although many cell membranes were broken, the free nuclei had large amounts of adhering cytoplasm which was almost impossible to

remove by repeated homogenisation. Thus a considerable amount of mitochondrial material was lost together with the nuclei on slow speed centrifugation.

Although prolonged exposure of mitochondria to water could conceivably be detrimental to their morphological structure and capacity for oxidative phosphorylation, it appeared from the experimental results with mitochondria prepared by osmotic shock treatment that no such damage had occurred. Separate tests failed to show any agglutination of mitochondria after standing for 10 minutes at 0°C. in distilled water. Fomesu and Davis (29) in experiments with rat liver mitochondria, observed that adenosine-monophosphate prevented swelling of the particulates. Certain experiments were therefore carried out in which washed Ehrlich ascites cells were lysed into water containing 0.001 M adenosine-monophosphate (pH 7.4). Mitochondria obtained by this modification did not, however, exhibit any difference in oxidative phosphorylation capacity from those isolated in the absence of adenosine-monophosphate.

## 2 OXIDATION AND ASSOCIATED PHOSPHORYLATIONS WITH SUCCINATE AND ASCORBATE AS SUBSTRATES

### 2.1 Method

Mitochondria were isolated in 0.25 M sucrose as described in the Experimental Section.

Measurement of oxidative phosphorylation. The following medium in a volume of 2.36 ml. was placed in the main compartment of each Warburg flask (molarities refer to the final volume of 3.0 ml. after addition of the side-arm contents and mitochondrial suspension): 0.045 M-sodium potassium phosphate buffer, pH 7.4; 15 mM-KF; 8 mM-MgCl<sub>2</sub>; 25 mM-adenosine-diphosphate (ADP);

mM-adenosine 5'-phosphate (AMP); 2 mM-ethylenediaminetetra-acetic acid (EDTA); 3 $\frac{1}{2}$  mM-D-glucose; 0.01 M-KCl; 62 mM-sucrose; 0.24 mM-diphosphopyridine nucleotide (DPN); 7 mM-substrate; 9.6  $\mu$ M-cytochrome c. (In measurement of  $\alpha$ -oxoglutarate oxidation 0.02 M-malonate was added as an inhibitor. AMP, ADP and all substrates were neutralised to pH 7.4 with KOH and stored at  $-30^{\circ}$  before use.) The side arms of each flask contained 70% of the total D-glucose, together with 1 - 3 mg. of crystalline hexokinase, in a volume of 0.24 ml.

To each Warburg vessel was added 0.4 ml. of a mitochondrial suspension in 0.25 M sucrose, giving a concentration of 0.4 - 1.5 mg. of protein/ml. of incubation medium after addition of the side-arm contents. The centre well of each flask contained 0.2 ml. of 40% (w/v) KOH for CO<sub>2</sub> absorption, and the gas phase was air. Oxygen uptake was measured for 25 - 30 minutes after 13 minutes equilibration at  $30^{\circ}$ . Phosphate uptake was determined by the method of Fiske and Subbarow (32), on cold trichloroacetic acid filtrates. The oxygen uptake of the mitochondria ( $Q_{O_2}$ ) was expressed as  $\mu$ l. of O<sub>2</sub>/mg. of protein/hour according to Slater and Holton (33).

## 2.2 Succinate and L-ascorbate oxidation

In preliminary experiments it was found that twice washed mitochondria showed satisfactory P:O ratios and oxygen uptakes, with a variety of substrates. Once washed mitochondria showed unimpaired respiration, but the associated phosphorylations were largely uncoupled (see Table 3). Twice washed mitochondria were accordingly used for all experiments, except where specifically mentioned. The results with succinate and L-ascorbate as substrates are given in Table 1. In most experiments EDTA was present at a final concentration of  $2.4 \times 10^{-4}$  M. Though this is obviously not required for oxidations associated with succinate and L-ascorbate,

it was included in the likelihood that it would serve to stabilise the isolated mitochondria.

In Experiment 1 of Table 1, P:O ratios with succinate are somewhat low. This may be due to use of a hexokinase with low activity. In the absence of potassium fluoride (ATF-ase inhibitor), the  $Q_{O_2}$  was increased from 78 to 99 (approximately 25%), while the P:O ratio remained essentially unchanged. These results are compatible with Slater's hypothesis (10) that formation of a phosphate-fluoride complex inhibits succinic dehydrogenase activity in mitochondria. DFN ( $2.4 \times 10^{-4}$  M) was without effect on the P:O ratio and  $Q_{O_2}$  with succinate as substrate.

The P:O ratios with succinate as substrate were measured for thirteen different mitochondrial preparations (Experiment 3) using the fortified medium of Table 1. An active hexokinase was used in all these experiments. These results compare favourably with the values of 1.4 obtained by Lindberg et al (4) and 1.2 by Kun et al (1). Lindberg and co-workers (4) measured phosphorylation by a very sensitive method employing radioactive  $P^{32}$ .

The variation in P:O ratio observed with succinate as substrate (Table 1), may be due to differences in the age of ascites fluid or mice, as well as to minor variations in experimental technique during the actual isolation procedure. It is noteworthy that the theoretical P:O ratio of 2.0 for succinate was never obtained with any preparation using the fortified medium of Table 1. Ehrlich ascites mitochondria showed  $Q_{O_2}$  values with succinate which, per mg. protein, were comparable to those of kidney, but which were in general above those of other normal cells such as liver and brain (11). The stability of the succinic dehydrogenase, succinic-oxidase series of enzymes appears to be high by comparison with the DFN-dependent dehydrogenase enzyme systems in Ehrlich ascites mitochondria.

TABLE 1

Oxidation and Associated Phosphorylations  
with Succinate and Ascorbate Respectively as Substrates

Each flask contained the following in a total volume of 3.0 ml.: mitochondrial suspension in 0.25 M-sucrose, 0.4 ml.; sodium potassium phosphate buffer (pH 7.4), 45 ml; D-glucose, 34 ml; KF, 15 ml;  $\text{MgCl}_2$ , 8ml; ADP, 2.5 ml; ATP, 2ml; EDTA (pH 7.4), 2 ml; KCl, 0.01 M; sucrose, 67 ml (in experiments without DFN), or 62 ml (in experiments with DFN); succinate, 0.01 M, or ascorbate, 0.6 ml; cytochrome c, 9.8  $\mu\text{M}$ ; 1 - 2 mg. of crystalline hexokinase. Temp. 30°. Equilibration time 12 - 15 min. Monometric flasks were maintained at 0° before each experiment.

| Expt.            | DFN additions (m-moles) | Incubation time (min.) | Qt. of protein/flask (mg.) | $\Delta O$ ( $\mu\text{g. atom}$ ) | $\Delta P_1$ ( $\mu\text{mole}$ ) | P:O             | $\text{O}_2$      | Remarks                                                                        |
|------------------|-------------------------|------------------------|----------------------------|------------------------------------|-----------------------------------|-----------------|-------------------|--------------------------------------------------------------------------------|
| <b>Succinate</b> |                         |                        |                            |                                    |                                   |                 |                   |                                                                                |
| 1                | 0.24                    | 32                     | 2.17                       | -7.36                              | -4.95                             | 0.67            | 78.8              | P:O ratios low owing to use of hexokinase with low activity                    |
|                  | 0.24                    | 32                     | 2.17                       | -7.16                              | -4.78                             | 0.67            | 76.9              |                                                                                |
|                  | 0.24<br>(No KF added)   | 32                     | 2.17                       | -9.26                              | -7.73                             | 0.84            | 99.6              |                                                                                |
| 2                | None                    | 33                     | 2.29                       | -6.64                              | -6.71                             | 1.01            | 65.6              | -                                                                              |
| 3                | 0.24                    | 32-4                   | 2.64 $\pm$ 0.78            | -(10.97 $\pm$ 1.63)                | -(15.82 $\pm$ 3.71)               | 1.44 $\pm$ 0.21 | 105.9 $\pm$ 22.2* | Average of 13 different mitochondrial preparations                             |
| 4                | 0.24                    | 35                     | 2.83                       | -11.15                             | -13.03                            | 1.17            | 83.9              | -                                                                              |
| <b>Ascorbate</b> |                         |                        |                            |                                    |                                   |                 |                   |                                                                                |
| 1                | None                    | 33                     | 2.29                       | -6.70                              | +0.93                             | -               | 66.2              | Same mitochondrial preparation as used for Expt. 2 with succinate as substrate |
| 2                | None                    | 33                     | 2.29                       | -6.58                              | +4.66                             | -               | 65.0              |                                                                                |
| 3                | 0.24                    | 35                     | 2.83                       | -7.14                              | +2.81                             | -               | 53.3              | Same mitochondrial preparation as used for Expt. 4 with succinate as substrate |

\*Average deviation from mean

With L-ascorbate as substrate, Ehrlich ascites mitochondria showed  $Q_{O_2}$  values of 53.8 - 66.2 in the phosphorylation medium of Table 1, with or without added IFN. In some cases oxygen uptake was equivalent to that of the succinate controls run simultaneously on the same mitochondrial preparation (see also Fig. 1). In no instance could phosphorylation with ascorbate as substrate, be demonstrated. Hence it was impossible to determine a P:O ratio with this substance. Instead of inorganic phosphate ( $P_i$ ) uptake and formation of ATP, a not positive increase of inorganic phosphate was consistently observed. Even the high concentration of fluoride present was without effect on this reaction. The not positive increase in  $P_i$  may be due to the operation of an active myokinase enzyme system, which produces ATP from ADP and ADP, present in the incubation medium.



The ATP formed in reaction (1) is then broken down by ATP-ase action.

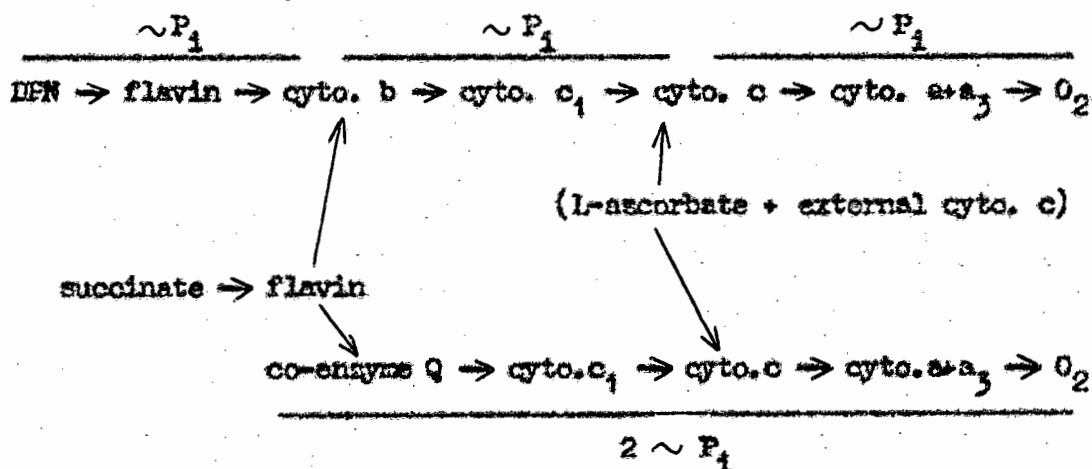


In the simple scheme presented above, two moles of ADP are used in reaction (1), and one mole generated in reaction (2). With time therefore, there will be a continuous decrease in ADP concentration with a concomitant increase in  $P_i$ .

It will be noted (Table 1) that in the ascorbate experiments, succinate controls were carried out on the same mitochondrial preparation. The P:O ratios obtained with succinate in these control experiments were 1.01 and 1.17 respectively. This result together with those of the ascorbate experiments, would suggest an uncoupling of phosphorylation somewhere in the succinic dehydrogenase-cytochrome oxidase region of the electron transfer chain. A dys function at the cytochrome c level of

phosphorylation is indicated, since this site is involved in both the oxidation of succinate and ascorbate. Its failure to function would reduce the P:O ratio associated with succinate oxidation from a theoretical value of 2.0 to unity, and would result in a P:O ratio of zero, as observed for ascorbate oxidation [Copenhaver and Lardy (12); Slater (13); Hunter (14); Nielsen and Lehninger (15); and Chance and Williams (16)].

However, recent investigations by Green and co-workers (30) have indicated that succinate oxidation in beef heart mitochondria occurs via a separate electron transfer pathway to that operating from the pyridine nucleotide-linked dehydrogenases. To illustrate the probable reactions occurring in this multienzyme complex, the following diagram is shown.



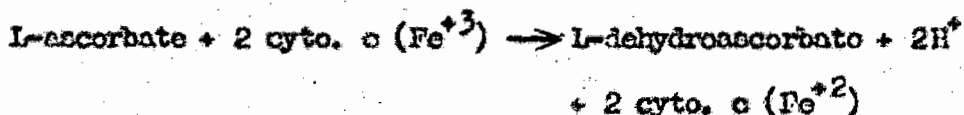
( $\sim P_1$  = generation of ATP)

The diagram clearly indicates that during either succinate or ascorbate oxidation, electrons may be transferred via a single pathway or through two pathways to oxygen. Therefore, any reasonable explanation accounting for the absence of phosphorylation associated with L-ascorbate oxidation in Ehrlich ascites cell mitochondria, would have to take into account the existence of these two pathways.

Uncoupling of the cytochrome c phosphorylation site has not been observed in normal cell mitochondria such as those of

liver (15). It must also be assumed that if there is indeed ATP formation due to myokinase activity, then the resulting ATP could be broken down at phosphorylation sites in the electron transport chain other than that at the cytochrome c level.

The reducing action of ascorbate on cytochrome c possibly occurs as follows:



This reaction is non-enzymatic and the cytochrome c involved is the externally added material. It is not certain how the reaction takes place, but it probably involves a two step reduction. The externally reduced cytochrome c reacts with the mitochondrial electron transfer chain, reducing carrier or endogenous cytochrome c.

### 3 OXIDATION AND ASSOCIATED PHOSPHORYLATIONS WITH $\alpha$ -KETOGLOUTARATE AND L-GLUTAMATE AS SUBSTRATES

#### 3.1 Method

Mitochondria were isolated in 0.25 M sucrose as described in the Experimental Section. All experiments were carried out with mitochondria which had stood for 2 to 3 hours at 0°C. after isolation, and were therefore depleted of endogenous pyridine nucleotides to some extent. Measurement of oxidative phosphorylation was carried out in Warburg manometers at 30°, employing the reaction medium shown in Table 2.

#### 3.2 $\alpha$ -Ketoglutarate and L-Glutamate Oxidation

Studies with  $\alpha$ -ketoglutarate and L-glutamate; substrates which require diphosphopyridine nucleotide for their oxidation,

revealed interesting data concerning the functioning of Ehrlich ascites cell mitochondria. The results of these studies are presented in Table 2.

In the absence of added DFN or the DFN-ase inhibitor nicotinamide, mitochondria oxidised  $\alpha$ -ketoglutarate with low  $Q_{O_2}$  values and P:O ratios. These values were little effected by addition of  $2.4 \times 10^{-4}$  M DFN to the medium. However, in experiments in which DFN was present at  $2.4 \times 10^{-3}$  M concentration with 0.02 M nicotinamide, a marked stimulation of the order of 100% in  $Q_{O_2}$  value was observed. The P:O ratio was also raised but not so markedly as the  $Q_{O_2}$ . The apparent negative effect on  $Q_{O_2}$  of  $2.4 \times 10^{-4}$  M DFN in the absence of nicotinamide, may be a reflection of the very rapid breakdown of DFN by tumour mitochondrial DFN-ases (11, 17). When nicotinamide and a high concentration of DFN were both present, the rates of phosphorylation and oxygen uptake were both approximately doubled. In the presence of nicotinamide alone, the P:O ratio with  $\alpha$ -ketoglutarate as substrate was slightly raised. In all experiments with  $\alpha$ -ketoglutarate, malonate was used as an inhibitor of succinic dehydrogenase.

In experiments with L-glutamate as substrate, malonate was not included in the incubation medium (see Table 2). Low values for oxygen uptake and P:O ratio were observed with this substrate in the absence of added co-factors. On fortification with  $2.4 \times 10^{-4}$  M DFN, a marked increase in  $Q_{O_2}$  was observed. An even more striking increase in oxygen uptake and rate of phosphorylation was achieved in the presence of  $1.2 \times 10^{-3}$  M DFN and 0.02 M nicotinamide. The increase in  $Q_{O_2}$  at the highest concentration of DFN was approximately 400%. The results demonstrate that isolated Ehrlich ascites mitochondria are capable of oxidising L-glutamate with coupled phosphorylation.

TABLE 2

Oxidation and Associated Phosphorylations with  $\alpha$ -Oxoglutarate  
and L-Glutamate respectively as Substrates

Each flask contained the following in a total volume of 3.0 ml.: mitochondrial suspension in 0.25 M-sucrose, 0.4 ml.; sodium potassium phosphate buffer (pH 7.4), 45 mM; D-glucose, 34 mM; KF, 15 mM;  $\text{HgCl}_2$ , 8 mM; ADP, 2.5 mM; ATP, mM; EDTA (pH 7.4), 2 mM; KCl, 0.01 M; sucrose, 62 mM;  $\alpha$ -oxoglutarate, 6.6 mM, or L-glutamate, 6.6 M; malonate, 0.02 M; cytochrome c, 9.8  $\mu\text{M}$ ; 1 - 2 mg. of crystalline hexokinase. (In experiments with L-glutamate as substrate, malonate was omitted from the incubation medium.) Temp. 30°. Equilibration time 13 min. Manometric flasks were maintained at 0° before each experiment.

| Expt.                                   | Special additions                | Incubation time (min.) | Wt. of protein/flask (mg.) | $\Delta O$ (ug. atom) | $\Delta P_i$ ( $\mu\text{mole}$ ) | PrO  | $Q_{O_2}$ |
|-----------------------------------------|----------------------------------|------------------------|----------------------------|-----------------------|-----------------------------------|------|-----------|
| <b><math>\alpha</math>-Oxoglutarate</b> |                                  |                        |                            |                       |                                   |      |           |
| 1                                       | -                                | 35                     | 1.98                       | -3.17                 | -5.87                             | 1.85 | 34.1      |
| 2                                       | 0.24 mM-DFH                      | 32                     | 4.35                       | -5.80                 | -11.23                            | 1.95 | 31.1      |
| 3                                       | 2.4 mM-DFH + 0.02 M-nicotinamide | 35                     | 1.98                       | -5.41                 | -12.97                            | 2.40 | 58.3      |
| 4                                       | 0.02 M-Nicotinamide              | 35                     | 2.12                       | -2.61                 | -6.43                             | 2.47 | 26.2      |
|                                         | 2.4 mM-DFH + 0.02 M-nicotinamide | 35                     | 2.12                       | -4.92                 | -11.59                            | 2.36 | 49.4      |
| <b>L-Glutamate</b>                      |                                  |                        |                            |                       |                                   |      |           |
| 1                                       | 1.2 mM-DFH + 0.02 M-nicotinamide | 30                     | 2.46                       | -6.03                 | -10.97                            | 1.83 | 60.9      |
|                                         | -                                | 30                     | 2.46                       | -1.48                 | -1.48                             | 1.00 | 15.0      |
| 2                                       | 0.24 mM-DFH                      | 30                     | 1.56                       | -2.36                 | -                                 | -    | 37.6      |

Oxygen uptake in  $\mu\text{l.}$  per mg. protein at  $30^\circ\text{C.}$

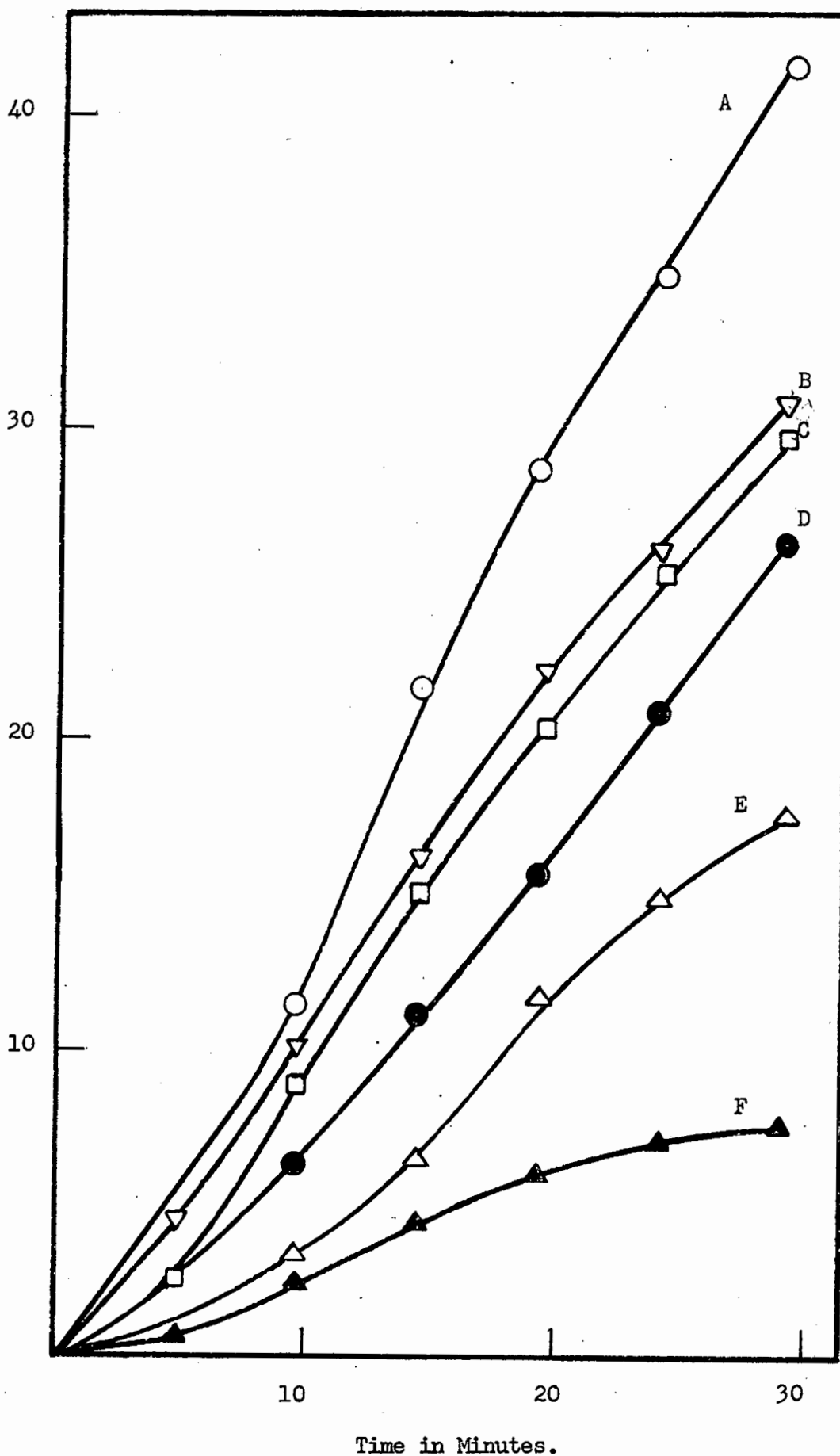


Fig. 1. Oxygen uptake of mitochondria in the presence of various substrates and co-factors.

Incubation medium as in Tables 1, 2 and 3 with:- A, 0.01 M succinate; B, 0.0066 M L-glutamate +  $1.2 \times 10^{-3}$  M DPN + 0.02 M nicotinamide; C, 0.0066 M  $\alpha$ -ketoglutarate +  $2.4 \times 10^{-3}$  M DPN + 0.02 M nicotinamide; D, 0.0066 M ascorbate; E, 0.0066 M  $\alpha$ -ketoglutarate; F, 0.0066 M L-glutamate.

tumour tissues, arriving at conclusions which support the postulate of differences between the mitochondrial membranes of normal and tumour cell mitochondria.

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## CHAPTER II

### FRACTIONATION OF CELL TYPES

#### PRESENT IN EHRLICH ASCITES FLUID

#### BY SUCROSE GRADIENT CENTRIFUGATION

## CHAPTER II

### FRACTIONATION OF CELL TYPES PRESENT IN EHRlich ASCITES FLUID BY SUCROSE GRADIENT CENTRIFUGATION

#### 1 FRACTIONATION OF CELL TYPES IN ASCITES FLUID

##### 1.1 Introduction

Ehrlich ascites fluid contains not only tumour cells, but also a variety of other cell types which are: polymorphonuclear leukocytes, monocytes, histiocytes, lymphocytes, erythrocytes and mesothelial cells.

Any method for removal of these contaminating cell types, would be of advantage in providing a uniform sample of tumour cells from which the mitochondrial fraction could be isolated in a homogeneous condition.

Erythrocytes may be removed by lysis with hypotonic saline leaving the ascites cells intact (1, 2). Centrifugation of the mixed cells in Krebs-Ringer bicarbonate or isotonic saline affords a rough separation between erythrocytes and tumour cells, whereby either layer may be scooped out with a glass spatula (3). The counter-streaming centrifugal procedure of Lindahl and Klein (4) yields considerably purified tumour cell preparations, but none in which contaminants are completely absent.

A method of purification was therefore devised which involves separation of the various cell types from each other, by slow speed centrifugation in a sucrose gradient. (The use of a sucrose gradient was suggested by Dr. Folson of the Virus Research Unit, Medical School, University of Cape Town.) By this method

it was found possible to obtain a reasonably pure suspension of ascites cells, free from the other contaminating cell types usually present in ascites fluid. The method is however applicable only to small amounts of cell material and is therefore inadequate for use in experiments designed to isolate mitochondria. For purposes of isolation comparatively large amounts of tumour cells are necessary. The method may nevertheless find application in the field of tissue culture in cases where it is desired to produce colonies from only a single cell type.

### 1.2 Method

A centrifuge tube containing a 10 - 50% gradient of sucrose in phosphate buffer was set up as described in the Experimental Section. After layering the ascites fluid onto the gradient, the tube was centrifuged at 250 - 300 r.p.m. for thirty minutes at 10 - 15°C.

### 1.3 Results of Sucrose Gradient Centrifugation

Following centrifugation, the sucrose gradient was examined against a suitable background source of light. Usually three distinct bands of cells were observed. The composition and positions of the various bands are shown in Fig. 1. Microscopic examination of the bands was carried out, following their careful removal with a sampling pipette. Occasionally a thin white band (Band A - Fig. 1) of unidentified material was seen above the erythrocyte layer. The main bulk of ascites cells was found to sediment at the 40% sucrose level. In certain cases, continued centrifugation (15 - 30 minutes) resulted in further separation of a narrow band of ascites cells below the main bulk, as indicated by a broken line at the 45% sucrose level (Fig. 1).

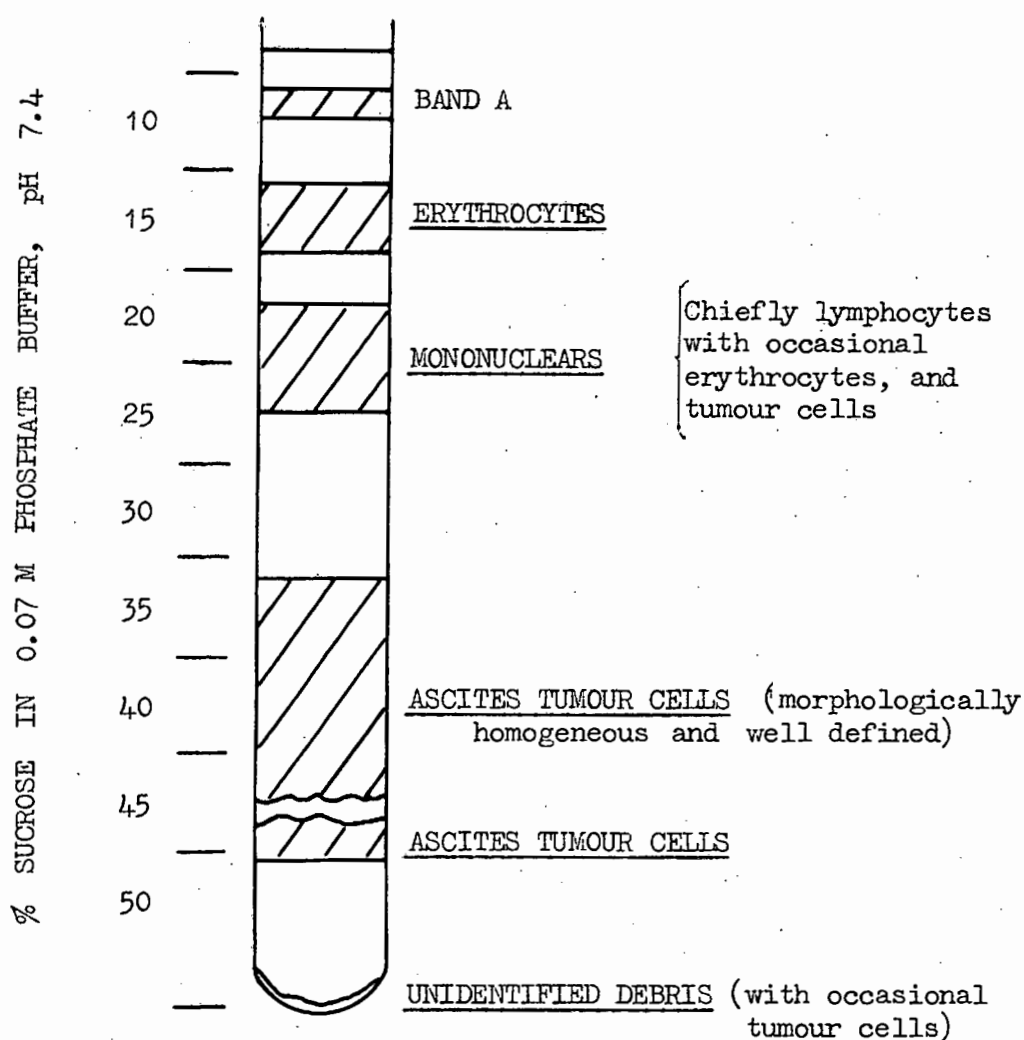


Fig. 1 Fractionation of Cell Types Present in Ehrlich Mouse Ascites Fluid by Means of Slow Speed Centrifugation in a Sucrose Gradient

In some experiments the mononuclear band at the 20-25% sucrose level was resolved into two separate bands. Optimum conditions of centrifugation were found to be 250-300 r.p.m. for 30 minutes at 10-15°C.

Ascites cells removed from the sucrose gradient appeared to be intact. The cell membrane was somewhat crinkled and the ratio of cytoplasmic to nuclear volume reduced owing to a certain amount of dehydration by the sucrose. The cells appeared to be more intensely stained by haematoxylin-eosin than fresh untreated ascites cells. Although large mononuclears were probably present in the main band of ascites cells, these were not directly sought by means of the special techniques required to distinguish them from ascites cells.

The respiratory function of ascites cells was apparently not seriously impaired after contact with strongly hypertonic 40% w/v sucrose solution. The  $Q_{O_2}$  of the cells maintained for forty minutes in 40% sucrose at 23°C. was -5.1, while that of cells maintained for the same time at 0°C. was -5.4 ( $Q_{O_2}$  values are expressed as  $\mu\text{l. } O_2/\text{mg. dry wt./hour at } 37^\circ\text{C.}$ ). Respiration remained undiminished in measurements of  $Q_{O_2}$  over sixty minutes. Both values of  $Q_{O_2}$  are within the limits of -5.0 to -10.0 quoted by Warburg (5) for ascites cells metabolising in ascites serum, and would indicate that viability of the cells is not greatly affected by contact with 40% w/v sucrose for the time required during centrifugation.

Intraperitoneal injection of 40% sucrose treated cells into mice, produced malignant tumours in 100% of cases. This provides additional evidence of cell viability after contact with hypertonic sucrose. Application of Schreck's staining test (6) to a sample of the 40% sucrose-treated cells showed that at least 50% were definitely viable.

Sucrose gradient centrifugation of the cell types present in ascites fluid, thus affords a satisfactory method for separation of the ascites cells from other varieties. The separated cells appear to be both viable and morphologically intact. It is

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## SUMMARY

1. A method has been devised for the separation of ascites tumour cells from other cell types present in Ehrlich mouse ascites fluid. The technique involves slow speed centrifugation in a sucrose gradient.

2. Ehrlich ascites mitochondria, capable of oxidative phosphorylation with a number of substrates have been prepared by a new method which offers several advantages over previous techniques.

3. Ehrlich ascites mitochondria prepared by the new technique, oxidise  $\alpha$ -ketoglutarate, succinate, L-glutamate and DL-isocitrate with coupled phosphorylation. L-Ascorbate and reduced diphosphopyridine nucleotide are rapidly oxidised but without coupled phosphorylation.

4. The oxidation of DL-isocitrate has been investigated in detail. Both diphosphopyridine nucleotide and triphosphopyridine nucleotide-linked isocitric dehydrogenases have been demonstrated in isolated Ehrlich ascites mitochondria. These dehydrogenases have separate electron transfer pathways to oxygen which are both coupled to phosphorylation. The activity of the DPK-dependent system is considerably greater than that of its TPN counterpart. Pyridine nucleotide transhydrogenase has been found to be completely absent from Ehrlich ascites mitochondria.

In contrast to that of ascites tumour cell mitochondria, the isocitric dehydrogenase system of normal tissue mitochondria has been shown to be TPN-specific.

5. The organic solvent dioxane (diethylene dioxide) stimulates DPN-isocitrate dehydrogenase activity to a remarkable extent with 2,6-dichlorophenol-indophenol as electron acceptor, but inhibits electron transport with cytochrome c or oxygen as acceptor. The DPN-isocitrate dehydrogenase was also stimulated by dioxane, but to a much smaller extent.
6. A new synthesis of n-octanoyl adenylyate has been achieved. The acyl adenylyate was prepared via the N,N'-di-cyclohexylcarbodiimide method and purified by paper chromatography and electrophoresis.
7. Swelling characteristics of Ehrlich ascites tumour cell mitochondria have been investigated and the results compared with those of normal tissue cell mitochondria.

ABBREVIATIONS

|                   |                                                      |
|-------------------|------------------------------------------------------|
| DHN, DHN          | = oxidised and reduced diphosphopyridine nucleotide  |
| TN, THN           | = oxidised and reduced triphosphopyridine nucleotide |
| AMP               | = adenosine monophosphate                            |
| ADP               | = adenosine diphosphate                              |
| ATP               | = adenosine triphosphate                             |
| IDP               | = inosine diphosphate                                |
| GDP               | = guanosine diphosphate                              |
| GTP               | = guanosine triphosphate                             |
| CoA, SH           | = co-enzyme A                                        |
| P <sub>i</sub>    | = inorganic phosphate                                |
| PP                | = inorganic pyrophosphate                            |
| cyto.             | = cytochrome                                         |
| O <sub>2</sub>    | = oxygen                                             |
| ~ P               | = high energy phosphate                              |
| α-Kg              | = α-ketoglutarate                                    |
| succinyl-S-CoA    | = succinyl co-enzyme A                               |
| Ac.Ac-S-CoA       | = acetoacetyl co-enzyme A                            |
| BH                | = β-hydroxybutyrate                                  |
| BH~S-CoA          | = β-hydroxybutyryl co-enzyme A                       |
| BH~ATP            | = β-hydroxybutyryl adenyate                          |
| FAD               | = oxidised flavin adenine dinucleotide               |
| FADH <sub>2</sub> | = reduced flavin adenine dinucleotide                |
| Q <sub>275</sub>  | = co-enzyme Q <sub>275</sub> or ubiquinone           |
| Tris              | = tris (hydroxymethyl) aminomethane                  |
| EDTA              | = ethylenediamine tetraacetate                       |
| n-octanoyl-ATP    | = octanoyl adenyate                                  |
| n-butyryl-AMP     | = butyryl adenyate                                   |

|                              |   |                               |
|------------------------------|---|-------------------------------|
| D-Glucose-6-PO <sub>4</sub>  | = | D-glucose-6-phosphate         |
| D-Ribulose-5-PO <sub>4</sub> | = | D-ribulose-5-phosphate        |
| DCLIP                        | = | 2,6-dichlorophenol-indophenol |
| ~                            | = | high energy bond              |
| TCA                          | = | trichloroacetic acid          |

## CHAPTER I

### PREVIOUS STUDIES CONCERNING

### TUMOR CELL MITOCHONDRIA

## CHAPTER I

### PREVIOUS STUDIES CONCERNING TUMOUR CELL MITOCHONDRIA

#### INTRODUCTION

Experiments at the Liesbeek Cancer Research Clinic in Cape Town, showed that X-irradiation of normal tissues produced unknown substances which affected the respiration of intact Ehrlich ascites tumour cells (1).

In order to study this effect in greater detail, more information was required concerning the cellular organelles responsible for respiration, namely, the "Mitochondria". This thesis reports such studies, as the result of which new knowledge has been acquired concerning the metabolism of Ehrlich ascites tumour cell mitochondria.

#### 1 MITOCHONDRIAL CONTENT AND LEVELS OF PYRIDINE NUCLEOTIDES IN TUMOUR CELLS

By comparison with the amount of research undertaken on normal cell mitochondria during the past ten years, very little has been published concerning the mitochondria of tumour cells. The biochemical mechanisms of mitochondrial function are now reasonably well understood as the result of work on normal cell particulates. However, the actual mechanisms of oxidative phosphorylation are as yet unknown, and their solution constitutes a major problem of contemporary biochemistry. The results of studies with normal cells have understandably proved of considerable value to the cancer research worker investigating similar problems involving the mitochondria of neoplastic cells.

In all studies with tumour cell mitochondria the following matters require careful consideration.

#### 1.1 Source of Tumour Material

This has to be carefully controlled and it is vitally important to use tissue that is genuinely neoplastic. The tumour used, should be as free as possible from normal tissue and necrotic tumour tissue.

#### 1.2 Availability of Tumour Material

Adequate amounts of tissue must be available for experimental purposes. This is especially important with regard to the Ehrlich ascites tumour, where rather large amounts of cell material are necessary in order to obtain a reasonable yield of twice washed mitochondria.

#### 1.3 Difficulty in Rupturing Malignant Cell Membranes

By comparison with those of normal cells, the membranes of tumour cells are extremely tough and difficult to rupture. This toughness necessitates the use of special homogenisation procedures to break neoplastic cells.

#### 1.4 Paucity of Mitochondria in Ehrlich Ascites Cells

The recovery of twice washed mitochondria from homogenates of Ehrlich ascites cells is extremely low by comparison with the yield from normal tissue cells.

In this connection Fiala (2) has observed that:

"Seemingly high respiration of ascites tumour cells (2.7 - 3.0 c.mm. oxygen uptake in one hour at 37°C. per cell in Krebs solution, pH 7.4), expressed on a physiologically meaningful basis of IMA unit (5 x 10<sup>8</sup> g. IMA), proves to be very low. (1.1 c.mm. oxygen per 1 x 10<sup>6</sup> units of IMA). Also mitochondria

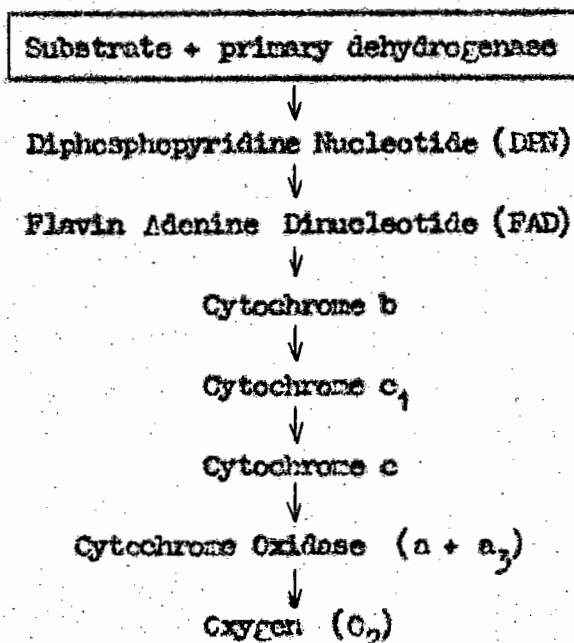
are scarce per unit of HIA. This indicates low respiratory capacity of the Ehrlich ascites tumour cells, in accord with the main feature of Warburg's concept of carcinogenesis."

#### 1.5 The Low Levels of Diphosphopyridine and Triphosphopyridine Nucleotides in Tumour Cells

It has been shown by Glock and Mclean (3) as well as by Weinhouse and co-workers (4,5) that the levels of DPN and TPN are lower in tumours than in normal tissues. The diminished levels of these co-enzymes might account for the lowered capacity of tumour cells for enzymic oxidation and reduction. However, this may not be the only reason for lowered redox activity in the tumour cell.

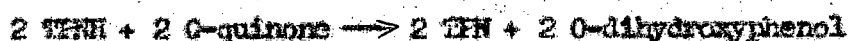
## 2 THE ELECTRON TRANSPORT CHAIN IN TUMOUR MITOCHONDRIA

Mitochondria contain the dehydrogenase enzymes involved in the operation of the Krebs Tricarboxylic Acid Cycle. The electrons liberated during the oxidation of each Krebs Cycle substrate are transferred to molecular oxygen along a respiratory chain which is usually represented as follows:



Previous studies of tumour mitochondria have suggested either a lowering in the enzymatic activities of various components along the electron transfer chain, or otherwise a decrease in the amounts of the various enzymes concerned. Greenstein and associates (6) have compared normal and tumour tissues in a study concerning the effect of added substrate plus cytochrome c on the relative rates of oxygen uptake. Tumours showed a greater increase in respiration than normal tissues. From these experiments, Greenstein concluded that malignant tissues possess relatively low quantities of cytochrome c. However, subsequent detailed spectrophotometric measurements by Chance and co-workers (7) with intact ascites cells (Ehrlich, Krebs-2 and Thyoma Cells) showed the presence of abnormally large quantities of cytochrome c relative to cytochromes a and a<sub>3</sub> (cytochrome oxidase). From their measurements, these authors concluded that Ehrlich ascites cells actually contain large amounts of cytochrome c.

In many tumours there is apparently a rate-limiting electron transfer step early on in the electron transport chain between substrate and oxygen. Experimental evidence (6, 8) shows this to be located somewhere between diphosphopyridine nucleotide and cytochrome b. Supporting evidence for this has been provided by Kertész and Albano (9) in their experiments with Ehrlich homogenates using Polyphenol Oxidase and O-dihydroxyphenol as artificial terminal electron accepting system in place of molecular oxygen. This reaction is shown below.



(In tumours, TPNH could be replaced by DPNH)

Ehrlich adenocarcinoma homogenates in the presence of the above electron accepting system, showed a marked increase in respiration, with a concomitant inhibition of aerobic glycolysis. Brilliant cresol blue, and other dyes such as the phenothiazines also increase the rate of electron transport in tumour homogenates (10).

### 3 THE CITRIC ACID CYCLE IN TUMOUR MITOCHONDRIA

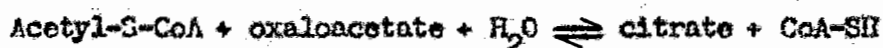
Much of the earlier work on enzymes of the citric acid cycle in tumour cells was carried out by Van R. Potter and his associates (11, 12, 13, 14, 15). These and subsequent investigations by a number of other workers (16, 17, 18, 19, 20, 21, 22), have shown that practically all the enzymes of the Tricarboxylic Acid cycle are located in tumour cells. However, the mechanism of citrate formation and oxidation is not completely understood. It is a fact, that some tumours accumulate citric acid while in others this metabolite is difficult to detect. Ambrose, Hellerman and Reiss (23) recently investigated the utilisation of pyruvate and formation of citrate in homogenates and isolated mitochondria of ascitic nodules from a strain of Walker Rat Mammary Carcinoma 256. They demonstrated the formation of substantial quantities of citrate in homogenates of the tumour when fortified with thiamine pyrophosphate, co-enzyme A and diphosphopyridine nucleotide. A requirement for added (+) lipoic acid was not demonstrated consistently.

Kit and Clark Griffin (24) state that tumours frequently, though not always, exhibit a diminished tendency to metabolise substrates through the citric acid cycle. To quote these authors:

"The primary metabolic pathway of pyruvate-2-C<sup>14</sup> leads to alanine, glutamate, aspartate and lactate in non-tumour tissues. In the tumours the ratio of isotope present in lactate to amino

acids was 20:1, while the ratio for other tissues was 1:1 or less. When tumour-bearing animals were poisoned with fluoroacetate, citrate accumulated in almost all normal tissues but not in a number of tumours with the exception of Hepatoma 98/15."

This failure to accumulate citrate in tumours blocked with fluoroacetate is difficult to explain. It may possibly be due to some subtle difference between tumour and normal tissue mitochondria, such as a different enzymatic structure, or perhaps instead due to a utilisation of citrate through some metabolic pathway other than that of the citric acid cycle. The fact that the condensing enzyme is present in tumours (24) suggests that this system might function in the formation of citrate from oxaloacetate and acetyl-CoA as follows:



Lindberg and his co-workers (21) showed that practically no oxidation of citrate occurs in isolated Ehrlich ascites cell mitochondria. Pyruvate oxidation was also found to be slow, but was considerably increased by the addition of a small quantity of succinate as "sparker".

Relevant to this matter is the research of D.E. Green and his co-workers at Wisconsin (25). This group studied the oxidation of isocitrate and citrate in intact and sonically disrupted beef heart mitochondria. Neither substrate was metabolised by intact mitochondria indicating a definite substrate permeability barrier. However, the sonically disrupted preparations rapidly oxidised both these substrates. The results explained the previous negative findings of Hogeboom and Schneider concerning the cytochemical distribution of isocitrate and citrate oxidase activities in liver homogenates. Permeability barriers may thus account for differences in the oxidation rates of various substrates in tumour mitochondria.

A considerable amount of research has recently been carried out with intact animals bearing a variety of tumours. Busch and co-workers (26, 27, 28), have been active in this field. Their method has been to administer pyruvate-2-C<sup>14</sup>, acetate-1-C<sup>14</sup> and labelled glucose to the animals, and to measure the characteristic labelling patterns of various compounds in different organs after necropsy. Considerable differences were observed between the normal tissues of the host and the tumour tissues.

#### 4. FATTY ACID OXIDATION IN NEOPLASTIC CELLS

Fatty acid oxidation by tumour mitochondria varies greatly according to the class of tumour employed. The Dutch workers, Emelot, Bos and their associates (29) have done interesting work in this field. In their studies of  $\beta$ -hydroxybutyrate oxidation they have found ATP and DEN to be essential co-factors. The necessity for these two co-factors is probably due to the high ATP-ase and DEN-ase activities known to be present in certain isolated tumour mitochondria. The well known ATP-ase activities of these particulates probably inhibits their capacity for fatty acid oxidation. The ATP-ase activities were however found to be suppressed by the inclusion of ethylenediamine-tetraacetate in the medium. n-Octanoate was oxidised only in the presence of DEN and  $\alpha$ -keto-octanoate as a spacer. Also, only certain tumours were found capable of oxidising this substrate.

Emelot, Bos and co-workers also carried out an interesting series of combination experiments, in which both liver and tumour mitochondria were present in the same medium. The following tumours were used:

- (1) Adrenal Cortex Carcinoma.
- (ii) Sarcomatoid Ovarian Tumour.
- (iii) Rutter yellow-induced Adenocarcinoma of Rat Liver.

In all oxidations studied, DEN was found to be a rate limiting factor. It was concluded that only one of the two isomers of DL-β-hydroxybutyrate undergoes oxidation by the tumour mitochondria studied. The evidence suggested that this is the isomer oxidised independently of CoA-SH and ATP (shown below).



It was assumed by Emsalet and his associates that the oxidation of L-β-hydroxybutyrate did not in fact take place in these tumour mitochondria. This isomer requires both ATP and CoA-SH for its oxidation as follows:



Failure to observe oxidation of L-β-hydroxybutyrate could have been due to the breakdown of ATP by endogenous ATP-ase action, thus preventing the initial activation of the fatty acid carboxyl group.

Weinhouse (8) in his review, "Oxidative Metabolism in Neoplastic Tissues", has discussed the oxidation of fatty acids by tumours in some detail. He states that very little work has been done on this matter. It has generally been assumed that glucose is the major fuel for neoplastic cells, but as the result of numerous studies conducted by the Weinhouse school, it has now been established that fatty acid oxidation is also of considerable importance in energy production. The problem has been approached mainly by indirect methods. Oxidation of C<sup>14</sup>-carboxy labelled fatty acids, such as palmitate occurred in tumours at approximately the same rates found with liver and kidney. The rate of oxidation of the fatty acids appeared to be dependent upon their chain length. Short chain fatty acids were not readily oxidised and appeared to act as powerful

inhibitors of respiration. Long chain fatty acids such as palmitate were rapidly oxidised.

Ciaranfi (30) showed that the methyl esters of  $C_4 - C_8$  fatty acids are rapidly oxidised in both normal and neoplastic tissues. The rate of oxidation was greater than when the sodium salts of the acids were used. Baker and Meister (31) found that in a variety of mouse and rat hepatoma homogenates, oxidation of hexanoic and octanoic acids proceeded at only 0 to 14% of the rate observed with normal liver. It appeared from this investigation that tumours might contain some inhibitor of fatty acid oxidation, since addition of hepatoma homogenate to liver homogenate decreased the capacity of the latter for fatty acid oxidation. It may be observed that early studies by Dickens and Sizer (32) established a low Respiratory Quotient for tumour slices, which was interpreted by them to indicate that fat may supply some of the energy needs of the tumour cell.

It is of interest to note that there is an absence of ketogenesis in hepatoma as compared to liver. This is of particular importance as it represents a marked difference between the metabolism of a normal liver cell and its neoplastic counterpart.

Reid and O'Neal (33) have studied the changes in "free" and "total" activity of ATP-ase and of acid phosphatase during azo-dye carcinogenesis. The mitochondrial fractions used in their study were isolated from, precancerous liver; tissue from rats which had been fed azo-dye for a prolonged period, but had not developed tumours; mixed tumours (hepatoma and cholangioma); hepatoma with little necrosis, and from hepatoma with marked necrosis.

Mutolo and Abrignani (34), investigated the effect of anionic detergents and trypsin on mitochondria of normal and